Hair nicotine concentration measurement in cats: its relationship to owner-reported environmental tobacco smoke exposure; and its measurement in cats with gastrointestinal lymphoma and unaffected control cases

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Abstract

The negative health effects of smoking and exposure to second-hand smoke are well documented in people. Several biomarkers of environmental tobacco smoke (ETS) exposure have been identified in human medical research. Relatively little research has been performed in cats to investigate possible biomarkers of ETS and disease associations, however an association between ETS and the development of several anatomical variants of lymphoma has been suggested.

The objectives of this thesis were to: (1) investigate the utility of hair nicotine concentration (HNC) in cats as a biomarker of ETS; (2) investigate the association between HNC in cats and owner reported exposure; (3) identify a cut-off HNC to differentiate between exposed and unexposed cats; and (4) further investigate the association between exposure to ETS and gastrointestinal lymphoma, using HNC as a biomarker.

Nicotine was extracted from cat hair by sonification in methanol, followed by hydrophilic interaction chromatography with mass spectrometry. Owner questionnaires were used to quantify ETS exposure. The HNC of reportedly exposed and unexposed groups were compared and a cut-off value to differentiate these groups was created. To investigate associations between ETS and gastrointestinal lymphoma, the HNC of cats affected by this condition was compared to the HNC of control cases.

The HNC of reportedly exposed cats was significantly higher than that of unexposed cats. When the cats were grouped according to the intensity of exposure and the number of products smoked each day at home, the median HNCs were significantly different. A hair nicotine concentration of 0.1ng/mg had a specificity of 98% for detecting exposure to ETS. Using HNC as a biomarker of exposure to ETS, there was no significant difference in HNC between cats with gastrointestinal lymphoma and control cases, although the group of cats with the highest HNC was composed of nearly two-thirds lymphoma cases. A further study with larger case numbers would help confirm whether cats with exposure to ETS do or do not have an increased risk of gastrointestinal lymphoma.

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Authors declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

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Definitions and abbreviations

AA	Absolute abundance
AUC	Area under the curve
ETS	Environmental tobacco smoke
FeLV	Feline leukaemia virus
FNA	Fine needle aspiration
HNC	Hair nicotine concentration
HPLC	Hydrophilic interaction liquid chromatography
IBD	Inflammatory bowel disease
LGAL	Low grade alimentary lymphoma
LOD	Limit of detection
LPE	Lymphoplasmacytic enteritis
MS	Mass spectrometry
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-glucs	Glucuronide metabolite of NNAL
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
PAH	Polycyclic aromatic hydrocarbons
PARR	PCR of antigen receptor rearrangements
ROC	Receiver Operator Characteristics
SCC	Squamous cell carcinoma
SHS	Second-hand smoke
THS	Third-hand smoke
TSNA	Tobacco-specific nitrosamines

1 Introduction

It is widely accepted that humans exposed to environmental tobacco smoke (ETS), also known as second-hand smoke or passive smoking, have an increased risk of pulmonary and cardiovascular disease, in addition to many cancers (General Report of the Surgeon General 1989, Linet *et al*. 1992, Witschi *et al*. 1997, Hecht 2003, Besaratinina & Pfeifer 2008, Öberg *et al.* 2011). The close physical relationships cats typically maintain with their owners results in indirect exposure to tobacco smoke, and also to contaminated clothing and skin. The frequent and extensive self-grooming that is typical of cats could lead to ingestion of ETS toxins and carcinogens from the environment. Compared to the abundance of human data, relatively little is known regarding the effects of such ETS exposure in cats, but it would be expected that it would pose similar health risks.

By developing a novel method of quantifying exposure to ETS in cats, associations between such exposure and disease development could be investigated. The development of a suitable biomarker for exposure to ETS in our pets would reflect long-term, cumulative exposure and would be strongly associated with exposure in terms of dose. Raising awareness of ETS exposure among pet owners could motivate changes in owner smoking behaviours. The potentially positive impact on the health of both owners and their pets is in line with the One Health initiative. The American Veterinary Medicine Association defines One Health as "the integrative effort of multiple disciplines working locally, nationally and globally to attain optimal health for people, animals and the environment" (American Veterinary Medicine Association 2016). A web-based survey found that 28.4% of smoking pet owners would try to quit smoking if they learnt that they were potentially harming their pets (Milberger *et al.* 2009). Therefore veterinarians may be an important influence on encouraging smoking cessation if they are able to educate owners with regards to the potentially harmful effects (Hodgson & Darling 2011).

1.1 Environmental tobacco smoke

The smoke emitted directly from a burning cigarette is referred to as sidestream smoke whereas the smoke exhaled from the lungs of smokers whilst smoking is referred to as mainstream smoke (Matt *et al.* 2011). It is estimated that ETS contains approximately 85% sidestream smoke and 15% mainstream smoke (Witschi *et al.* 1997). The compounds present in sidestream and mainstream smoke are qualitatively similar; therefore exposure

via either passive smoking or from being an active smoker poses similar risks (Jaakkola *et al.* 2001, Besaratinia & Pfeifer 2008). It has been shown, however, that sidestream and mainstream smoke are quantitatively different, with sidestream smoke being richer in some carcinogens such as aromatic amines (Besaratinia & Pfeifer 2008). Research in mice has shown a significantly increased risk of developing malignant skin tumours when directly exposed to sidestream smoke, in comparison to those exposed to mainstream smoke (Mohtashamipur *et al.* 1990).

Environmental tobacco smoke contains greater than 4,000 chemicals including over 40 mutagens and carcinogens, many of which are associated with carcinogenesis in humans (Hoffman *et al.*1994, Hsu *et al.* 1997, Romano *et al.* 1997, Husgafvel-Pursiainen *et al.* 2000, U.S. Department of Health and Human Services 2006). In vitro studies have shown that tobacco smoke has a direct carcinogenic effect on human gastric mucosal cells (Tayler & Piper 1977). There are three major classes of carcinogens present in ETS: tobacco-specific nitrosamines (TSNA), polycyclic aromatic hydrocarbons (PAH), and aromatic amines (Hoffman & Hoffman 1997, Hecht 2003). Of the TSNAs, the most notable are N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the latter is one of the most potent carcinogens to have been tested in laboratory animals (Prokopczyk *et al.* 2002). In addition to these pre-formed nitrosamines, tobacco smoke also contains nitrosamine precursors such as nicotine, which can lead to endogenous nitrosamine formation within the body (Hoffman *et al*. 1994). Although nicotine is not in itself carcinogenic, its presence has been shown to confer a survival advantage on mutated tumour cells induced by tobacco smoke carcinogens (Catassi *et al.* 2008). Nicotine provides both growth and proliferation advantages in addition to inhibiting apoptosis of these damaged cells (Catassi *et al.* 2008). In addition, it has been shown that nicotine stimulates the malignant behaviour of glioma cells in vitro, and also increases the radio-resistance of these tumours (Khalil *et al*. 2013).

In addition to the risks associated with traditional forms of tobacco smoking, evidence is also emerging of the human health effects of using electronic cigarettes (e-cigarettes) (Callahan-Lyon 2014). These are battery-powered devices that do not involve tobacco combustion but instead aerosolise nicotine and the other components prior to inhalation (Callahan-Lyon 2014). The chemical propylene glycol is added to the liquid to create artificial “smoke” to simulate the experience of smoking a normal cigarette (Kuschner *et al.* 2011). The level of nicotine exposure from e-cigarettes is said to be highly variable due

to wide ranges in the amount of nicotine in products, variability in aerosolisation and inconsistent nicotine delivery during use (Callahan-Lyon 2014).

1.2 Environmental tobacco smoke exposure

Non-smokers, children and pets living with a smoker will be exposed to ETS. This includes exposure to both: (1) the airborne products of tobacco combustion present at the time a cigarette is lit, commonly referred to as second-hand smoke (SHS); and (2) the pollutants remaining in an indoor environment long after a cigarette is extinguished, known as third-hand smoke (THS) (Winickoff *et al.* 2009, Matt *et al.* 2010). Smokers in a household can also directly contaminate objects such as toys and food with ETS pollutants from their hands, providing a further avenue of exposure to their infants and pets (Matt *et al.* 2011). Clothing of people, both smokers and non-smokers, which have been exposed to ETS are an additional source of exposure in the home environment (Matt *et al.* 2011).

Tobacco smoke is able to move between adjacent properties via wall and floor cracks, even along plumbing and electrical routes (Spengler 1999), therefore people, and their pets, may be inadvertently exposed to the harmful effects of ETS from smoking neighbours. Research into this phenomenon has shown that children from non-smoking households living in multiunit housing had higher serum cotinine levels, a metabolite of nicotine, than those living in detached homes, presumably due to the aforementioned migration of ETS (Wilson *et al.* 2011).

Second-hand smoking and passive smoking are commonly used terminology, however the phenomenon of THS has only relatively recently been recognised having first appeared in the medical literature in 2009 (Winickoff *et al.* 2009). It is now known that the harmful residues of smoking remain in the environment for a very long time, resulting in involuntary exposure to chemicals, including nicotine and carcinogens, many months or years after smoking cessation (Kuschner *et al.* 2011, Matt *et al.* 2011). It has been shown that when non-smokers move into housing previously occupied by a smoker, the amount of nicotine present on their skin and the amount of a nicotine metabolite in their urine increases due to THS (Matt *et al.* 2010).

Some of the compounds present in THS are odourant and are cited as the source of the unpleasant, stale, tobacco smoke odour that clings to smokers or is present in enclosed

areas in which smoking has occurred (Matt *et al.* 2010). Of greater concern are the harmful toxins that develop within this residue of tobacco smoke (Kuschner *et al.* 2011). The nicotine in THS can react with ambient nitrous acid resulting in the production of TSNAs, many of which as previously mentioned are potent carcinogens (Kuschner *et al.* 2011, Matt *et al.* 2011). It has been suggested that the hand-to-mouth activity of young children, in addition to their close contact whilst crawling on contaminated floors, puts them at an increased risk from such THS toxins (Dreyfuss 2010, Kuschner *et al.* 2011, Matt *et al.* 2011). This concern could also be raised regarding pets in our homes, as they demonstrate similar behaviours. It could be assumed that improving ventilation by way of opening windows or using air conditioners would reduce or eliminate the risk of contamination with THS, however these measures have been shown not to be protective (Kuschner *et al.* 2011). In fact it would be necessary to remove furniture, carpets, curtains and wall boards to eliminate the toxins associated with THS (Kuschner *et al.* 2011).

1.3 Methods of quantifying exposure to environmental tobacco smoke in humans

1.3.1 Questionnaires

Self-reporting questionnaires to document ETS exposure have the advantages of being low cost and simple to prepare, therefore allowing data to be collected from a large number of subjects (Jaakkola & Jaakkola 1997, Glasgow *et al.* 1998, Al-Delaimy *et al.* 2002a). The results provided, however, can be subject to recall bias and may not account for variability in smoking habits, external or unknown sources of exposure and ventilation at home (Nafstad *et al.* 1995, Al-Delaimy *et al* 2002b, Besaratinia & Pfeifer 2008). There is also a degree of trust that the respondents are answering the questions honestly.

Quantifying exposure can be difficult using questionnaire responses alone; although a subject may be knowingly exposed to ETS, it is difficult to account for temporal associations (Besaratinia & Pfeifer 2008). A further concern is that respondents are very unlikely to be aware of potential exposure to the aforementioned phenomenon of THS, and therefore questionnaires are likely to fail to identify such exposure.

Despite the potential shortcomings of self-reporting questionnaires, they are a simple way to identify known exposure to ETS and may allow subsequent categorisation of subjects into differing exposure groups. However as they cannot provide a true measure of the

concentration of the exposure, questionnaires should ideally be considered as complimentary to the measurement of biomarkers for documenting exposure to ETS (Al-Delaimy 2002). The low sensitivity of questionnaires to identify exposure to ETS raises concerns for inaccuracy when interpreting associations between exposure and disease development (Al-Delaimy *et al* 2002a), and in such circumstances a biomarker of ETS would be more reliable.

1.3.2 Biomarkers of environmental tobacco smoke exposure

To confirm ETS is actually absorbed by people the measurement of a biomarker is required, which can also provide an index of the dose (Eliopoulos *et al.* 1994, Nilsen *et al.* 1994, Matt *et al.* 2008).

The ideal biomarker would have the following characteristics: be unique to ETS; easily detectable; detectable by analytical methods with reproducible results across laboratories; have a strong association with known exposures; and exhibit changed levels with a corresponding change in exposure (Avila-Tang *et al.* 2013). In addition a biomarker would ideally make use of non-invasive sample collection. Disadvantages of using biomarkers to quantify exposure to ETS include the cost and technical expertise required.

Biomarkers of ETS that have been used in people include TSNAs, nicotine, cotinine and hair nicotine concentration (HNC).

1.3.2.1 Tobacco specific nitrosamines and their metabolites

Measurement of carcinogenic TSNAs in pancreatic juice has been used in humans to investigate the association between smoking and pancreatic cancer (Prokopczyk *et al.* 2002). Subjects were classified as smokers or non-smokers by measurement of both urinary cotinine and hair nicotine concentration. The mean level of NNK was found to be significantly higher in the pancreatic juice of smokers compared to non-smokers; supporting the hypothesis that exposure to ETS may increase the risk of developing pancreatic cancer. Metabolites of NNK, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-glucs), have also been studied (Hecht 2003). Research has shown that NNAL and NNAL-glucs can be detected in the urine of smokers and non-smokers exposed to ETS (Hecht 1998). These metabolites are considered to be

urinary carcinogen biomarkers in humans (Hecht 2002). As with other urinary biomarkers, these metabolites are only reflective of short-term exposure.

1.3.2.2 Nicotine

Nicotine is found naturally in tobacco leaves as a botanical insecticide and is the principal alkaloid in tobacco (Benowitz *et al.* 2009). It is estimated that nicotine comprises 1.5% the weight of commercial cigarette tobacco (Benowitz *et al.* 2009).

The relationship between the number of cigarettes smoked in a household and the nicotine concentration in the air, as measured by nicotine monitors, is relatively linear (Glasgow *et al.* 1998). Nicotine is the principal identifying constituent of tobacco smoke and therefore has been used as a marker of the presence of other tobacco smoke constituents, including carcinogens (Al-Delaimy 2002, Benowitz 2010).

Nicotine is lipid soluble and therefore readily permeates cell membranes (Al-Delaimy 2002). It is well absorbed through the airways, mucous membranes, skin and gastrointestinal tract and is subsequently absorbed into the systemic circulation (Al-Delaimy 2002, Eliopoulos *et al.* 1994, Callahan-Lyon 2014). It is estimated that 60-80% of the nicotine present in inspired air is absorbed by the human body (Iwase *et al.* 1991). When ingested, nicotine is ionised by the acidic gastric fluid and therefore is poorly absorbed in the stomach (Hukkanen *et al.* 2005). Nicotine is, however, well absorbed in the small intestine due to the more alkaline pH and the large surface area (Hukkanen *et al.* 2005). Interestingly, studies investigating the effects of nicotine in the intestine have shown altered gut motility and smooth muscle contractility, decreased permeability, changes in colonic mucous and oxygen free radicals, and alterations in the microcirculation (Green *et al.* 2000, Karban & Eliakim 2007, Lakatos *et al.* 2007).

Nicotine has been measured in blood, urine, saliva, toenails or hair. With a half-life of only 2 hours, measurement of nicotine levels in blood, urine or saliva reflects only very recent exposure to tobacco smoke (Avila-Tang *et al.* 2013, Nilsen *et al.* 1994). Therefore these methods have limited use in studies assessing temporal exposure to ETS, due to the short period of exposure reflected by their presence.

In addition when assessing the risk of disease development in association to ETS exposure, alternative biomarkers reflecting exposure over a longer cumulative time period are recommended (Al-Delaimy *et al.* 2002b). Alternatives considered to be valid biomarkers for chronic ETS exposure include the concentration of nicotine in human toenails and hair (Nafstad *et al.* 1995, Eliopoulos *et al.* 1996, Al-Delaimy *et al.* 2000, Al-Delaimy 2002, Al-Delaimy *et al.* 2002a, Al-Delaimy *et al.* 2002b, Okoli *et al.* 2007).

1.3.2.3 Cotinine

An alternative biomarker for ETS is the measurement of cotinine in serum, urine or hair. Cotinine is formed exclusively by the biotransformation of nicotine in hepatic cells and is the main metabolite of nicotine (Al-Delaimy *et al.* 2000). Cotinine has a half-life of 10-14 hours and therefore urine cotinine represents exposure to nicotine in the proceeding 2-3 days. Therefore this is not useful for long-term exposure studies (Nilsen *et al.* 1994, Eliopoulos *et al.* 1996). In addition, there are concerns regarding metabolic variability in clearance rates (Eliopoulos *et al.* 1994, Al-Delaimy *et al.* 2000, Al-Delaimy *et al.* 2002b, Kim *et al.* 2014). A further disadvantage of these biomarkers is the necessity to collect liquid bio-samples, which may reduce patient or owner compliance (Kim *et al.* 2014).

Cotinine is known to accumulate in human hair, however the concentration tends to be lower than that for nicotine and therefore the latter is more commonly measured (Gerstenberg *et al.* 1995, Matt *et al.* 2008). In addition it has been suggested that cotinine has a lower affinity for hair than nicotine (Al-Delaimy *et al.* 2000).

A study comparing urinary cotinine and hair nicotine concentration in children found that the latter could be used to differentiate those with smoking and non-smoking parents, whereas urinary cotinine levels could not (Al-Delaimy 2002, Nafstad *et al.* 1995). In addition, urinary cotinine was less closely correlated with questionnaire estimates of ETS exposure than hair nicotine concentration (Al-Delaimy 2002).

1.4 Hair nicotine concentration

Nicotine can enter hair via two possible mechanisms: (1) by absorbing directly onto hair from the environment (Nilsen *et al.* 1994, Gerstenberg *et al.* 1995, Al-Delaimy 2002); and (2) by deposition into the hair shaft through the hair bulb blood supply following systemic

absorption ((Al-Delaimy 2002, Apelberg *et al.* 2012). In the latter situation, nicotine is able to passively diffuse into the growing hair cells, where it becomes incorporated into the hair shaft during keratogenesis (Al-Delaimy 2002). Absorption of nicotine from the environment directly onto hair results in an increasing hair nicotine concentration (HNC) from the scalp outwards due to increased time of exposure (Nilsen *et al.* 1994, Al-Delaimy 2002). Therefore, in humans, the older distal ends of hair contain higher concentrations of nicotine than the newer proximal ends and it is recommended that the proximal 1-2 cm of hair should be used when estimating ETS exposure in people (Nilsen *et al.* 1994, Al-Delaimy 2002). There is no metabolism of nicotine once it is incorporated into hair (Al-Delaimy 2002). The HNC is reflective of relatively long-term exposure of several months and therefore is beneficial for epidemiological studies of disease aetiology (Al-Delaimy 2002). Each centimetre of hair represents greater than one month of exposure, as human hair grows uniformly at a rate of 1±0.3cm each month (Uematsu *et al*. 1995). In people it is recommended that the back of the scalp is used as the site for hair sampling when measuring HNC as this is the area with the most uniform growth pattern (Al-Delaimy 2002).

Hair nicotine as a biomarker for ETS has several advantages. These include: the non-invasive nature of sample collection; the lack of trauma to the donor; the ease of transport (Al-Delaimy 2002, Eliopoulos *et al.* 1996, Tzatzarakis *et al.* 2011, Antunes *et al.* 2015); and the ability to store samples for up to five years, as HNC remains stable (Zahlsen & Nilsen 1994). In addition, HNC is sufficiently sensitive to detect alterations in both active and passive exposure to ETS and has shown a high reproducibility over time (Mizuno *et al.* 1993, Mizuno *et al.* 1997, Zahlsen & Nilsen 1994).

1.4.1 Association of hair nicotine concentration with environmental tobacco smoke in people

The use of HNC has been extensively investigated in people and is well correlated with ETS exposure (Nafstad *et al.* 1995, Eliopoulos *et al.* 1996, Al-Delaimy *et al.* 2000, Al-Delaimy 2002, Al-Delaimy *et al*. 2002a, Okoli *et al.* 2007). Hair nicotine concentrations can clearly distinguish between smokers and non-smokers (Okoli *et al*. 2007).

Many human studies have used self-reporting questionnaires to document smoking behaviours, including the number of cigarettes smoked, and compared this information to

the subjects' HNC (Eliopoulos *et al.* 1994, Eliopoulos *et al.* 1996, Knight *et al.* 1996, Al-Delaimy *et al.* 2000, Apelberg *et al.* 2012). These studies have consistently shown that HNC is significantly higher in people that are reportedly exposed to ETS, compared to those that are reportedly unexposed. In comparison to HNC, hair cotinine levels have been found to be less strongly associated with ETS (Al-Delaimy *et al.* 2000).

Hair nicotine concentration has been found to be strongly associated with the number of cigarettes smoked per day (Eliopoulos *et al.* 1996, Al-Delaimy *et al.* 2000, Apelberg *et al*. 2012). Apelberg *et al* (2012) found that for every additional cigarette smoked each day, the mean HNC increased by 4%. This association was lost when the number of cigarettes smoke each day was greater than 20 (considered commonly to be one packet). Interestingly the same study showed that people, who reported smoking their first cigarette within 30 minutes of waking up in the morning, had twice the HNC of those that did not. Results have shown that HNC in children is strongly associated with parental smoking habits and that HNC increases with an increased daily number of cigarettes smoked by parents (Nafstad *et al.* 1995, Okoli *et al* 2007). Overall these studies show that HNC is a reliable method of determining human exposure to ETS.

Hair nicotine concentration can also be used to document temporal exposure to ETS; in people participating in a smoking cessation programme, there is a cm-by-cm reduction in HNC, which approximates with the monthly reduction in the number of products smoked (Mizuno *et al.* 1993).

Other factors affecting variations in HNC in people exposed to ETS include differences in room size and ventilation, in addition to the distance from the smoker and the number of hours of exposure (Al-Delaimy *et al.* 2000).

1.4.2. Factors other than environmental tobacco smoke exposure that can affect hair nicotine concentration

Darker haired smokers have been found to have higher HNCs (Mizuno *et al.* 1993, Gerstenberg *et al.* 1995, Uematsu *et al.* 1995, Claffey *et al.* 2001, Apelberg *et al.* 2012). Nicotine is known to have a high affinity for melanin, which is produced by melanocytes in the hair bulb and incorporated into the cortex of darker hair (Al-Delaimy 2002). However others studies have suggested that the effect of dark hair on nicotine uptake is not

consistent (Knight *et al.* 1996, Zahlsen *et al.* 1996). Furthermore it has been have found in people that there are racial differences in HNC; Black smokers had substantially higher levels of nicotine in their hair compared to White smokers, after controlling for the number of cigarettes smoked per day (Apelberg *et al.* 2012).

It is known that hair washing in people will not affect the internal hair nicotine but will wash away externally attached nicotine (Al-Delaimy 2002). An additional concern for measuring HNC in humans is the requirement to document cosmetic treatments used on hair such as bleaching (Al-Delaimy 2002). Such treatments can alter the integrity of the outer cuticle of the hair shaft potentially allowing nicotine leakage (Al-Delaimy 2002). Perhaps a more viable concern for measuring HNC in pets is the routine coat washing, although this is expected to be a rare concern with cats. A study has shown that washing of hair samples from laboratory animals that had been directly exposed to ETS, removed 71-90% of nicotine from the hair (Gerstenberg *et al.* 1995).

1.4.3 Measuring hair nicotine concentration

The first step in measuring HNC is to extract the nicotine from the hair. Ultrasonic washing of hair (sonification) in methanol has been shown to remove all nicotine in the hair (Bawazeer *et al.* 2012), both that adherent to the surface and that incorporated into the hair shaft following systemic absorption. The resultant nicotine-containing supernatant can be used for subsequent analysis.

Several analytical methods have been described for measuring nicotine concentrations in human hair including gas chromatography, high-performance liquid chromatography, electrochemical detection and radioimmunoassays (Chetiyanukornkul *et al.* 2004). It is said that mass spectrometry is the most accurate of all (Chetiyanukornkul *et al.* 2004). Mass spectrometry (MS) separates organic molecules according to their molecular mass and allows their detection and quantification with extremely high sensitivity (Sargent 2013). An additional separation technique, such as hydrophilic interaction or liquid chromatography, is typically used prior to MS to permit the isolation and measurement of analytes from highly complex mixtures (Sargent 2013), such as that produced following methanol extraction of nicotine from hair. The chromatography differentiates compounds based on their physico-chemical properties and the MS differentiates compounds by their mass-to-charge ratio (Sargent 2013). This dual selectivity gives confidence that the correct

component is being measured (Sargent 2013). The technique is rapid and deuterated analytes can be used as internal standards (Chetiyanukornkul *et al.* 2004). These deuterated analytes are isotopically labeled internal standards. The internal standard and the analyte of interest may not separate by liquid chromatography, but can be distinguished following MS due to their mass difference (Sargent 2013). The use of such internal standards helps to control variability in a quantitative assay when run in tandem with the samples of interest (Sargent 2013).

1.5 Environmental tobacco smoke exposure in cats and dogs

Pet cats and dogs share a living environment with humans and therefore are exposed to the same environmental contaminants, such as ETS (National Research Council 1991). In circumstances where pet cats are kept continuously indoors, it has been suggested that they may have higher exposure levels to environmental contaminants in the home, compared to their owners who can spend extended periods of time outside of the house (Bertone *et al.* 2002). Uptake of ETS constituents can be inhalational, trans-dermal or via oral ingestion, particularly during grooming of contaminants on fur (Bertone et al. 2002, Bertone-Johnson *et al.* 2008). In addition to exposure via contaminated skin and clothing, contact with ETS also occurs via carpets and soft furnishings (Bertone-Johnson *et al.* 2008).

1.5.1 Questionnaire based studies

Previous studies investigating the association between ETS and disease in cats and dogs have largely been based on retrospective, self-reported owner questionnaires (Reif *et al.* 1992, Reif *et al.* 1998, Bertone *et al.* 2002, Snyder *et al*. 2004). A previous study investigating the use of HNC as a biomarker in dogs used owner based questionnaires as the gold standard reference for estimating exposure to ETS (Knottenbelt *et al.* 2012).

In addition to the shortcomings identified when questionnaires are used to estimate ETS in people, owners acting as proxy respondents for their pet cannot account for exposure during free roaming (Kelsey *et al.* 1998). In addition, due to the known associations between ETS and cancer in humans, owners may feel guilty and their subsequent reporting of smoking behaviour may be subject to recall bias when a malignancy has been diagnosed in their pets (McNiel *et al.* 2007).

1.5.2 Biomarkers for detecting environmental tobacco smoke exposure in cats and dogs

Previous studies investigating ETS exposure in pet cats and/or dogs have investigated the use of cotinine, nicotine and TSNAs.

1.5.2.1 Urinary cotinine, nicotine and tobacco specific nitrosamines

Urinary cotinine concentration has been used in canine and feline studies (Roza & Viegas 2007, Bertone-Johnson *et al.* 2008). Roza & Vegas (2007) compared bronchoalveolar lavage cell samples from dogs that lived with smokers to dogs that did not. Urinary cotinine levels were subsequently used to confirm exposure to ETS. All dogs that were known to live with smokers had cotinine detected in their urine, whereas dogs that did not live with smokers did not. A further canine study found that urinary cotinine concentrations were approximately twice as high in dogs exposed to ETS in the prior 24 hours when compared to unexposed dogs (Bertone-Johnson *et al.* 2008). In addition, the urinary cotinine level also increased significantly as the number of cigarettes smoked at home in the 24-hour period prior to urine collection increased (Bertone-Johnson *et al.* 2008).

A similar study in cats determined urinary nicotine and cotinine levels in cats exposed to ETS and cats unexposed to ETS (McNiel *et al.* 2007). The authors also looked at urinary levels of NNAL, a primary metabolite of NNK. The study population consisted of 19 cats exposed to ETS and 42 cats not exposed. The cats that had been exposed to ETS had lived with their owners for a mean of 4.4 years, ranging from 1 to 14 years. Significantly higher urinary concentrations of nicotine, cotinine and NNAL were found in cats reportedly exposed to ETS. Cut-off values for these biomarkers were not determined. Interestingly three cats that were reportedly not exposed to ETS had measurable NNAL concentrations in their urine, suggesting that these cats were actually exposed to ETS but the owners were unaware of this or provided false information. The conclusion was that cats absorb and metabolise tobacco-associated chemicals from the home environment.

A disadvantage of using urinary biomarkers in cats, as has been shown in people, is that they reflect only short-term rather than cumulative exposure (McNiel *et al.* 2007). Obtaining urine from cats can be challenging as owners often find collection of free catch urine samples difficult and the alternative method of obtaining urine via cystocentesis is an

invasive procedure. In addition declining renal function in aged cats could lead to variability in the clearance of such metabolites (McNiel *et al.* 2007).

1.5.2.2 Hair nicotine concentration

Hair nicotine concentrations have been shown to have a strong positive association with the magnitude of owner-reported ETS exposure in dogs (Bawazeer *et al.* 2012, Knottenbelt *et al.* 2012). Owners were asked to complete a brief questionnaire to document known ETS exposure in the prior three-month period. There was a statistically significant difference in HNC between the three study groups: those dogs unexposed to ETS, those exposed occasionally, and those exposed regularly.

Hair nicotine concentration has not been investigated in cats. The dogs' hair samples were collected from the neck, at the site of routine blood sampling. There are no known recommendations as to which sites to obtain hair samples from cats, however it is known that the turnover of hair is similar on any area of a cats' body (Hendriks *et al.* 1997). Interestingly adult cats have been shown to have comparable rates of hair growth to those of man, growing approximately 300 microns per day (Baker 1974).

1.6 Associations between environmental tobacco smoke and disease in cats and dogs

Canine studies have suggested an increased risk of lung cancer in brachycephalic dogs (Reif *et al.* 1992) and nasal cavity and paranasal sinus cancer in dolichocephalic dogs (Reif *et al.* 1998), when their owners smoke. Both studies used owner-based questionnaires to categorise exposure to ETS. Unfortunately no biomarkers were used to confirm the level of ETS exposure in those dogs, which could have lead to inaccurate risk estimates (Reif *et al.* 1992). A study by Roza & Vegas (2007) used urinary cotinine as a biomarker in dogs to confirm exposure to ETS, concluding that dogs that are exposed have significantly higher macrophage and lymphocyte populations in bronchoalveolar lavage samples than those that are unexposed.

A study in cats identified increased expression of p53 in oral squamous cell carcinoma (SCC) (Snyder *et al.* 2004) in cats that were exposed to ETS, suggesting that p53 is a potential site for carcinogen-related mutation in this type of tumour. Exposure to ETS was identified by the use of an owner questionnaire. This association was not statistically

significant and therefore there is the likelihood that chance played a role in this finding (Snyder *et al.* 2004). A similar questionnaire based feline study suggested an increased risk of developing several anatomical forms of lymphoma, including gastrointestinal lymphoma (Bertone *et al.* 2002) when cats are exposed to ETS. The duration of exposure to ETS was significantly associated with the risk of lymphoma; cats that were exposed for 5 or more years had a relative risk of 3.2 compared to those in non-smoking households. The majority of malignant lymphomas in that study were gastrointestinal and nasal in origin, which would be consistent with the hypothesis that ingestion and inhalation of ETS is involved in the aetiology of such diseases (Bertone *et al.* 2002).

1.7 Gastrointestinal lymphoma in cats

Alimentary lymphoma is characterised by infiltration of the gastrointestinal tract with neoplastic lymphocytes, with or without mesenteric lymph node involvement (Louwerens *et al.* 2005, Lingard *et al.* 2009, Barrs & Beatty 2012a). It is the most common anatomical form of lymphoma in cats; a study of 1129 feline intestinal tumours found that 55% were lymphoma, 32% were adenocarcinomas and 4% were mast cell tumours (Rissetto *et al.* 2011). There are several nomenclatures for the sub-classification of alimentary lymphoma. Some authors recognise three histological grades, namely low, intermediate and high, as based on the frequency of mitoses (Barrs & Beatty 2012a). Others more simply recognise two histopathological variants: (1) a small cell lymphocytic villous lymphoma associated with older cats; and (2) a large cell lymphoblastic lymphoma which is seen in cats of any age and is more aggressive in nature (Washabau *et al.* 2010). In addition, alimentary lymphoma can be categorised depending upon immunophenotyping (B or T cell). A separate sub-classification of lymphoma, which can be of any grade, is large granular lymphocyte lymphoma (Barrs & Beatty 2012b).

The most common clinical signs of alimentary lymphoma include weight loss (≥80%), vomiting (≥70%), diarrhoea (≥60%) and inappetence (≥50%) (Barrs & Beatty 2012a). Abdominal palpation may reveal thickened intestines, the presence of mesenteric lymph node enlargement or a focal intestinal mass (Barrs & Beatty 2012a). Abdominal ultrasound can be used to assess intestinal wall thickness and to identify disruption of normal intestinal wall layering and for the presence of abdominal lymph node enlargement, although these abnormalities are not consistently seen.

Definitive diagnosis of gastrointestinal lymphoma can be made by either cytology of intestinal wall masses or enlarged mesenteric lymph nodes; or histopathology of endoscopic or full-thickness gastrointestinal biopsies (Barrs and Beatty 2012a). Obtaining full-thickness biopsies has shown to be advantageous and is recommended when diagnosing low-grade alimentary lymphoma (LGAL), which could be incorrectly classified as inflammatory bowel disease (IBD) if not all layers of the intestine are examined histologically (Kleinschmidt *et al.* 2010, Barrs & Beatty 2012b). Low-grade alimentary lymphoma shares many histological features with benign lymphoplasmacytic enteritis (LPE), which is a chronic mononuclear inflammatory process (Barrs & Beatty 2012b). Newer diagnostic modalities including immunophenotyping and PCR for antigen receptor rearrangements (PARR) may be required if histopathological examination is insufficient to confirm the presence of lymphoma (Washabau *et al.* 2010).

Endoscopic biopsies only permit sampling from the mucosal layer and therefore may not allow detection of lymphoma in deeper tissues, resulting in an incorrect or inconclusive diagnosis in 9 out of 11 cats (Evans *et al.* 2006). Therefore obtaining transmural, full thickness biopsies provide greater confidence in distinguishing LPE from LGAL. However, intermediate and high-grade alimentary lymphoma can often be diagnosed simply by aspiration cytology (Barrs & Beatty 2012b).

1.7.1 Aetiology of gastrointestinal lymphoma in cats

Although there has long been a causal link between lymphoma and Feline Leukaemia Virus (FeLV), alimentary lymphoma is said to have the weakest association with FeLV antigenaemia (Cotter *et al.* 1975, Barrs & Beatty 2012). The prevalence of FeLV infection has significantly reduced in recent years due to increased frequency of vaccination and testing for the infection (Vail *et al.* 1998). The reduction in the incidence of lymphoma in anatomical sites more strongly associated with FeLV, such as thymic lymphoma, has led to a relative increase in the incidence of alimentary lymphoma (Francis *et al.* 1979, Barrs & Beatty 2012a) and it is now considered the most common anatomical form (Rissetto *et al.* 2011). The aetiology of non-retroviral-induced lymphomas in cats, such as gastrointestinal lymphoma, is poorly understood (Louwerens *et al.* 2005) and a number of genetic, lifestyle and environmental factors have been suggested. The presence of chronic lymphoplasmacytic inflammation of the alimentary mucosa, such as that seen in IBD, has been suggested to be a precursor to the development of gastrointestinal lymphoma in

people, dogs and cats (Bertone *et al.* 2002, Louwerens *et al.* 2005, Washabau *et al.* 2010, Morrison 2012).

Cats exposed to ETS have been shown to be at an increased risk of developing lymphoma (Bertone *et al.* 2002). Owners of cats diagnosed with lymphoma were asked to recall exposure to ETS over the preceding seven years. Cats with any history of smoke exposure had a 2.4-fold greater risk of developing lymphoma compared to those without exposure. Cats presenting to the same hospital for renal failure during that same time period were used as controls. Cats with more than five years exposure to ETS had a 3.2-fold increased risk. Of the cats with lymphoma that were reportedly exposed to ETS, 56% had a gastrointestinal form of the disease, compared to 39% of lymphoma cases in reportedly unexposed cats. Although this study presented some interesting results, the study conclusions have been questioned due to the concern that a biomarker for ETS exposure was not used (Denson 2003).

Chapter 2:

Hair nicotine concentration measurement in cats and its relationship to owner-reported environmental tobacco smoke exposure

2.1 Aims

Hair nicotine concentration (HNC) has previously been investigated in humans, dogs and rats (Gerstenberg et al. 1995, Nafstad et al. 1995, Eliopoulos et al. 1996, Al-Delaimy et al. 2000, Al-Delaimy 2002, Al-Delaimy et al. 2002a, Okoli et al. 2007, Knottenbelt *et al.* 2012). The first aim of the study was to investigate whether this biomarker could be measured in cats.

The second aim was to investigate the association between measured HNC in cats and owner-reported exposure to ETS.

The third aim was to identify a cut-off HNC which had optimal specificity to allow differentiation between exposed and unexposed cats, enabling use of this biomarker in future studies investigating the effect of exposure on disease in cats.

2.2 Materials and Methods

The University of Glasgow's School of Veterinary Medicine's Ethics and Welfare Committee granted ethical approval prior to commencement of the studies.

2.2.1 Power and sample size calculation

A power calculation was performed using nQuery Advisor (Version 3.0. Statistical Solutions Ltd, Boston, Massachusetts; 1999). The power calculation was based on data obtained in a previous study measuring HNC in dogs (Knottenbelt *et al.* 2012).

A sample size of at least 35 cats unexposed to ETS and 35 cats exposed to ETS would give 80% power to detect an observed difference in mean HNC of 0.237ng/mg (unexposed) and 2.038ng/mg (exposed), assuming a common standard deviation of 2.5ng/mg, at $p<0.05$.

Based on this data, the aim was to recruit at least 35 unexposed and 35 exposed cats to investigate HNC in cats and its relationship to owner-reported ETS exposure.

2.2.2 Recruitment of cases

The study population was a convenience sample of cats presenting to the University of Glasgow's Small Animal Hospital, or a local first opinion practice, for a wide variety of clinical problems. Cats belonging to either staff or students at the Small Animal Hospital were also volunteered. Data collection began in July 2014.

Initially hair samples were collected from all cats whose owners gave consent for them to take part. Latterly cats were selected based on the owner-reported exposure to ETS; therefore ensuring that approximately even numbers of exposed and unexposed cats were recruited. Sufficient numbers of both unexposed and exposed cats were achieved by January 2015 and March 2015 respectively.

2.2.3 Exclusion criteria

Cats were excluded if they were owned for a period of less than three months, an insufficient hair sample was collected (less than 30mg), the owners documented exposure to electronic cigarettes only, or they reported washing of the cat's fur.

2.2.4 Owner questionnaire

Owners were asked to consent to collection of a hair sample from their cat and to completion of the study questionnaire. A questionnaire was designed for participating owners to complete (Figure 2.1). The questionnaire was modelled on that previously used in a similar study measuring hair nicotine concentration in dogs exposed to ETS (Knottenbelt *et al.* 2012).

Owners were asked to document their cat's exposure to ETS in the previous three months. A period of three months was considered a reasonable time period for owners to recall as accurately as possible sources of ETS exposure. Additionally, this was the same time period used for the aforementioned study in dogs (Knottenbelt *et al.* 2012).

If owners were unable to provide the number of tobacco products smoked per day in the home environment, the reason for this was noted.

The owners' postcode was requested and used to categorise the cats' environments as either urban or rural using a web-based service (Google Maps 2015). Towns and cities were classified as urban, whereas areas with low population density and abundant local countryside were classified as rural.

Figure 2.1: Owner questionnaire

University of Glasgow | School of Veterinary Medicine

All information provided in this questionnaire will be used for research purposes only and will remain confidential.

1. How old is your cat? _____ years/months (delete as appropriate)

2. Is your cat (please circle) male / female neutered / entire

3. What breed is your cat? _____

4. What colour is your cat's coat? _____

5. For approximately how long have you lived with your cat? _____

6. Does your cat go outside? Yes / No (delete as appropriate)

7. In the last three months has vomiting or diarrhoea been a particular concern with your cat?
Yes / No (delete as appropriate)

8. Do you ever wash / shampoo your cats fur? Yes / No (please circle)

9. Has your cat been in contact with cigarette or tobacco smoke in the last 3 months? Please circle:

Regularly **Occasionally** **Never**

10. If you do smoke, but only away from your cat (i.e. in your garden or through an open window) does this occur:

Regularly **Occasionally** **Never**

11. If you answered yes to question 9 or 10, approximately how many cigarettes or tobacco products are smoked each day at home?

_____ tobacco products

Thank you very much for taking the time to complete this questionnaire. Your assistance is greatly appreciated.

2.2.5 Categories of owner reported environmental tobacco smoke exposure

Cats were categorised into different exposure groups based upon the answers provided by the owner questionnaire.

Cats were categorised as unexposed if there was no reported exposure to ETS (their owners answered 'never' to both Q9 and Q10). They were defined as exposed if the owners documented either direct (Q9) or indirect (Q10) exposure to ETS.

Cats defined as being exposed to ETS were further subdivided:

Exposure group 1: These cats were reportedly only indirectly exposed to ETS. The owners of these cats answered 'never' for direct exposure (Q9) and either 'regularly' or 'occasionally' for indirect exposure (Q10).

Exposure group 2: These cats were reportedly directly exposed to ETS, but only on an occasional basis. These cats belonged to owners who answered 'occasionally' for direct exposure (Q9).

Exposure group 3: These cats were reportedly directly exposed on a regular basis. The owners of these cats answered 'regularly' for direct exposure (Q9).

2.2.6 Hair sampling and sample storage

For each cat, a 3x3cm area of hair was clipped from the ventral neck over the jugular vein. This could be from either the right or left side. Clippers were cleaned before and after use to prevent cross contamination. The neck was chosen as the site of sampling as it has previously been investigated in dogs when measuring HNC (Knottenbelt *et al.* 2012). This area is also commonly clipped for blood sample collection in feline patients, and therefore avoided the need to clip additional sites from the cat's coat. This site also has the benefit of having minimal impact on the appearance of the cat after collection.

Hair samples were stored in labelled, individually sealed paper envelopes, which were then placed inside sealed plastic wallets. All samples were kept in a smoke-free environment prior to analysis, to avoid environmental contamination.

The colour of the hair sample taken was noted, and would be used in the statistical analyses. Coat colour was later categorised as black, white or other.

2.2.7 Preparation of a calibration curve

Nicotine was obtained from Sigma Aldrich Dorset UK. Deuterated nicotine ($^2H_4$-nicotine; 1mg/ml in methanol) was obtained from CK gases, UK. In order to allow quantification of the concentration of an unknown analyte, in this case nicotine from the hair samples, samples containing known concentrations must first be prepared (Sargent 2013).

A 0.2 µg/ml solution of nicotine was prepared by serial dilution of pure nicotine liquid (chemical formula $C_{10}H_{14}N_2$). Similarly, a stock 2 ng/µl solution of deuterated nicotine ($^2H_4$-nicotine; chemical formula $C_{10}H_{10}D_4N_2$) was prepared. For the calibration standards, 100ng of $^2H_4$-nicotine was spiked into methanol with 0.1% formic acid in addition to 0, 4, 8, 16, 32, 64 and 128 ng amounts of nicotine. The contents of each of these calibration standards are shown in Table 2.1.

The samples were each prepared in 1.8ml glass autosampler vials (Kinesis, UK) and injected into the mass spectrometer. Using the output from the mass spectrometer, a calibration curve was created by plotting the ratio of the nicotine: $^2H_4$-nicotine signal as a function of the nicotine concentration of the standards.

The calibration curve established the relationship between the response of the instrument (which is the output of the measurement system) and the values of the nicotine calibration standards (which is the amount of analyte present). The response of the instrument in this study was described as the ratio of the absolute abundance of nicotine to the absolute abundance of the deuterated nicotine (internal standard).

An equation that best described this linear relationship was created by linear regression. This equation would enable calculation of the nicotine content of the hair samples. The coefficient of variation (R^2) was determined to assess the goodness of fit of the regression line. An R^2 of 1 would indicate that the regression line perfectly fits the data.

Table 2.1: Composition of solutions used to prepare a calibration curve

Calibration solution (ng/ml of nicotine)	Nicotine (µl of 0.2µg/ml solution)	$^{2}H_{4}$-nicotine (µl of 2ng/µl solution)	Methanol with 0.1% formic acid (µl)
0	0	50	950
4	20	50	930
8	40	50	910
16	80	50	870
32	160	50	790
64	320	50	630
128	640	50	310

2.2.8 Extraction of nicotine from cat hair

Each sample of cat hair for analysis required to be ~30mg in weight. The weight for each sample was accurately measured and recorded. The individual samples of weighed hair were placed into 4ml glass autosampler vials (Kinesis, UK). To each of these, 900µl (0.9 ml) of methanol with 0.1% formic acid was added in addition to 50µl of $^{2}H_{4}$-nicotine (2 ng/µl) solution as the internal standard. The vials were sonicated in a water bath for 30 minutes at room temperature. The supernatant (the extract solution) was filtered using Acrodisc syringe filters (Sigma-Aldrich, UK).

2.2.9 Measurement of hair nicotine concentration using hydrophilic interaction chromatography with mass spectrometry

The filtered supernatant from each hair sample was placed into individual 1.8ml glass autosampler vials (Kinesis, UK). The samples were automatically injected into an Orbitrap Exactive mass spectrometer (Thermoelectron, UK) (Figure 2.2). The mass spectrometer was operated in positive ion ESI mode with a needle voltage of 4.5kV, a heated capillary temperature of 275°C, a sheath gas flow of 50 arbitrary units and an auxillary gas flow of 17 arbitrary units. The instrument was operated at 50,000 resolution and scanned from 75 to 1000 amu.

Hydrophilic interaction chromatography was subsequently performed using a Surveyor hydrophilic interaction liquid chromatography (HPLC) pump (Figure 2.3) fitted with a ZIC® HILIC column (150mm x 4.6mm, 3.5µm particle size; Hichrom UK) (Figure 2.4). A gradient was used with a flow rate of 0.5ml per minute. Mobile phase A was 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. The gradient was: 60% B at 0 minutes to 20% B at 20 minutes followed by re-equilibration for 10 minutes. HPLC grade methanol and acetonitrile were obtained from Fisher Scientific, UK. HPLC grade water was prepared in the lab using a Milli Q purification system.

The calibration curve stock solutions (as shown in Table 2.1) were run in tandem with the hair sample extracts.

Figure 2.2: Orbitrap Exactive Mass Spectrometer

Figure 2.3: Surveyor High Performance Liquid Chromatography pump

Figure 2.4: ZIC® HLIC column

2.2.10 Calculation of the amount of nicotine present in the hair samples

The output from the mass spectrometer was presented as a chromatogram (Figure 2.5). This displayed the absolute abundance of nicotine and deuterated nicotine (the internal standard) in each sample. The ratio of these two values was input into the equation of the line from the calibration curve, allowing calculation of the amount of nicotine present in ng. This was converted to ng/mg by dividing the result by the exact weight of hair used.

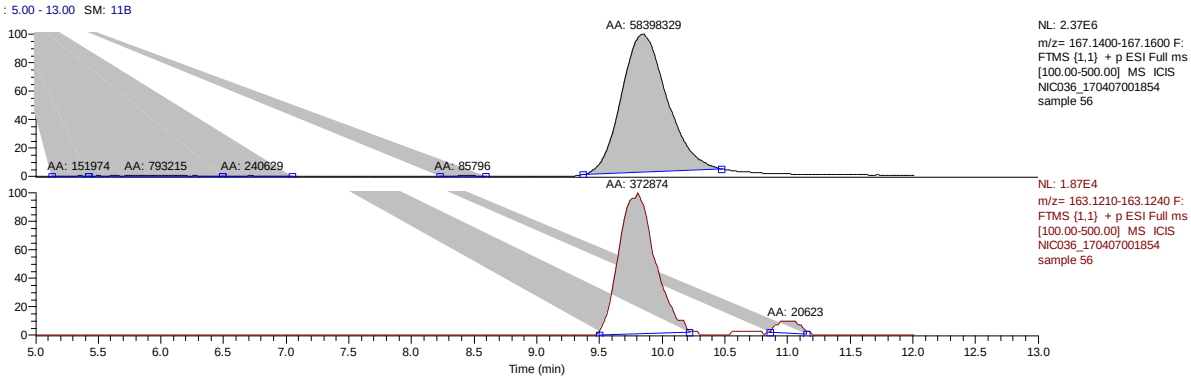
2.2.11 Intra-assay variability

One large sample of cat hair with an HNC in the upper quartile of the data set was selected. This hair sample was of sufficient size to allow preparation of five extract solutions, each requiring ~30mg of hair.

The extract solutions were then prepared and analysed as previously described. The standard deviation and coefficient of variation were calculated for these five repeated measurements to give some indication as to the repeatability of the assay at this high nicotine concentration.

Figure 2.5: Mass chromatogram

The x-axis represents time and the y-axis represents the signal intensity (relative abundance). The tallest peaks seen demonstrate the peaks for nicotine (bottom graph) and deuterated nicotine (internal standard; top graph). The area under each peak is the absolute abundance (AA) and is presented above each peak.



2.2.12 Statistical analysis

Statistical analysis was performed using both Minitab® Statistical Software (version 17.1.0) and SPSS Statistics (Version 22).

Variables were tested for normality using the Kolmogorov-Smirnov normality test. As the HNC data were not normally distributed, non-parametric tests were used. Mann-Whitney and Kruskal-Wallis tests were used for assessing associations between HNC and owner-reported ETS exposure. Non-parametric 1-sample sign tests were used to determine confidence intervals. For all analyses, a p value of <0.05 was considered to be significant. Descriptive statistics were performed for the variables of: length of ownership; coat colour; outdoors access; and the cats' local environment (urban or rural).

Receiver operator characteristics (ROC) curve analysis was performed in order to identify a cut-off HNC value to distinguish between exposed and unexposed cats. The area under the curve (AUC), sensitivity and specificity were all calculated. The aim was to determine a cut-off HNC with maximal specificity.

2.3 Results

2.3.1 Calibration curve

The calibration curve created is shown in Figure 2.6. The correlation coefficient (R^2) was 0.999, indicating an excellent goodness of fit of the regression line. The equation of the line was determined as: $y = 0.0117x – 0.0185$.

2.3.2 The study population

All owners approached agreed to take part in the study. Hair samples were obtained from 40 cats that were reportedly exposed to ETS and 40 cats that were reportedly not exposed. Three exposed cats were excluded from the final data analysis. One of these was excluded as the owner reported that the cat was exposed to electronic cigarettes only (HNC 0.071 ng/mg). Two other cats were excluded as their owners reported washing of the cats' coat (HNCs of 0.097 ng/mg and 1.217 ng/mg). Descriptive data for the final study population of 37 exposed and 40 unexposed cats are presented in Table 2.2.

2.3.3 Hair nicotine concentration of cats and its relationship to length of time lived with owners and coat colour

The HNC was successfully measured in all hair samples. The measured HNC from all 77 cats, including both those exposed and unexposed, ranged from 0 to 5.968 ng/mg (median 0.090 ng/mg).

The length of time that cats were reported to have lived with their owners did not appear to have an effect on the median HNC (Table 2.3).

Coat colour representation was similar between hair samples from cats reportedly unexposed and those exposed to ETS. Of the 37 exposed cats, five hair samples were black, 18 were white and 14 were other colours. Of the 40 unexposed cats, five hair samples were black, 14 were white and 21 were other colours. There was no obvious difference in the median HNC between the different hair colours of all 77 cats (Table 2.4).

Figure 2.6: Calibration curve

Calibration curve demonstrating the relationship between: (1) the response of the instrument (which is the output of the measurement system – y axis); and (2) the values of the nicotine calibration standards (which is the amount of analyte present – x axis). An equation that best described this linear relationship was created by linear regression (y=0.0117x – 0.0185).

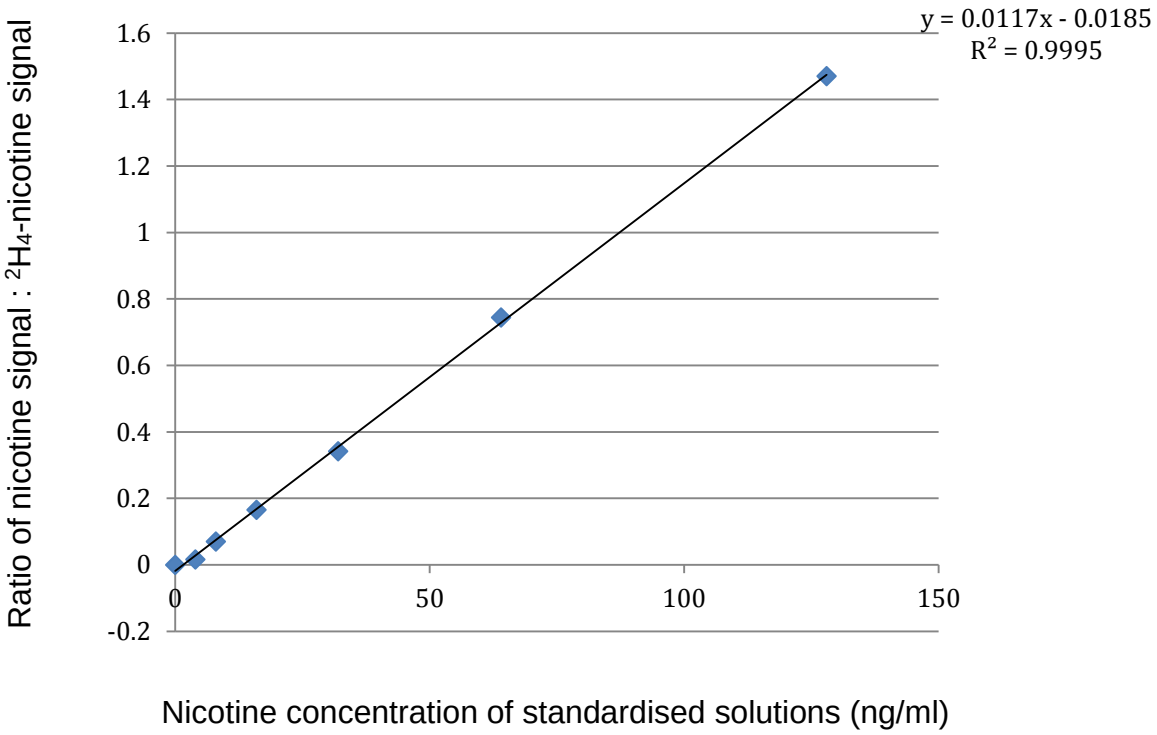


Table 2.2: Descriptive data for 37 reportedly exposed and 40 reportedly unexposed cats

Descriptive Category	Exposed Cats	Unexposed Cats
Number of cats	37	40
Number of male and female cats	23 male (62.2%), 14 female (37.8%)	17 male (42.5%), 23 female (57.5%)
Number of neutered cats	36 (97.3%)	39 (97.5%)
Median age of cat in months (range)	96 (6 to 204)	96 (8 to 216)
Breed (number of cats)	Domestic Shorthair (32), Maine Coon (2), Domestic Longhair (1), British Shorthair (1), American Domestic Shorthair (1)	Domestic Shorthair (33), Siamese (1), Maine Coon (1), Domestic Longhair (1), La Perma (1), Tiffany (1), Bengal (1), Siberian Forest Cat (1)
Median number of months the cat had lived with owner(s) (range)	70 (3 to 192)	72 (6 to 214)
Colour of cat hair sample (Number of cats)	Black (5), White (18), Other (14)	Black (5), White (14), Other (21)
Number of cats with access to outdoors	21 (56.8%)	28 (70%)
Number of cats from an urban environment	35 (94.6%)	29 (72.5%)
Number of cats from a rural environment	2 (5.4%)	13 (32.5%)

Table 2.3: Hair nicotine concentration and its relationship with how long cats had lived with their owners

Length of time lived with owner (years)	Number of cats in this category	Median HNC (ng/mg)	Range of HNC (ng/mg)	Confidence interval (95%)
0-4	28	0.081	0-1.911	0.062-0.096
4-8	22	0.093	0-2.330	0.068-0.398
8-12	15	0.090	0-5.968	0.067-0.467
>12	12	0.094	0-1.269	0.014-0.238

Table 2.4: Hair nicotine concentration in cats and its relationship to coat colour

Colour of hair sample	Number of cats	Median HNC (ng/mg)	Range of HNC (ng/mg)	Confidence interval (95%)
Black	10	0.093	0-1.269	0.044-0.535
White	32	0.094	0-2.962	0.077-0.269
Other	35	0.082	0-5.968	0.059-0.093

2.3.4 Hair nicotine concentration in cats reportedly unexposed to environmental tobacco smoke

The HNC of the 40 unexposed cats ranged from 0 - 0.269 ng/mg with a median of 0.064 ng/mg (CI_{95} 0.055-0.074). Ten (25%) of the unexposed cats had an HNC of 0 ng/mg.

2.3.4.1 Hair nicotine concentration in unexposed cats and its relationship to indoor and outdoor lifestyle

Unexposed cats with outdoor access had a similar median HNC to unexposed cats that were kept indoors only. The median HNC of unexposed cats with outdoor access was 0.065 ng/mg, with a range of 0 - 0.980 ng/mg (CI_{95}: 0.054-0.076). The median HNC of unexposed cats that were indoors only was 0.060 ng/mg with a range of 0 - 0.269 ng/mg (CI_{95}: 0.053-0.076).

2.3.4.2 Hair nicotine concentration in unexposed cats and its relationship to urban or rural environments

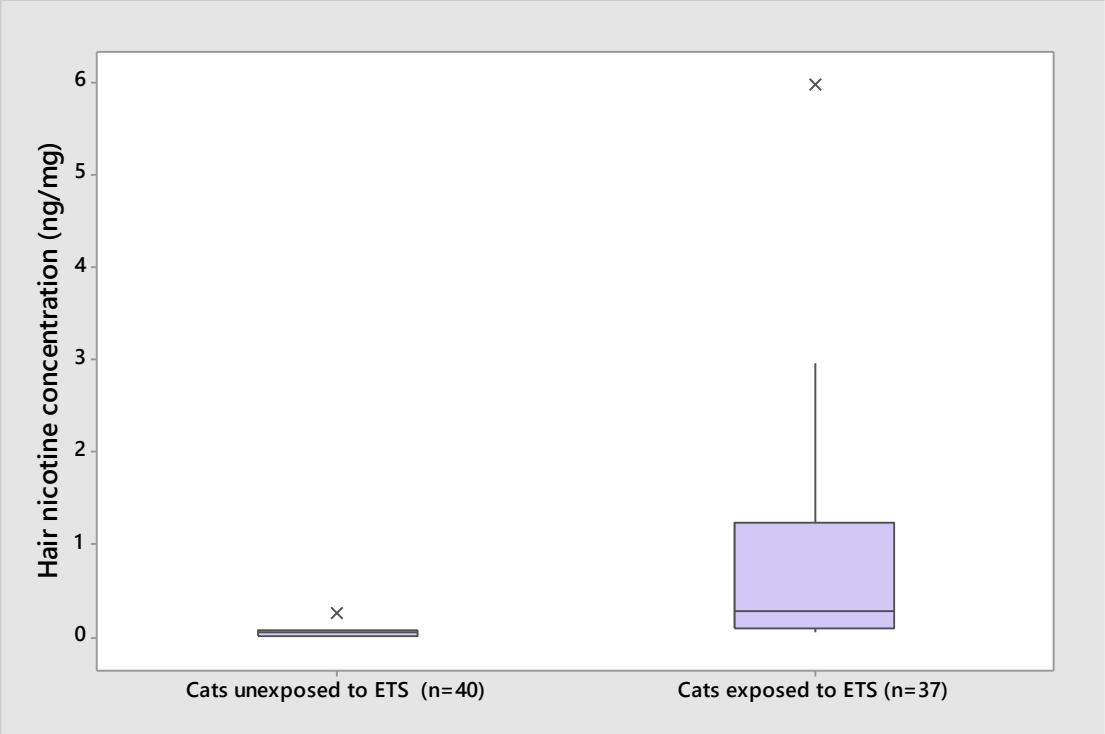
There did not appear to be an effect on the HNC of unexposed cats when comparing those living in an urban area to those living in a rural area. Unexposed cats living in an urban area had a median HNC of 0.065 ng/mg with a range of 0 - 0.269 ng/mg (CI_{95}: 0.056-0.076) and those from a rural area had a median HNC of 0.057 ng/mg with a range of 0 - 0.091 ng/mg (CI_{95}: 0-0.077).

2.3.5 Hair nicotine concentration in cats exposed to environmental tobacco smoke

The median HNC of the 37 reportedly exposed cats was 0.281 ng/mg with a range of 0.055 - 5.968 ng/mg (CI_{95}: 0.133-0.766). This was significantly higher than the median HNC of 0.064 ng/mg found for the unexposed cats ($p<0.001$) (Figure 2.7).

Figure 2.7: Box plot showing the hair nicotine concentrations of cats reported by their owners to be either unexposed or exposed to environmental tobacco smoke

Observations that are greater than 1.5 times the interquartile range (Q3-Q1) from the edge of the box are defined as outliers and are represented by crosses (×).



2.3.5.1 Hair nicotine concentration in exposed cats and its relationship to indoor and outdoor lifestyle

Similarly to unexposed cats, there did not appear to be an effect on outdoor access on the HNC of reportedly exposed cats. Exposed cat with outdoor access had a median HNC of 0.146 ng/mg with a range of 0.055 - 2.962 ng/mg (CI_{95}: 0.094 - 0.822). In comparison, exposed cats living indoors had a median HNC of 0.502 ng/mg with a range of 0.082 - 5.968 ng/mg (CI_{95}: 0.251-1.524). The difference between the median HNC of these two groups was not significant (p=0.085).

2.3.5.2 Hair nicotine concentration in exposed cats and its relationship to urban or rural environments

The number of exposed cats that lived in a rural environment was small (n=2). The HNC of these cats were 0.396 ng/mg and 0.784 ng/mg. These were not greatly different from the HNC of the 35 exposed cats that lived in an urban environment, which had a median HNC of 0.237 ng/mg, with a range of 0.055 - 5.968 ng/mg (CI_{95}: 0.113-0.741).

2.3.5.3 Hair nicotine concentration of exposed cats categorised according to owner reported exposure

The 37 exposed cats were categorised according to the level of owner-reported exposure to ETS: eight (21.6%) were categorised as exposure group 1; fourteen (37.8%) as exposure group 2; and fifteen (40.5%) as exposure group 3. There was a significant difference between the median HNCs of these groups (p<0.001) (Table 2.5 and Figure 2.8).

The exposed cats were re-categorised depending on the reported number of products smoked at home per day. Five (13.5%) owners of exposed cats were unable to provide a number of products, commenting that there was intermittent and variable exposure to ETS from neighbours and visitors. The median HNC for these five cats was 0.091 ng/mg with a range of 0.082 – 0.185 ng/mg (CI_{95}: 0.082-0.185).

Sixteen out of 37 exposed cats (43.2%) were reportedly exposed to 10 or fewer tobacco products per day. The median HNC for these sixteen cats was 0.259 ng/mg with a range of

0.055 – 2.130 ng/mg (CI_{95}: 0.104-0.591). Sixteen cats (43.2%) were reportedly exposed to greater than 10 products per day with a median HNC of 0.833 ng/mg and a range of 0.073 – 5.968 ng/mg (CI_{95}: 0.154-1.533). The difference between the medians of these three groups was significant (p=0.024) (Figure 2.9).

2.3.6 Calculation of a hair nicotine concentration cut-off to predict exposure to environmental tobacco smoke

The ROC curve created to assess hair nicotine concentration as a predictor of ETS exposure had an AUC of 0.945 (CI_{95}: 0.893 - 0.993) (Figure 2.10). The most discriminatory HNC based on this was 0.091 ng/mg, which had 87% sensitivity (CI_{95}: 72-100%) and 88% specificity (CI_{95} :73-100%). In order to maximise specificity, an HNC of 0.1 ng/mg was selected as a cut-off for detecting exposure to ETS. This cut-off had a higher specificity of 98% (CI_{95}: 83-100%) at the expense of a reduced sensitivity of 69% (CI_{95}: 54-84%).

2.3.7 Intra-assay variability

Five 30mg samples of hair from one cat were used to prepare five individual extracts. The HNCs ranged from 0.717 - 1.101 ng/mg with a mean of 0.883 ng/mg and a standard deviation of 0.151 ng/mg. The intra-assay coefficient of variation was 17%.

Table 2.5: Hair nicotine concentrations of exposed cats categorised into different exposure groups

Exposure group	Number of cats	Median HNC (ng/mg)	Range of HNC (ng/mg)	Confidence interval (95%)
1	8	0.092	0.073-0.146	0.081-0.098
2	14	0.170	0.055-2.130	0.099-0.548
3	15	1.223	0.131-5.968	0.521-1.886

Figure 2.8: Box plot showing the hair nicotine concentrations of cats exposed to environmental tobacco smoke categorised into different exposure groups on the basis of the magnitude of owner reported ETS exposure.

Observations that are at least 1.5 times the interquartile range (Q3-Q1) from the edge of the box are defined as outliers and are represented by stars (*).

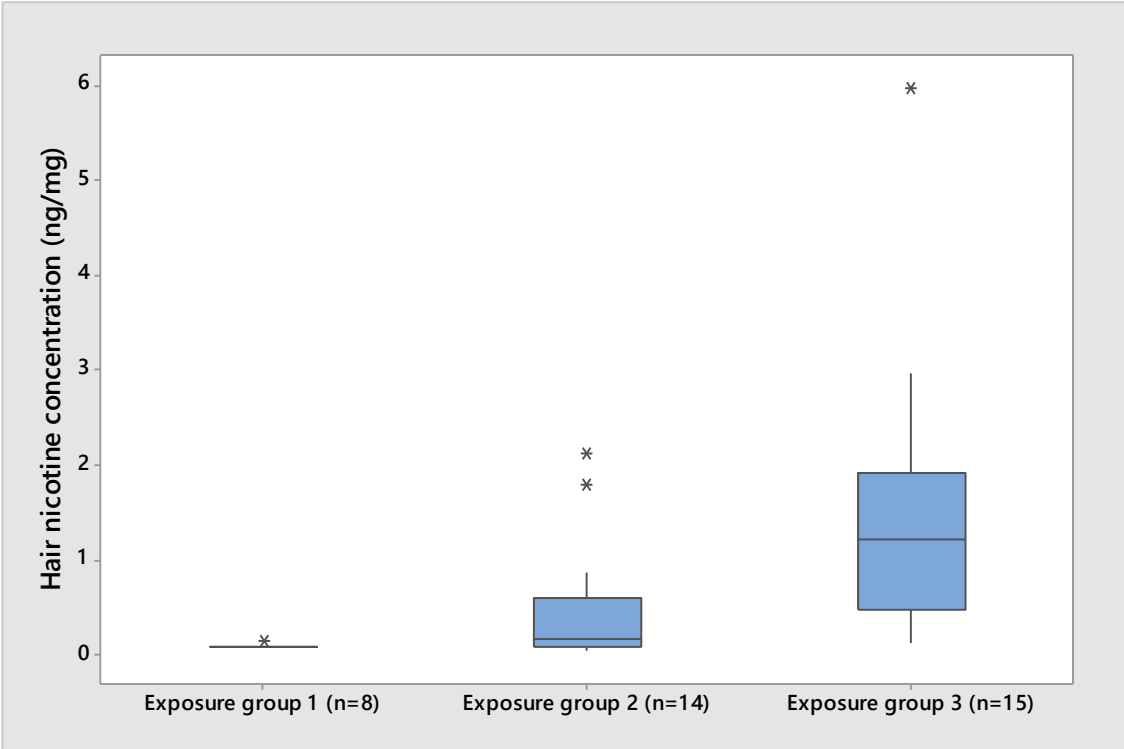


Figure 2.9: Box plot showing hair nicotine concentrations of cats exposed to environmental tobacco smoke categorised by the reported number of tobacco products smoked per day.

Observations that are at least 1.5 times the interquartile range (Q3-Q1) from the edge of the box are defined as outliers and are represented by stars (*).

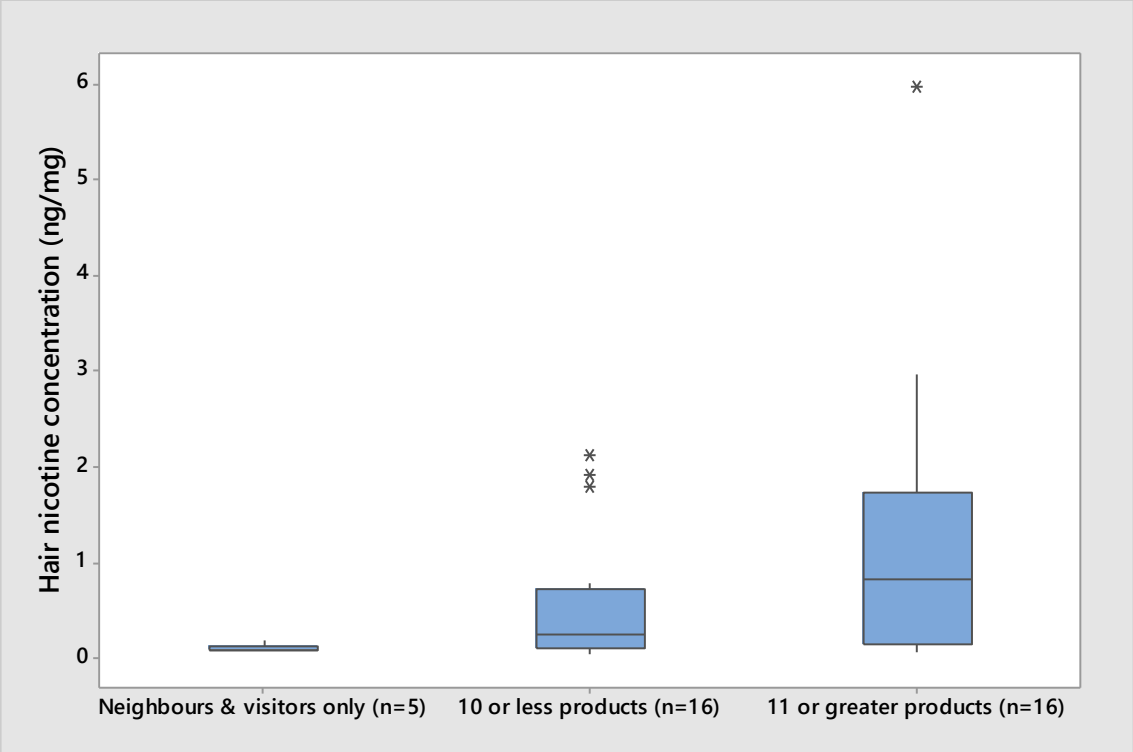
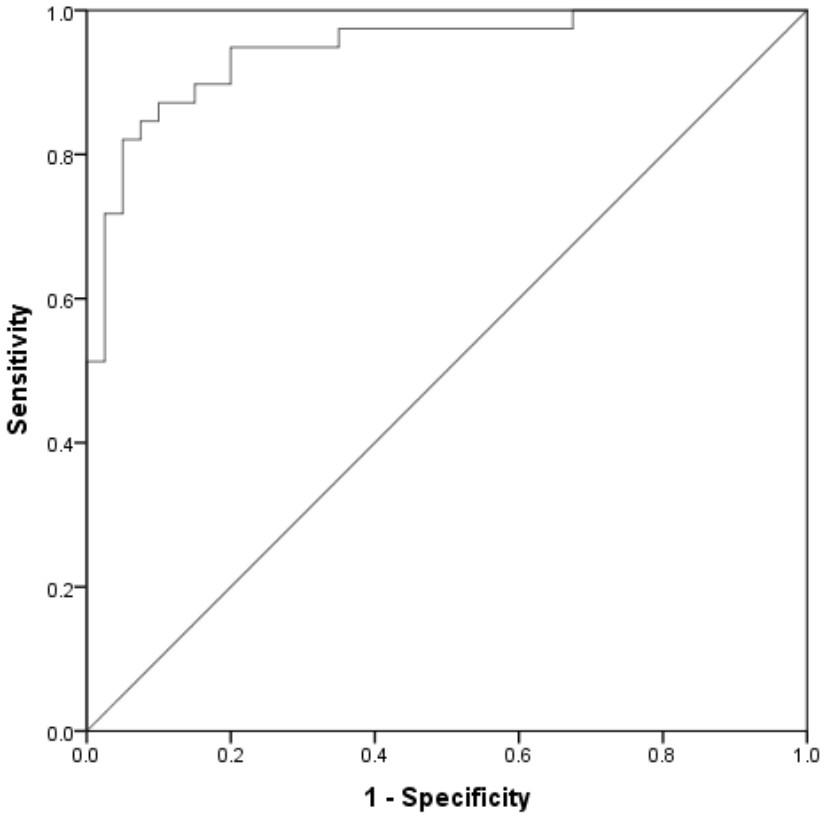


Figure 2.10: Receiver operating characteristics (ROC) curve to assess hair nicotine concentration in cats as a predictor of exposure to environmental tobacco smoke



2.4 Discussion

This study showed that HNC could be measured in cats using the technique described.

2.4.1 Hair nicotine concentration in reportedly exposed cats

Cats with owner-reported exposure to ETS had significantly higher HNC than those cats that were reportedly unexposed. Similar findings have been found in dogs (Knottenbelt *et al.* 2012) and in people, both children (Al-Delaimy *et al.* 2001) and adults (Kintz 1992). The latter study found that the HNC of known passive smokers was higher than that of people with no known ETS exposure. As would be expected, none of the reportedly exposed cats in this study had an HNC of 0 ng/mg.

2.4.2 Measurable hair nicotine concentration in reportedly unexposed cats

Only 25% of the reportedly unexposed cats had an HNC of 0 ng/mg. Interestingly this is comparable to a previous study in dogs assessing HNC as a biomarker for ETS: out of 15 reportedly unexposed dogs, only 4 (26.6%) had hair samples with no measurable nicotine content (Knottenbelt *et al.* 2012).

A similar discrepancy, with reported absence of exposure but positive detection of an ETS biomarker, has been found in a previous study evaluating urinary ETS biomarkers in cats (McNiel *et al.* 2007). That study looked at the presence of two metabolites of TSNAs in cat urine. The authors found that three cats reportedly not exposed to ETS had measurable concentrations of NNAL in their urine, a metabolite of NNK. Similarly, some people who do not consider themselves as being exposed to ETS have been shown to have measurable HNCs (Kintz 1992). Although a study by Kintz (1992) used a different technique to measure HNC (gas chromatography combined with mass spectrometry), the range of HNC found in reportedly unexposed people was similar to the cats used in this study (people: 0.06–0.33 ng/mg, cats: 0-0.269 ng/mg) and it should be noted that none had 0 ng/mg. This apparent discrepancy between measurable HNC and reported absence of exposure is most likely due to an underestimation of potential sources of exposure.

There are a variety of reasons why subjects in such studies, be it people reporting their own exposure to ETS or owners reporting on behalf of their pets, may underestimate exposure.

Firstly, the phenomenon of recall bias can lead to under-reporting of exposure to ETS (Jaakkola & Jaakkola 1997, Besaratinia & Pfeifer 2008). Recall bias occurs as subjects are being relied upon to honestly and accurately recall events in the past, and the involved parties may feel guilt due to the negative connotations associated with smoking. It has been suggested, however, that intentional under-reporting of exposure may be reduced when parents or pet owners are made aware that the purpose of the study is to assess a novel technique, such as the measurement of HNC in cats, and not to investigate their smoking habits (Nafstad *et al*. 1995). Owners of cats in this study were made aware that their cat's HNC would be measured, their awareness of this potentially confirmatory test might further reduce the likelihood of false reporting in this particular study.

A second and possibly more likely explanation for the discrepancy between owners reporting no exposure to ETS yet finding measurable HNC, is exposure of the cat to ETS from sources unknown to the owners. Such unknown sources of ETS exposure can occur when a cat is outside, particularly in heavily populated urban areas, or if the cat were to visit other people's homes, where smoking may take place. The data from this study did not however find any obvious effect of outdoor access or indeed urban dwelling on feline HNC in this population of cats.

A further source of ETS exposure that owners may have been unaware of is that of THS. As mentioned previously, THS refers to the ETS pollutants that remain in an indoor environment for months or even years after smoking cessation. Owners may be unaware of this ongoing exposure to ETS related chemicals, such as carcinogens and nicotine. This could potentially result in discrepancies between HNC and reported ETS exposure. The data from this study cannot account for any discrepancy created by cats and their owners having moved into properties previously inhabited by smokers. In addition, ETS is known to be able to move between adjacent properties (Spengler 1999) and therefore people and their pets may be unwittingly exposed from neighbouring houses. This could potentially account for some indoor cats from households that have never smoked being exposed to ETS, however as owners were not questioned regarding the smoking behaviours of neighbouring properties this cannot be assessed from the study data.

Overall the data from this study suggest that owner reporting underestimates the true level of exposure to ETS. This substantiates the importance of using a valid objective measure

such as HNC when investigating particular associations between ETS exposure and adverse health effects (Okoli *et al.* 2007).

2.4.3 Creating a cut-off hair nicotine concentration

The AUC of the ROC curve suggested excellent accuracy in distinguishing unexposed cats from exposed cats, based on their HNC (Figure 2.10). This would indicate that HNC is a suitable biomarker for detecting ETS exposure in cats. From the ROC curve, an HNC cut-off of 0.1 ng/mg in cats was selected for detecting ETS exposure. This had a high specificity of 98%. A high specificity was selected to minimise the number of false positives (i.e. incorrectly labelling unexposed cats as exposed). Similar studies in people have suggested varying cut-off HNCs for distinguishing smokers from non-smokers; including 2 ng/mg (Kintz 1992), 2.77 ng/mg (Kim *et al.* 2014), and between 2-5 ng/mg (Zahlsen and Nilsen 1994). Although these values are higher than the HNC cut-off of 0.1 ng/mg suggested by this study, these results cannot be directly compared as human studies include subjects who are active smokers and will be exposed to greater levels of nicotine than the passive smoking of pets.

2.4.4 Magnitude of environmental tobacco smoke exposure and the effect on hair nicotine concentration

As would be expected, cats whose owners reported greater levels of exposure to ETS, according to the frequency (occasional or regular) and proximity (direct or indirect) of exposure, had significantly higher HNCs than those with lower levels of exposure. Such findings have also been demonstrated in people (Kintz 1992, Okoli *et al.* 2007, Kim *et al.* 2014) and in dogs (Knottenbelt *et al.* 2012). Interestingly, with regards to the proximity of smoking in people, children of parents who smoke but only outside the house do not have lower HNC levels than those whose parents permit smoking inside the house (Al-Delaimy *et al.* 2001). This finding is in agreement with the recommendations of a similar study of families with infants, which found that smoking outside of the home, and therefore away from the child, did not completely protect the infant from ETS exposure (Matt *et al.* 2004). The HNC was also positively associated with the number of cigarettes reportedly smoked daily by the cats' owners, again as expected and similar to reports in humans (Nafstad *et al.* 1995, Al-Delaimy *et al.* 2000, Okoli *et al.* 2007, Tzatzarakis *et al.* 2011, Kim *et al.* 2014).

2.4.5 Hair nicotine concentration in cats compared to dogs

In a study in dogs using a similar technique to measure HNC, the HNC was lower than that of the cats in this study with comparable ETS exposure. For cats reportedly unexposed to ETS, the median HNC was 0.064 ng/mg. The equivalent result for dogs was lower at 0.010 ng/mg. Similarly for cats regularly exposed to ETS, the median HNC was 1.223 ng/mg whereas again for dogs it was lower at 0.910 ng/mg. The reasons for this are unknown but it could be that the HNC of cats is higher due to their regular and extensive self-grooming which may result in greater internalisation of nicotine, which is subsequently deposited into the hair shaft via the systemic circulation. An alternative hypothesis is that cats maintain a closer physical relationship with their owners, possibly resulting in a more direct exposure when compared to dogs. It is also possible that there are inter-species differences in hair composition that affect HNC, similar to the inter-racial differences documented in humans.

2.4.6 Factors affecting hair nicotine concentration

It was interesting to note that no significant difference was found between the median HNC of reportedly exposed cats kept exclusively indoors and those with outdoor access. It could be assumed that cats exposed in the home environment, which also spend time outside in fresh air, would have a relatively lower HNC, however this was not the case.

Coat colour of the cats in this study did not appear to have an obvious effect on HNC. It is thought that nicotine has a high affinity for the hair pigment melanin, which is incorporated into the cortex of darker hair (Al-Delaimy 2002). Some investigations into the effect of hair colour on HNC in other species have shown that HNC is higher in darker haired subjects (Mizuno *et al.* 1993, Gerstenberg *et al.* 1995, Uematsu *et al.* 1995). Results from other studies are conflicting and have not found hair colour to have an effect on HNC (Knight *et al.* 1996, Zahlsen *et al.* 1996). It should be noted that only a small number of black cats (five unexposed to ETS and five exposed) were included in this study, which could influence this finding.

No comment can be made about HNC following e-cigarette exposure or about the effect of washing cats on HNC as these were excluded from this study. With regards to hair washing, it has been shown in people that washing of hair will remove externally attached

nicotine (Al-Delaimy 2002) and it can be assumed that the same is true with other species' hair. To the authors knowledge there are no reports of a similar effect when hair becomes wet such as when exposed to rain, as could occur in cats with outdoor access. However, the cats' hair was sampled from the ventral neck, a site that would in theory likely be protected from exposure to rain.

2.4.7 Limitations

A limitation of this study was the incomplete assessment of instrument precision and repeatability. Intra-assay variability was assessed by calculating the co-efficient of variation from five samples of hair taken from one cat's sample. This was calculated at 17%, which is considered to be acceptable precision; for bioanalytical method validation this should not exceed 20% (United Nations Office on Drugs and Crime 2009). It could be argued that five times repeat analysis was insufficient and a greater number of repeats would improve the assessment of the precision of the technique. In addition, repeat analysis of samples with varying HNCs would have been interesting (for example from a low, mid and high range HNC sample). Assessment of the sensitivity of the assay at very low levels of ETS exposure was also not performed.

It should be noted that this study was performed using a particular instrument in a single laboratory, by a single investigator at one time point. Therefore the performance of the assay and optimal HNC cut-offs may vary when using different experimental set-ups.

Although it is useful to establish cut-off values for such a biomarker as HNC, some potential concerns regarding the reliability of these values should be mentioned. Firstly, the standard for comparison used in this study was information provided by owners, which cannot be confirmed or proven. Additionally, the population of cats investigated here was relatively small and larger scale studies may be needed to verify the reliability of the suggested cut-off values.

It is also possible that the small number of cats recruited, particularly in the subgroups, could have resulted in failure to detect significant differences. The categorisation of cats' local environment as urban or rural was subjective; therefore this could limit any conclusions drawn with regards to associations between this and HNC.

2.4.8 Conclusion

The results of this study indicate that HNC reflects exposure to ETS in pet cats, however exposure tends to be greater than that reported by owner questionnaires. Therefore measurement of HNC may be more reliable than the sole use of owner questionnaires, and is a suitable biomarker for ETS exposure in cats. The difference in HNC between the various exposure groups would suggest that owners that smoke might minimise feline HNC by avoiding direct contact with their cat whilst smoking and minimising the number of cigarette products smoked. A cut-off HNC of 0.1 ng/mg may be used to distinguish between cats unexposed to ETS and those significantly exposed, thus enabling HNC to be used as a biomarker of exposure to ETS in feline studies investigating the effect of exposure on disease development.

Chapter 3:

Hair nicotine concentration of cats with gastrointestinal lymphoma and unaffected control cases

3.1 Aims

Previous research has suggested that cats exposed to ETS are at an increased risk of developing several anatomical variants of lymphoma, including gastrointestinal lymphoma (Bertone *et al.* 2002). The principal aim of this study was to further investigate the association between exposure to ETS and gastrointestinal lymphoma, using HNC as a biomarker. A further aim was also to address some of the limitations from the previous study; namely calculation of the limit of detection (LOD) in addition to further assessment of both instrument precision (repeatability) and within assay variability.

3.2 Materials and Methods

The University of Glasgow's School of Veterinary Medicine's Ethics and Welfare Committee granted ethical approval prior to commencement of the studies.

3.2.1 Power and sample size calculation

A power calculation was performed using nQuery Advisor (Version 3.0. Statistical Solutions Ltd, Boston, Massachusetts; 1999). Calculations were based on the assumption that 10% of control cats may be exposed to ETS. A sample size including 38 cases and 38 controls would have 80% power to detect an odds ratio of 6 as significant at $p<0.05$.

3.2.2 Recruitment of cases and controls

The cases and controls were collected as part of a prospective, multi-centre case-control study. Five veterinary referral centres were involved in data collection: University of Glasgow's Small Animal Hospital, University of Bristol's Small Animal Hospital, University of Liverpool's Small Animal Teaching Hospital, Northwest Veterinary Specialists, and Cave Vet Specialists.

Cases and controls were recruited over a two-year period from March 2015 until March 2017. Owners provided consent for study inclusion and hair sample collection. Contributing institutions were asked to provide a control case for each gastrointestinal lymphoma case recruited.

Cats over 6 months of age that had lived with the same owner for at least 6 months were eligible for inclusion. Only newly diagnosed cases of gastrointestinal lymphoma were eligible. A brief clinical history was requested to allow documentation of clinical signs including vomiting and diarrhoea. Cats with no history of vomiting or diarrhoea within the previous six months and no clinical suspicion of lymphoma were recruited as control cases.

3.2.3 Diagnosis of gastrointestinal lymphoma

The attending clinician decided the method of diagnosis of gastrointestinal lymphoma. A diagnosis was confirmed either by: a) consistent cytology from fine needle aspirations of abnormal areas of the gastrointestinal tract or local lymph nodes, or b) consistent histopathology of the gastrointestinal tract or local lymph nodes. The method of diagnosis was recorded. Histopathological samples were collected endoscopically, via Tru Cut biopsy, surgically or during post mortem examination. In some cases additional testing with PCR for Antigen Receptor Rearrangement (PARR) or immunohistochemistry was performed, at the discretion of the attending clinician.

3.2.4 Hair samples

Hair samples were collected on a clinical suspicion of gastrointestinal lymphoma and subsequently excluded if this was not confirmed. Hair samples were only included if collected within 30 days of confirming the diagnosis. The hair samples were collected and stored as described in section 2.2.6.

3.2.5 Preparation of a calibration curve

Stock nicotine and deuterated nicotine solutions were prepared as previously described (section 2.2.7). For this study, additional nicotine solutions were prepared with lower concentrations of 1 and 2 ng/ml to assist in calculation of the lower limit of detection. The contents of these solutions are shown in Table 3.1.

The samples were prepared in 1.8ml glass autosampler vials and injected into the mass spectrometer. Using the output from the mass spectrometer, a calibration curve was created which plotted the ratio of the nicotine: $^{2}H_{4}$-nicotine signal as a function of the nicotine concentration of the standards (see section 2.2.7).

3.2.6 Limit of detection

In order to determine the LOD, the six lowest points on the calibration curve were used in order to prevent weighting towards the higher values. The standard deviation of the

response of the curve and the slope of the calibration curve were determined, allowing calculation of the LOD.

Table 3.1: Composition of solutions used to prepare a calibration curve

Calibration solution (ng/ml of nicotine)	Nicotine (µl of 0.2µg/ml solution)	$^{2}H_{4}$-nicotine (µl of 2ng/µl solution)	Methanol with 0.1% formic acid (µl)
0	0	50	950
1	5	50	945
2	10	50	940
4	20	50	930
8	40	50	910
16	80	50	870
32	160	50	790
64	320	50	630
128	640	50	310

3.2.7 Extraction of nicotine from cat hair

The cat hair samples were weighed and sonicated with methanol and the internal standard, as described in section 2.2.8.

3.2.8 Measurement of hair nicotine concentration using hydrophilic interaction chromatography with mass spectrometry

As described in section 2.2.9, extracts from the hair samples were placed into 1.8ml glass autosampler vials (Kinesis, UK) from which the samples were automatically injected into an Orbitrap Exactive mass spectrometer (Thermoelectron, UK) (Figure 3.1).

The mass spectrometer was operated in positive/negative ion switching ESI mode with a needle voltage of 4.5kV in positive mode and 4.0 kV in negative ion mode, a heated capillary temperature of 320°C, sheath gas flow of 50 arbitrary units and auxillary gas flow of 17 arbitrary units. The instrument was operated at 50,000 resolution and scanned from 75 to 1000 amu.

Hydrophilic interaction chromatography was performed using a Surveyor HPLC pump fitted with a Supelco F5 PFP column (150mm x 4.6mm, 3µm particle size; Sigma-Aldrich, UK). An isocratic method was used with a flow rate of 0.4ml per minute. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The system was run in isocratic mode with 55% A: 45% B with 10 minutes for each run.

3.2.9 Calculation of the amount of nicotine present in the hair samples

The output from the mass spectrometer was presented as a chromatogram (Figure 2.5). This displayed the absolute abundance of nicotine and deuterated nicotine (the internal standard) in each sample. The ratio of these two values was input into the equation of the line from the calibration curve, allowing calculation of the amount of nicotine present in ng. This was converted to ng/mg by dividing the result by the exact weight of hair used.

Figure 3.1: Orbitrap Exactive mass spectrometer and Surveyor HPLC pump.

The mass spectrometer is on the left of the image and the HPLC pump is on the right.

3.2.10 Intra-day instrument precision (repeatability)

To assess the precision of the instrument in this study, two extract solutions were subjected to repeat analysis. For these two samples, six times repeat analysis within the same run through the instrument was performed.

3.2.11 Intra-day within assay variability

Two large samples of cat hair (from two separate cats) were selected for assessment of within assay variability. One of these hair samples had a relatively low HNC and the other had a relatively high HNC. Each selected hair sample required to be of sufficient size to enable the preparation of ten extract solutions, each of which required ~30mg of hair. The extract solutions from these multiple hair samples were analysed as previously described in section 3.2.8.

3.2.12 Statistical analysis

Statistical analysis was performed using Minitab Statistical Software (Version 17.1.0). Variables were tested for normality using the Kolmogorov-Smirnov normality test. A p value of <0.05 was considered significant.

The HNC data for cases and controls were not normally distributed therefore non-parametric tests were used. Confidence intervals were determined using non-parametric 1-sample sign tests. Descriptive statistics were performed for HNC in relation to both the coat colour and age of all the cats.

Mann-Whitney and Kruskal-Wallis tests were used to assess associations between the median HNC of gastrointestinal lymphoma cases and controls, and between the median HNC of gastrointestinal lymphoma cases diagnosed by cytology or histopathology. The odds ratio for a cat being diagnosed with lymphoma if exposed to ETS was calculated and the 95% CI was reported. An association would be considered significant if the 95% CI did not contain 1.

Chi-square by association was used to assess relationships between rank ordered hair nicotine concentration divided into quartiles, and the proportion of cats diagnosed with gastrointestinal lymphoma. Chi-square by association was also used to assess the relationship between cats with an HNC ≥ 0.1ng/mg and the proportion of cats diagnosed with gastrointestinal lymphoma.

The data for the intra-day within assay variability and intra-day instrument precision (repeatability) were normally distributed. The mean, standard deviation and coefficient of variation were calculated. The modified Thomson Tau test was used to identify and exclude outliers.

3.3 Results

3.3.1 Calibration curve

The calibration curve created is shown in Figure 3.2. The correlation coefficient (R^2) was 0.996, indicating an excellent goodness of fit of the regression line. The equation of the line was determined as: $y = 0.0091x – 0.0058$.

3.3.2 Limit of detection

The LOD was calculated as 0.425 ng. As the hair nicotine levels were subsequently corrected for the weight of the hair samples used (~30mg per sample), the LOD could be alternatively considered as ~0.014 ng/mg. Three samples had an HNC of 0 ng/mg which was below the LOD. The remaining 64 samples all had an HNC above the LOD.

3.3.3 The study population

By the end of the data collection period, 35 cats with gastrointestinal lymphoma and 32 controls were eligible for inclusion. Descriptive data for the two groups are presented in Table 3.2.

3.3.3.1 Control cases

Control cases included 32 cats presenting for: routine health checks (11), oncological conditions other than gastrointestinal lymphoma (8), cardiac disease (5), endocrine disease (2), neurological conditions (2), haematological disease (1), hepatic disease (1), urinary disease (1) and nasal disease (1).

3.3.3.2 Clinical signs of gastrointestinal lymphoma cases

Twenty-eight lymphoma cases (80%) had a presenting complaint of vomiting and 15 (42.8%) had diarrhoea. Five cats (14.3%) had neither vomiting nor diarrhoea. These patients did however present with a history of weight loss (3 cats), inappetence (5 cats) or a palpable abdominal mass (4 cats).

Figure 3.2: Calibration curve.

Calibration curve demonstrating the relationship between: (1) the response of the instrument (which is the output of the measurement system – y axis) and; (2) the values of the nicotine calibration standards (which is the amount of analyte present – x axis). An equation that best described this linear relationship was created by linear regression (y = 0.0091x – 0.058).

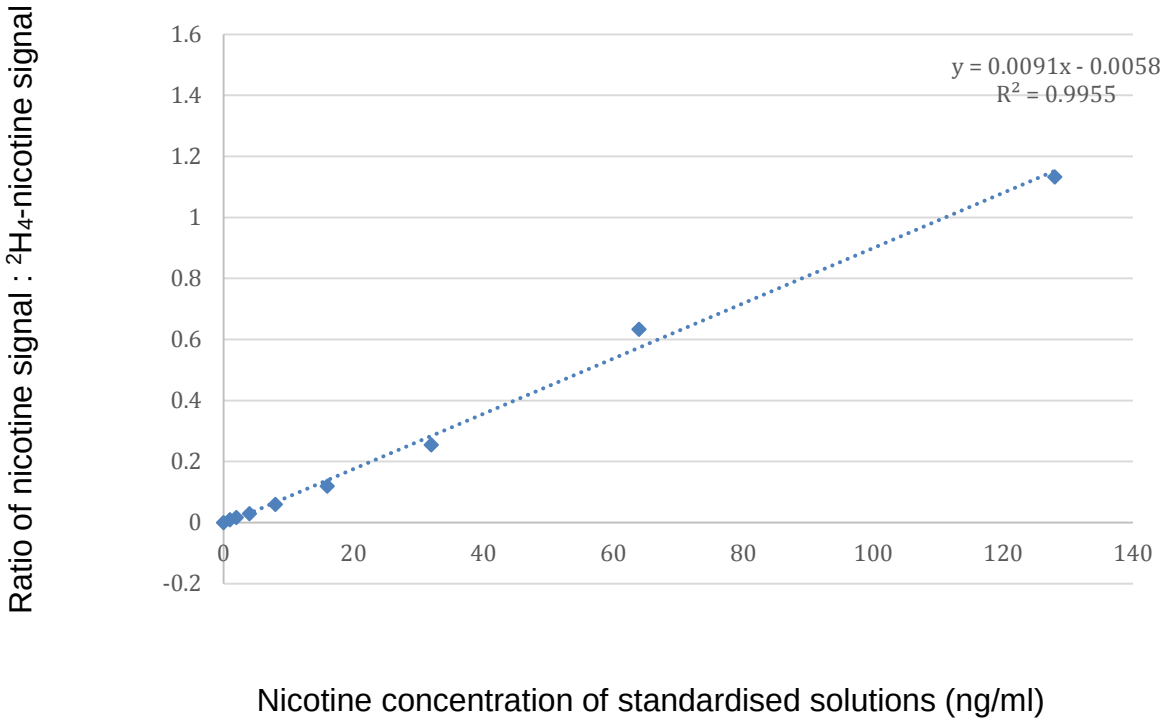


Table 3.2: Descriptive details of the gastrointestinal lymphoma cases and controls

Category	Lymphoma cases	Controls
Number of cats	35	32
Number of male and female cats	19 males (54.3%), 16 females (45.7%)	20 males (62.5%), 12 females (37.5%)
Number of neutered cats	35 (100%)	32 (100%)
Breeds of cat included (number)	Domestic Shorthair (25), British Shorthair (3), Siamese (2), Bengal (1), Birman (1), Ragdoll (1), Maine Coon (1), Russian Blue (1)	Domestic Shorthair (26), Domestic Longhair (3), British Shorthair (1), Ragdoll (1), La Perma (1)
Median age of cats in months (range)	122 (48-225)	108 (8-215)
Colour of cat hair sample (number of cats)	Black (5), White (9), other (21)	Black (5), White (10), other (17)
Number of cases recruited from each of the five centres	Centre A (24), Centre B (4), Centre C (3), Centre D (2), Centre E (2).	Centre A (27 cases), Centre B (1), Centre C (2), Centre D (1) and Centre E (1).

3.3.3.3 Diagnosis of gastrointestinal lymphoma cases

The median time from diagnosis of gastrointestinal lymphoma to hair sample collection was 6 days (range 1 to 30).

Cytology obtained via fine needle aspiration (FNA) was used to diagnose 21 (60%) gastrointestinal lymphoma cases. The site in which lymphoma was detected was: gastric wall (3 cases); the small intestinal wall (3 cases); abdominal lymph node (12 cases); and intestinal wall and mesenteric lymph node (3 cases). Additional testing with PARR was performed in one case to confirm the diagnosis.

The other 14 cases (40%) were diagnosed using histopathology. Samples were obtained by: exploratory laparotomy (11 cases); upper gastrointestinal endoscopy (1 case); ultrasound guided TruCut biopsy (1 case); or post mortem (1 case). A histopathological diagnosis of gastrointestinal lymphoma was reached by assessment of tissue from the stomach (2 cases), the intestine (7 cases), both the stomach and intestine (1 case) or abdominal lymph nodes (6 cases). Eight of these 14 cases had immunohistochemistry performed to confirm the diagnosis of lymphoma.

3.3.4 Hair nicotine concentrations of all 67 cats and its relationship to coat colour and age

The HNC was successfully measured in the samples from all 67 cats and ranged from 0 to 2.694 ng/mg (median 0.030 ng/mg; CI_{95}: to 0.028 to 0.035).

There was no obvious difference in median HNC between black (n=10), white (n=19) and other (n=38) colours of hair (Table 3.3). In addition, the age group of the cats did not appear to have an association with median HNC (Table 3.4).

Table 3.3: Colour of hair sample and hair nicotine concentration in all 67 cats

Colour of hair sample	Number of cats	Median HNC (ng/mg)	Range of HNC (ng/mg)	Confidence interval (95%)
Black	10	0.026	0-0.182	0.022-0.033
White	19	0.035	0.023-1.511	0.028-0.095
Other	38	0.029	0-2.270	0.027-0.040

Table 3.4: Hair nicotine concentration and age group

Age group (years)	Number of cats in this category	Median HNC (ng/mg)	Range of HNC (ng/mg)	Confidence interval (95%)
0-4	9	0.040	0-0.224	0.025-0.122
4-8	12	0.034	0.025-1.963	0.029-0.087
8-12	27	0.028	0-0.903	0.025-0.029
>12	19	0.033	0.023-2.269	0.027-0.072

3.3.5 Comparing hair nicotine concentration between gastrointestinal lymphoma cases and control cats

The difference in median HNC between lymphoma cases and controls was not significant (p=0.463) (Figure 3.3). The HNC of the lymphoma cats ranged from 0.021 to 2.269 ng/mg (median 0.030 ng/mg; CI_{95}: 0.028 to 0.045). The HNC of control cats ranged from 0 to 1.511 ng/mg (median 0.029 ng/mg; CI_{95}: 0.027 to 0.038).

When the HNC of all 67 cats was rank ordered and divided into quartiles, there was no significant difference in the proportion of lymphoma cases or controls within these groups (p=0.631). The lowest quartile (≤0.025 ng/mg) included 8 lymphoma cases and 9 controls; the second quartile (> 0.025 to ≤0.030 ng/mg) included 9 lymphoma cases and 8 controls; the third quartile (>0.030 to ≤0.063 ng/mg) included 7 lymphoma cases and 9 controls; and the highest quartile (>0.063 to ≤2.269 ng/mg) included 11 lymphoma cases and 6 controls (Figure 3.4).

Figure 3.3: Box plot showing the hair nicotine concentration of cats with gastrointestinal lymphoma compared to control cases.

Observations that are at least 1.5 times the interquartile range (Q3-Q1) from the edge of the box are defined as outliers and represented by stars (*).

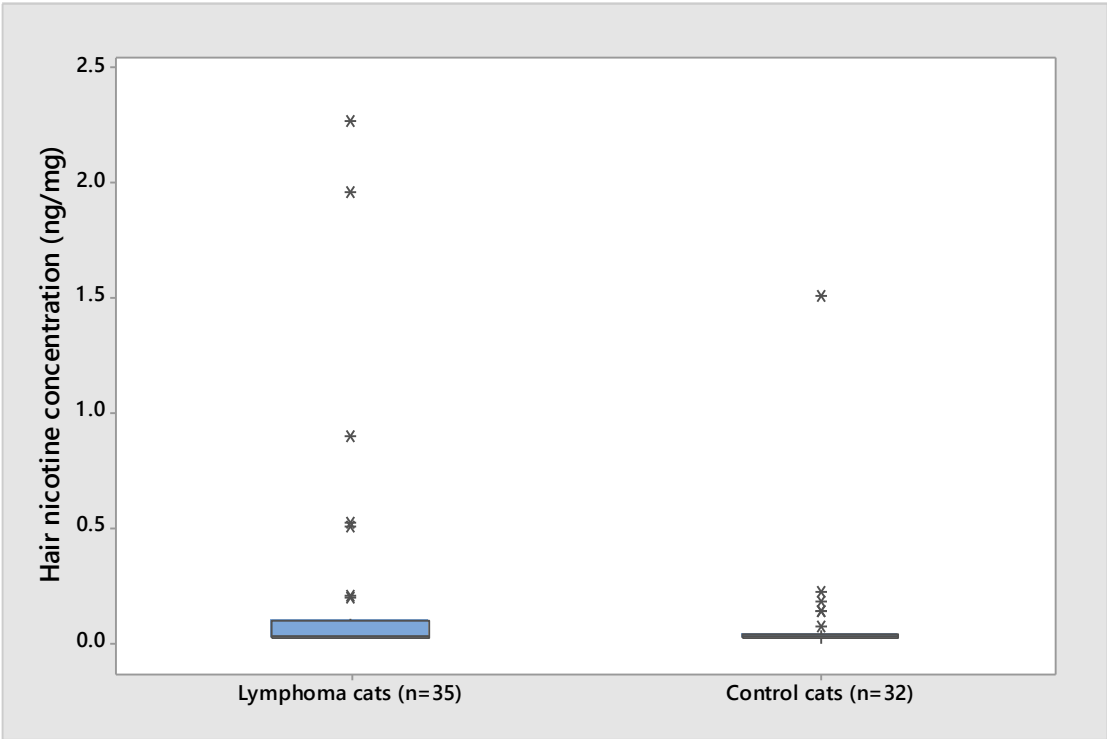
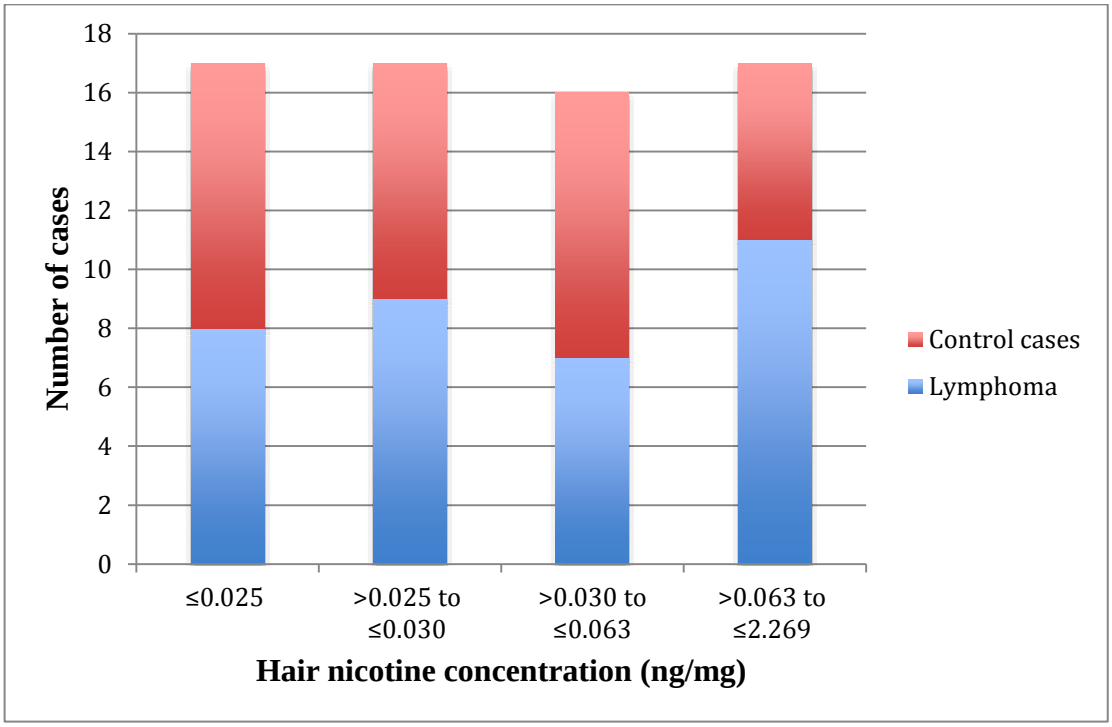


Figure 3.4: Stacked bar chart demonstrating the number of lymphoma and control cases in each quartile of hair nicotine concentration



3.3.6 Hair nicotine concentration ≥ 0.1ng/mg as an indicator of presumed exposure to environmental tobacco smoke

The number of cats with an HNC ≥0.1ng/mg, and therefore considered exposed to environmental tobacco smoke, was similar between the lymphoma cases and the control cats. Eight out of 35 lymphoma cases (22.9%) had an HNC ≥ 0.1ng/mg and five out of 32 control cats (15.6%) had an HNC ≥ 0.1ng/mg. The median HNC of these presumably exposed cats was not significantly different between lymphoma cases (median 0.514 ng/mg, range to 0.108 to 2.269; CI_{95}: 0.193 to 1.980) and controls (median 0.182 ng/mg, range 0.136 to 1.511; CI_{95}: 0.136 to 1.511) (p=0.272).

The odds ratio for a cat being diagnosed with lymphoma given exposure to ETS (HNC ≥0.1ng/mg) was calculated as 1.6 (95% CI: 0.46 to 5.52) (p=0.46).

3.3.7 Comparing the hair nicotine concentration of cats with gastrointestinal lymphoma diagnosed by cytology or histopathology

The median HNC of cats with gastrointestinal lymphoma diagnosed with cytology was 0.028 ng/mg (range 0.023 to 2.269 ng/mg; CI_{95}: 0.026 to 0.064). The median HNC of those diagnosed by histopathology was 0.033 ng/mg (range to 0.021 to 0.903; CI_{95}: 0.027 to 0.094). The difference between these two groups was not significant (p=0.674).

3.3.8 Intra-day instrument precision (repeatability)

The mean HNCs for six times repeat analyses of the two extracts were 0.028 ng/mg and 0.022 ng/mg, with a coefficient of variation of 6.1% and 3.3%, respectively.

3.3.9 Intra-day within assay variability

Two extracts from the cat hair sample with a relatively low HNC failed to run due to machine error. The HNC of the remaining eight repeats ranged from 0.028 to 0.034 ng/mg, with a mean of 0.031 ng/mg and no outliers identified. The standard deviation was 0.002 ng/mg with a coefficient of variation of 5.9%.

All ten extracts from the cat hair sample with a relatively high HNC were run successfully. One outlier was identified and removed from the data set. The HNC of the remaining nine repeats ranged from 1.80 to 2.683 ng/mg, with a mean of 2.229 ng/mg. The standard deviation was 0.364 ng/mg with a coefficient of variation of 16.3%.

3.4 Discussion

3.4.1 Hair nicotine concentration

Hair nicotine concentrations were successfully measured in all 67 samples. It is interesting to note that out of all 67 cases in this study, only 13 (19.4%) were classified as exposed, which is a relatively low proportion. In addition, the median HNC of all 67 cats was 0.030 ng/mg (range: 0 – 2.694 ng/mg; CI_{95}: 0.028 to 0.035), which is considerably lower than the reported median HNC of 0.281 ng/mg (range 0.055 – 5.968; CI_{95}: 0.133-0.766) of 37 exposed cats in the previous study (Smith *et al*. 2017). It is also lower than the reported median HNC of 0.064 ng/mg (range 0 – 0.269; 95% CI: 0.055 – 0.074) in the 40 unexposed cats of that study. This might suggest that even the exposed cats in the current study population had a relatively low level of exposure to ETS, making associations with smoking related disease less likely to be identified. An alternative explanation, however, is that although the methodology was similar between these two studies, due to technical difficulties it was not possible to use the same instrument and column as was used in the first study. Although unlikely, this could possibly have reduced the proportion of hair nicotine detected in the samples in this study.

3.4.2 Hair nicotine concentration of gastrointestinal lymphoma cases and controls

The proportion of cats with an HNC ≥ 0.1ng/mg, and therefore considered as being exposed to ETS on the basis of results from the previous study (Smith *et al.* 2017), was similar between the gastrointestinal lymphoma and control cases. Furthermore, no significant difference was found between the median HNC of the gastrointestinal lymphoma cases and that of the control cats, nor between the median HNC of the exposed lymphoma cases and that of the exposed control cases. As HNC is an accepted biomarker of exposure to ETS in people, dogs and cats (Al-Delaimy 2002, Al-Delaimy *et al.* 2002a, Okoli *et al.* 2007, Knottenbelt *et al.* 2012, Smith *et al.* 2017) and increased HNC concentrations have been detected in cats with greater ETS exposure (Smith *et al.* 2017), these findings suggest therefore that there was no difference in exposure to ETS between cats with or without gastrointestinal lymphoma. The odds ratio for a cat having lymphoma given ETS exposure was estimated to be 1.6, suggesting that an exposed cat was 1.6 times more likely to have lymphoma than an unexposed cat, however as the 95% confidence interval spans 1 and the p value was >0.05, this may be a chance observation and many

more cats would be needed to narrow the confidence interval and determine if this effect is real.

This contradicts the findings of Bertone *et al.* (2002), who identified an association between exposure to ETS and the development of lymphoma in cats, and a relative risk of developing malignant lymphoma of 2.4 in exposed cats. There are many possible reasons for the differing findings between these studies including: the anatomical variants of lymphoma being investigated; methodology; case numbers and statistical power; differing smoking behaviours between the study periods; as well as geographical and demographic differences.

The study by Bertone *et al.* (2002) included not only cats affected by gastrointestinal lymphoma but also several other anatomical variants. Although the majority of cases were reported to be of gastrointestinal and nasal origin, the actual number of gastrointestinal cases was not reported. As the current study focused solely on gastrointestinal lymphoma, conclusions cannot be drawn on possible associations between ETS exposure and other anatomical variants of lymphoma.

The differences in methodology between the two studies might also have affected the study conclusions. Firstly, questionnaire-based studies such as that performed by Bertone *et al.* (2002) are at risk of recall bias and likely do not correspond to the actual exposure of the cat. For example, if a cat considered as exposed to ETS in the home environment spends a significant amount of time outside or in a distant part of the house, it's relative ETS exposure would be lower than a cat that maintains more direct contact whilst the owner is smoking, in this respect HNC measurement likely reflects the actual ETS exposure more accurately. Secondly, HNC in cats likely reflects ETS exposure over a different time frame than that documented by the owner questionnaire used in the Bertone *et al.* study (2002). Hair nicotine concentration in cats likely reflects exposure to ETS over the proceeding few months as, in this species, hairs are gradually replaced following the quiescent phase of telogen, which is of several months duration (Baker 1974). In contrast, respondents in the study by Bertone *et al.* (2002) were asked to recall ETS exposure over the past five years and beyond, therefore if a cat were exposed to ETS for many years, but this exposure ceased 6 months prior to the study, HNC results would suggest that the cat had a low level of ETS exposure whereas the aforementioned epidemiological study of Bertone *et al* (2002) would lead to a classification of high ETS exposure.

The small number of cases recruited in this study may have prevented an association between lymphoma and HNC from being found. In contrast, the study by Bertone *et al* (2002) included a much larger group of cats; its retrospective methodology achieved final case numbers of 80 cats with lymphoma and 114 control cases.

The use of electronic cigarettes, also known as e-cigarettes have become increasingly popular in recent times and hence would not have been a confounding factor in the Bertone *et al* (2002*)* study, as this was performed more than a decade earlier. The cats investigated by Bertone *et al.* (2002) were diagnosed with lymphoma between the years 1993 and 2000, whereas the current study was performed between 2015 and 2017. These battery-powered devices do not involve tobacco combustion but instead aerosolise nicotine prior to inhalation (Callahan-Lyon 2014). The possibility that some cats in the current study were exposed to e-cigarettes, and therefore not exposed to traditional tobacco smoke carcinogens, unfortunately cannot be excluded. This could affect the relationship between HNC and the concentration of carcinogens on the cats' coats, which therefore could also affect the study conclusion regarding a lack of association with gastrointestinal lymphoma.

Another possible reason for the difference in findings is that the current study was performed in the United Kingdom whereas the investigation by Bertone *et al* (2002) was performed in the United States. There may possibly also have been genetic or other environmental differences between these two study populations.

3.4.3 Risk of lymphoma at different levels of hair nicotine concentration

Although no association was found between HNC and gastrointestinal lymphoma in this study, it is particularly interesting to note that the group of cats with the highest HNC was composed of nearly two-thirds gastrointestinal lymphoma cases. Bertone *et al* (2002) identified that the risk of lymphoma increased with the quantity of exposure, however the proportion of affected and unaffected cats in each quartile of HNC was not significantly different in this study. The reasons for this difference are similar to those described above. A further study with much larger numbers of cats, and therefore more statistical power, would help confirm whether or not cats exposed to higher levels of ETS have an increased risk of gastrointestinal lymphoma.

3.4.4 Method of diagnosis of gastrointestinal lymphoma

The gastrointestinal lymphoma cases in this study were diagnosed by either aspiration cytology (60%) or histopathology (40%). Cases diagnosed by aspiration cytology were considered eligible for study inclusion, as this is a common and accepted diagnostic technique, especially for intermediate and high-grade alimentary lymphoma (Barrs & Beatty 2012). In addition it is has the advantages of being minimally invasive and a rapid diagnostic method (Gieger 2011). As part of the study data analysis, the HNC of cats with lymphoma diagnosed by cytology were compared to those diagnosed by histopathology. The group numbers were small, therefore although no significant difference was detected with regards to ETS exposure between those cats diagnosed by cytology and those diagnosed by histopathology, the lack of power means that firm conclusions cannot be made.

3.4.5 Hair nicotine concentration and coat colour

Nicotine has a high affinity for melanin (Yerger & Malone 2006) and some studies in both people and rats have found a higher HNC in darker hair (Mizuno *et al.* 1993, Gerstenberg *et al.* 1995, Uematsu *et al.* 1995). The current study showed no associations between the hair colour and HNC, this is similar to the findings of the first study. The failure to find an association may be due to the relatively small numbers in each group of cats and the effect of several other confounding variables, or because there is no effect of coat colour on HNC in cats.

3.4.6 Repeat analyses

Hair nicotine concentrations were successfully measured in all 67 samples. To attempt to address some of the limitations of the first study, additional repeat analyses were performed. This allowed assessment of intra-day within assay variability, which demonstrated an acceptable coefficient of variation for both low and high level HNCs. A good coefficient of variation for intra-day instrument precision (repeatability) was found at a low HNC, however similar repeat analyses of extracts with a high HNC were not performed.

3.4.7 Limitations

To reduce the likelihood of inadvertently including a cat with undiagnosed gastrointestinal lymphoma in the control group, any cats with a clinical suspicion of gastrointestinal lymphoma or with vomiting or diarrhoea in the six months prior to examination were excluded. Recent weight loss in control cats was not documented. This could be considered as a limitation of this study as weight loss can be a non-specific sign of gastrointestinal lymphoma. It was noted that 14.3% of cats diagnosed with gastrointestinal lymphoma had no reported vomiting or diarrhoea; therefore it is possible that some control cats were misclassified. However, it is likely that the prevalence of gastrointestinal lymphoma is low in the healthy cat population and so this is unlikely to have significantly affected the results. The cases of gastrointestinal lymphoma included in this study were not further sub categorised into the various histological subtypes. Therefore no comment can be made with regards to potential associations between these subtypes and HNC in cats.

The technique used was similar to that described for the first study of this thesis (described in Chapter 2.2) and both studies were performed in the same laboratory. However, technical difficulties with the original instrument at the time of performing the second study required that an alternative instrument and column be used. Therefore there is the potential for inter-assay variability between these two studies. An HNC of 0.1ng/mg was used in the second study as the cut-off to discriminate between unexposed and exposed cats on the basis of the results from the first study. As the instrument and column used in this study were different, this cut-off may be inappropriate. Despite this, the median HNC of the cats in both the lymphoma and control groups were not significantly different, nor were the proportion of cats in each quartile of HNC different, therefore it is unlikely that using this cut-off has affected the conclusions of this study.

Cats that had lived with their current owner for at least 6 months were considered eligible for inclusion in this study. As such it is possible that some cats had lived in more than one household in their lifetime, with varying exposure to ETS. Therefore it is should be considered that the HNC at the time of diagnosis of lymphoma in this study may not be reflective of a cat's long term exposure to ETS and it's associated carcinogens.

The small number of cases recruited may have prevented an association between lymphoma and HNC being found in this study. The assumption in the power calculation that 10% of control cats would be exposed to ETS was nearly correct (actually 15.6%), however the magnitude of the effect of ETS on the development of lymphoma (the odds ratio) was much smaller than 6 (estimated at 1.6) and this study was underpowered to detect an effect of this magnitude.

3.4.8 Conclusion

In conclusion, no significant difference in HNC was found between the cats with gastrointestinal lymphoma and the control cases in this study population. As HNC is a biomarker for ETS exposure, this would suggest that these two groups were similarly exposed over the period measurable by HNC. Therefore an association between ETS exposure and gastrointestinal lymphoma has not been found in this study, which may be attributable to the study being underpowered or the aforementioned limitations. An odds ratio of 1.6 suggests that ETS exposed cats may be 1.6 times more likely to be diagnosed with gastrointestinal lymphoma, however due to the small number of cats in this study the confidence interval was wide and this may just be a chance finding. It is not yet understood how the level of nicotine in hair relates to the biologically effective dose of ETS (Al-Delaimy 2002). In addition, the number of interacting factors and the protracted time period over which mutations may accumulate makes it difficult to identify individual risk factors and associations, therefore future studies with much larger case numbers would be required to identify such an association if it exists.

4 Conclusion

The studies performed in this thesis have confirmed that constituents of tobacco smoke are present in samples of hair collected from pet cats exposed to ETS. As would be expected, the HNC was significantly higher in reportedly exposed cats than in reportedly unexposed cats. Providing outdoor access to an exposed cat does not appear to affect HNC, however it appears that smoking away from the cat and also minimising the number of cigarettes smoked in their environment may significantly reduce feline HNC. An HNC of 0.1 ng/mg was found to be a useful cut-off to distinguish between exposed and unexposed cats in the first study, and was utilised in the second study to investigate the association between exposure to ETS and the development of gastrointestinal lymphoma.

Using HNC as a biomarker for exposure to ETS, it was shown that there was no significant difference in median HNC between cats with gastrointestinal lymphoma and control cases. This would suggest that these two groups of cats were similarly exposed to ETS. Although no association was found between HNC and gastrointestinal lymphoma, it is particularly interesting to note that the group of cats with the highest HNC was comprised of nearly two-thirds gastrointestinal lymphoma cases. This suggests that there may be a higher risk in the cats with the highest ETS exposure. As this study was underpowered, a further study with larger numbers of cats, and therefore increased statistical power, would help in further investigating whether cats exposed to ETS have a higher risk of gastrointestinal lymphoma.

5 List of references

Al-Delaimy, W. K., Crane, J. & Woodward, A. (2000) Questionnaire and hair measurement of exposure to tobacco smoke. *Journal of Exposure Analysis and Environmental Epidemiology* 10, 378-384

Al-Delaimy, W. K. (2002). Hair as a biomarker for exposure to tobacco smoke. *Tobacco Control* 11, 176-182

Al-Delaimy, W. K., Crane, J. & Woodward, A. (2002a). Is the hair nicotine level a more accurate biomarker of environmental tobacco smoke exposure than urine cotinine? *Journal of Epidemiology and Community Health* 56, 66-71

Al-Delaimy, W. K., Mahoney, G. N., Speizer, F. E., *et al.* (2002b). Toenail nicotine levels as a biomarker of tobacco smoke exposure. *Cancer Epidemiology,* Biomarkers *and Prevention* 11, 1400-1404

American Veterinary Medicine Association. One health – it's all connected. Available at: https:// www.avma.org/KB/Resources/Reference/Pages/One-Health94.aspx Accessed July 14, 2016.

Antunes, M. V., da Silva, C. F. R., Finger, M. A., *et al.* (2015). Correlation analysis between cotinine hair concentrations from active smokers and nicotine intake and dependence. *Therapeutic Drug Monitoring* 37(3), 405-407

Apelberg, B. J., Hepp, L. M., Avila-Tang, E., *et al.* (2012). Racial differences in hair nicotine concentrations among smokers. *Nicotine and Tobacco Research* 14(8), 933-941

Avila-Tang, E., Al-Delaimy, W. K., Ashley, D. L., *et al.* (2013). Assessing secondhand smoke using biological markers. *Tobacco Control* 22, 164-171

Baker, K. P. (1974). Hair growth and replacement in the cat. *British Veterinary Journal* 130, 327-328

Barrs, V. & Beatty, J. (2012a). Feline alimentary lymphoma 1. Classification, risk factors, clinical signs and non-invasive diagnostics. *Journal of Feline Medicine and Surgery* 14, 182-190

Barrs, V. & Beatty, J. (2012b). Feline alimentary lymphoma: 2. Further diagnostics, therapy and prognosis. *Journal of Feline Medicine and Surgery* 14, 191-201

Bawazeer, S., Watson, D. G. & Knottenbelt, C. (2012). Determination of nicotine exposure in dogs subjected to passive smoking using methanol extraction of hair followed by hydrophilic interaction chromatography in combination with Fourier transform mass spectrometry. *Talanta* 88, 408-411

Benowitz N.L., Hukkanen J. & Jacob, P. (2009). Nicotine chemistry, metabolism, kinetics and biomarkers. *Handbook of Experimental Pharmacology* 192, 29-60
Benowitz, N. L. (2010) Nicotine addiction. *The New England Journal of Medicine* 362(24), 2295-2303

Bertone, E. R., Snyder, L. A. & Moore, S. A. (2002) Environmental tobacco smoke and risk of malignant lymphoma in pet cats. *American Journal of Epidemiology* 156 (3), 268-273

Bertone-Johnson, E. R., Procter-Gray, E., Gollenberg, A. L., *et al.* (2008) Environmental tobacco smoke and canine urinary cotinine level. *Environmental Research* 106, 361-364

Besaratinia, A. & Pfeifer, G. P. (2008) Second hand smoke and human lung cancer. *Lancet Oncology* 9, 657-66

Callahan-Lyon, P. (2014) Electronic cigarettes: human health effects. *Tobacco Control* 23, 36-40

Catassi, A., Servent, D., Paleari, L., *et al.* (2008) Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: implications on lung carcinogenesis. *Mutation Research* 659, 221-231

Chetiyanukornkul T, Toriba A, Kizu R., *et al.* (2004). Hair analysis of nicotine and cotinine for evaluating tobacco smoke exposure by liquid chromatography-mass spectrometry. *Biomedical chromatography* 18, 655-661

Claffey, D. J., Stout, P. R. & Ruth, J. A. (2001) ^{3}H-Nicotine, ^{3}H-Flunitrazepam, and ^{3}H-Cocaine incorporation into melanin: a model for the examination of drug-melanin interactions. *Journal of Analytical Toxicology* 25, 607-611

Cotter, S. M., Hardy, W. D. & Essex, M. (1975) Association of feline leukaemia virus with lymphosarcoma and other disorders in the cat. *Journal of the American Veterinary Medical Association* 166, 449-454

Denson, K. W. E. (2003) Letter to the editor. *American Journal of Epidemiology* 158, 1227-1230

Dreyfuss, J, H. (2010). Thirdhand smoke identified as potent, enduring carcinogen. *CA: A Cancer Journal for Clinicians.* 60 (4), 203-204.

Eliopoulos, C., Klein, J., Phan, M. K., *et al.* (1994) Hair concentrations of nicotine and cotinine in women and their newborn infants. *Journal of the American Medical Association* 271, 621-623

Eliopoulos, C., Klein, J. & Koren, G. (1996) Validation of self-reported smoking by analysis of hair for nicotine and cotinine. *Therapeutic Drug Monitoring* 18(5), 532-536

Evans, S. E., Bonczynski, J. J., Broussard, J. D., *et al.* (2006) Comparison of endoscopic and full-thickness biopsy specimens for diagnosis of inflammatory bowel disease and alimentary tract lymphoma in cats. *Journal of the American Veterinary Medical Association* 229, 1447-1450

Francis, D. P., Cotter, S. M., Hardy, W. D., *et al.* (1979) Comparison of virus-positive and virus-negative cases of feline leukaemia and lymphoma. *Cancer Research* 39, 3866-3870

General Report of the Surgeon General (1989) Reducing the health consequences of smoking: 25 years of progress. Washington, D. C: US Department of Health and

Human Services Publication. https://profiles.nlm.nih.gov/ps/access/NNBBXT.pdf (accessed 1st November, 2015)

Gerstenberg, B., Schepers, G., Voncken, P. *et al.* (1995). Nicotine and cotinine accumulation in pigmented and unpigmented rat hair. *Drug Metabolism and Disposition* 23(1), 143-148

Gieger, T. (2011). Alimentary lymphoma in cats and dogs. *Vet Clinics of North America Small Animal Practice* 41, 419-432

Glasgow R., Foster L., Lee M., et al. (1998). Developing a brief measure of smoking in the home: description and primary evaluation. *Addiction Behaviours* 23, 567-571

Green, J. T., Richardson, C., Marshall, R.W. *et al.* (2000) Nitric oxide mediates a therapeutic effect of nicotine in ulcerative colitis. *Alimentary Pharmacology and Therapeutics* 14, 1429-1434

Hecht, S. (1998) Biochemistry, Biology, and Carcinogenicity of Tobacco-Specific N-Nitrosamines. *Chemical Research in Toxicology* 11, 559-603

Hecht, S. (2002) Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. *Carcinogenesis* 23, 907-922

Hecht, S. (2003) Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nature Reviews Cancer* 3, 733-744

Hendriks, W.H., Tarttelin, M., F. & Moughan. P. J. (1997) Seasonal hair growth in the adult domestic cat (*Felis catus)*. *Comparative Biochemistry and Physiology* 116, 29-35

Hodgson, K. & Darling, M. (2011). Zooeyia: An essential component of "One Health". *Canadian Veterinary Journal* 52, 189-191

Hoffmann, D., Brunnemann, K. D., Prokopczyk, B., *et al.* (1994) Tobacco-specific N-nitrosamines and Areca-derived N-nitrosamines: Chemistry, biochemistry, carcinogenicity, and relevance to humans. *Journal of Toxicology and Environmental Health* 41, 1-52

Hoffmann, D. & Hoffman, I. (1997) The changing cigarette, 1950-1995. *Journal of Toxicology and Environmental Health* 50, 307-364

Hsu, T., Zhang, Y. & Santella, R. M. (1997) Immunoperoxidase quantitation of 4-aminobiphenyl- and polycyclic aromatic hydrocarbon-DNA adducts in exfoliated oral and urothelial cells of smokers and nonsmokers. *Cancer Epidemiology, Biomarkers and Prevention* 6, 193-199

Hukkanen, J., Jacob, P. & Benowitz, N. L. (2005) Metabolism and disposition kinetics of nicotine. *Pharmacological Reviews* 57, 79-115

Husgafvel-Pursiainen, K., Boffetta, P., Kannio, A., *et al.* (2000) P53 mutations and exposure to environmental tobacco smoke in a multicenter study on lung cancer. *Cancer Research* 60, 2906-2911

Iwase, A., Aiba, M. & Kira, S. (1991) Respiratory nicotine absorption in non-smoking females during passive smoking. *International Archives of Occupational and Environmental Health* 63, 139-143

Jaakkola, M. S. & Jaakkola, J. J. K. (1997). Assessment of exposure to environmental tobacco smoke. *European Respiratory Journal* 10, 2384-2397

Jaakkola, J. J. K., Jaakkola, N. & Zahlsen, K. (2001). Fetal growth and length of gestation in relation to prenatal exposure to environmental tobacco smoke assessed by hair nicotine concentration *Environmental Health Perspectives* 109, 557-561

Karban A. & Eliakim R. (2007). Effect of smoking on inflammatory bowel disease: is it disease or organ specific? *World Journal of Gastroenterology* 13, 2150-2152

Kelsey, J. L., Moore, A. S. & Glickman, L. T. (1998). Epidemiologic studies of risk factors for cancer in pet dogs. *Epidemiologic Reviews* 20(2), 204-217

Khalil, A.A., Jameson, M. J., Broaddus, W. C. *et al.* (2013). Nicotine enhances proliferation, migration, and radioresistance of human malignant glioma cells through EGFR activation. *Brain Tumour Pathology* 30, 73-83

Kim, S., Apelberg, B. J., Avila-Tang, E. *et al* (2014) Utility and cutoff value of hair nicotine as a biomarker of long-term tobacco smoke exposure, compared to salivary cotinine. *International Journal of Environmental Research and Public Health* 11, 8368-8382

Kintz, P. (1992) Gas chromatographic analysis of nicotine and cotinine in hair. *Journal of chromatography* 580, 347-353

Kleinschmidt, S., Harder, J., Nolte, I., *et al.* (2010) Chronic inflammatory and non-inflammatory diseases of the gastrointestinal tract in cats: diagnostic advantages of full-thickness intestinal and extra-intestinal biopsies. *Journal of Feline Medicine and Surgery* 12, 97-103

Knight, J. M., Eliopoulos, C., Klein, J., *et al.* (1996). Passive smoking in children. Racial differences in systemic exposure to cotinine by hair and urine analysis. *CHEST* 109, 446-450

Knottenbelt, C., Bawazeer, S., Hammond, J. *et al.* (2012) Nicotine hair concentrations in dogs exposed to environmental tobacco smoke: a pilot study. *Journal of Small Animal Practice* 53, 623-626

Kuschner, W. G., Reddy, S., Mehrota, N., *et al.* (2011) Electronic cigarettes and thirdhand tobacco smoke: two emerging health care challenges for the primary care provider. *International Journal of General Medicine* 4, 115-120

Lakatos P.L., Szamosi T. & Lakatos L. (2007). Smoking in inflammatory bowel diseases: Good, bad or ugly? *World Journal of Gastroenterology* 13, 6134-6139
Linet, M. S., McLaughlin, J. K., Hsing, A. W., *et al.* (1992) Is cigarette smoking a risk factor for non-hodgkin's lymphoma or multiple myeloma? Results from the Lutheran Brotherhood cohort study. *Leukemia Research* 16, 621-624

Lingard, A. E., Briscoe, K., Beatty, J. A., *et al.* (2009). Low-grade alimentary lymphoma: clinicopathological findings and response to treatment in 17 cases. *Journal of Feline Medicine and Surgery* 11, 692-700

Louwerens, M., London, C. A., Pederson, N. C., *et al.* (2005) Feline lymphoma in the post-feline leukaemia virus era. *Journal of Veterinary Internal Medicine* 19, 329-335

Matt, G. E., Bernert, J. T. & Hovell, M. F. (2008) Measuring secondhand smoke exposure in children: An ecological measurement approach. *Journal of Pediatric Psychology* 33(2), 156-175

Matt, G. E., Quintana, J. E., Zakarian, J. M., *et al.* (2010). When smokers move out and non-smokers move in: residential thirdhand smoke pollution and exposure. *Tobacco Control* 20, e1-e8

Matt, G. E., Quintana, P. J. E., Destaillats, H. *et al.* (2011). Thirdhand tobacco smoke: emerging evidence and arguments for a multidisciplinary research agenda. *Environmental Health Perspectives* 119(9), 1218-1226

McNiel, E. A., Carmella, S.G., Heath, L.A. *et al* (2007). Urinary biomarkers to assess exposure of cats to environmental tobacco smoke. *American Journal of Veterinary Research* 68, 349-353

Milberger, S. M., Davis, R. M. & Holm, A. L. (2009). Pet owners' attitudes and behaviours related to smoking and second-hand smoke: a pilot study. *Tobacco Control* 18, 156-158

Mizuno, A., Uematsu, T., Oshima, A., *et al.* (1993). Analysis of nicotine content of hair for assessing individual cigarette-smoking behaviour. *Therapeutic Drug Monitoring* 15(2), 99–104

Mizuno, A., Uematsu, T., Ishiwaka, T., *et al.* (1997). Clinical outcome of a smoking-cessation trial of nicotine chewing gum evaluated by analysis of nicotine in hair. *Therapeutic Drug Monitoring* 19, 407-412

Mohtashamipur, E., Mohtashimipur, A., Germann, P., *et al.* (1990). Comparative carcinogenicity of cigarette mainstream and sidestream smoke condensates on the mouse skin. *Journal of Cancer Research and Clinical Oncology* 116: 604-608

Morrison, W. B. (2012) Inflammation and Cancer: A Comparative View. *Journal of Veterinary Internal Medicine* **26**, 18-31

Nafstad, P., Botten, G., Hagen, J. A., *et al.* (1995). Comparison of three methods for estimating environmental tobacco smoke exposure among children aged between 12 and 36 months. *International Journal of Epidemiology* 24(1), 88-94

National Research Council (1991). Animals as sentinels of environmental health hazards. Washington, DC: National Academy Press. Page 69. http://www.nap.edu/read/1351/chapter/6 (accessed 2nd November 2015)

Nilsen, T., Zahlsen, K. & Nilsen, O. G. (1994) Uptake of nicotine in hair during controlled environmental air exposure to nicotine vapour: evidence for a major contribution of environmental nicotine to the overall nicotine found in hair from smokers and non-smokers. *Pharmacology and Toxicology* 75, 136-142

Öberg, M., Jaakkola, M.S., Woodward, A., *et al.* (2011). Worldwide burden of disease from exposure to second-hand smoke: a retrospective analysis of data from 192 countries. *The Lancet* 377, 139-146

Okoli, C. T. C., Hall, L. A., Rayens, M. K. *et al.* (2007). Measuring tobacco smoke exposure among smoking and nonsmoking bar and restaurant workers. *Biological Research for Nursing* 9(1), 81-89

Prokopczyk, B., Hoffmann, D., Bologna, M., *et al*. (2002) Identification of tobacco-derived compounds in human pancreatic juice. *Chemical Research in Toxicology* 15, 677-685

Reif, J. S., Dunn, K., Ogilvie, G. K, *et al.* (1992). Passive smoking and canine lung cancer risk. *American Journal of Epidemiology* 135 (3), 234-239

Reif, J. S., Bruns, C. & Lower, K. S. (1998) Cancer of the nasal cavity and paranasal sinuses and exposure to ETS in pet dogs. *American Journal of Epidemiology* 147(5), 488-492

Rissetto, K., Villamil, J. A., Selting, K. A., *et al.* (2011) Recent trends in feline intestinal neoplasia: an epidemiological study of 1129 cases in the Veterinary Medicine Database from 1964 to 2004. *Journal of the American Animal Hospital Association* 47, 28-36

Romano, G., Mancini, R., Fedele, P., *et al.* (1997) Immunohistochemical analysis of 4-aminobiphenyl-DNA adducts in oral mucosal cells of smokers and nonsmokers. *Anticancer Research* 17, 2827-2830

Roza, M. R. & Viegas, C. A. A. (2007) The dog as a passive smoker: Effects of exposure to environmental cigarette smoke on domestic dogs. *Nicotine and Tobacco Research* 9(11), 1171-1176

Sargent, M (Ed.) (2013) Guide to achieving reliable quantitative LC-MS measurements. RSC Analytical Methods Committee.ISBN 978-0-948926-27-3

Smith, V. A., McBrearty, A.R., Watson, D.G., *et al*. (2017) Hair nicotine concentration measurement in cats and its relationship to owner-reported environmental tobacco smoke. *Journal of Small Animal Practice* 58, 3-9

Snyder, L. A., Bertone, E. R., Jakowski, M. S., *et al.* (2004) P53 expression and environmental tobacco smoke exposure in feline oral squamous cell carcinoma. *Veterinary Pathology* 41, 209-214

Spengler, J. D. (1999). Buildings operations and ETS exposure. *Environmental Health Perspectives* 107 (2), 313-317

Tayler, R. & Piper, D.W. (1977). The carcinogenic effect of cigarette smoke: The effect of cigarette smoke on human gastric mucosal cells in organ culture. *Cancer* 39, 2520-2523

Tzatzarakis, M. N., Vardavas, C. I., Kokkinakis, M. *et al.* (2011). Hair nicotine/cotinine concentrations as a method of monitoring exposure to tobacco smoke among infants and adults. *Human and Experimental Toxicology* 31(3), 258-265

Uematsu, T., Mizuno, A., Nagashima, S., *et al.* (1995). The axial distribution of nicotine content along hair shaft as an indicator of changes in smoking behaviour: evaluation in a smoking-cessation programme with or without the aid of nicotine chewing gum. *British Journal of Clinical Pharmacology* 39, 665-669

United Nations Office on Drugs and Crime (2009). Guidance for the Validation of Analytical Methodology and Calibration of Equipment Used for Testing of Illicit Drugs in Seized Materials and Biological Specimens: A Commitment to Quality and Continuous Improvement (New York: United Nations), https://www.unodc.org/documents/scientific/ validation_E.pdf [accessed 26th October 2015]

U.S. Department of Health and Human Services (2006). The Health Consequences of Involuntary Exposure to Tobacco Smoke: A Report of the Surgeon General. Atlanta, GA: U.S. Department of Health and Human Services, Centers of Disease Control and Prevention, Coordinating Center for Health Promotion, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, p. 12–136 www.ncbi.nlm.nih.gov/books/NBK44324/pdf/Bookshelf_NBK44324.pdf (accessed 1st November 2015).

Vail, D. M., Moore, A. S., Ogilvie, G. K., *et al.* (1998) Feline lymphoma (145 cases): proliferation indices, cluster of differentiation 3 immunoreactivity, and their association with prognosis in 90 cats. *Journal of Veterinary Internal Medicine* 12, 349-354

Washabau, R. J., Day, M. J., Willard, M. D., *et al.* (2010) ACVIM consensus statement: endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. *Journal of Veterinary Internal Medicine* 24, 10-26

Wilson, K. M., Klein, J. D., Blumkin, M. S., *et al.* (2011). Tobacco-smoke exposure in children who live in multi-unit housing. *Pediatrics* 127, 85-92

Winickoff, J. P., Friebely, J., Tanski, S. E., *et al.* (2009). Beliefs about the health effects of "thirdhand" smoke and home smoking bans. *Pediatrics* 123, e74-e79

Witschi, H., Joad, J. P. & Pinkerton, K. E. (1997). The toxicology of environmental tobacco smoke. *The Annual Review of Pharmacology and Toxicology* 37, 29-52

Yerger, V. B. & Malone, R. E. (2006). Melanin and nicotine: A review of the literature. *Nicotine & Tobacco Research* 8, 487-498

Zahlsen, K. & Nilsen, O. G. (1994) Nicotine in hair of smokers and non-smokers: sampling procedure and gas chromatographic/mass spectrometric analysis. *Pharmacology and Toxicology* 75, 143-149

Zahlsen, K., Nilsen, T. & Nilsen, O. G. (1996). Interindividual differences in hair uptake of air nicotine and significance of cigarette counting for estimation of environmental tobacco smoke exposure. *Pharmacology and Toxicology* 79, 183-190