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"New perspectives in Veterinary Forensic Sciences"

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List of Abbreviations

ASA (Animal sexual abuse)

AI (Accidental injuries)

NAI (Non accidental injuries)

a-SMA (a- smooth muscle actin)

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Abstract

Over the last years, the knowledge on the veterinary forensic sciences has experienced a rapid increase as evidenced by number of peer- reviewed publications, textbooks, and inclusion of the topic in many veterinary medical conferences. However, most of the information in veterinary forensic medicine is still acquired by human forensic literature. This lack of information is currently considered a serious problem in veterinary forensic medicine. Indeed, although it is undeniably true that the mechanisms of forensic injuries as well as the post-mortem cadaveric changes are similar between humans and animals, the different morphology, weight, and tissue resistance of animals compared to human anatomy and other species-specific factors make the information validated in human forensic medicine not always applicable in the veterinary forensic field. Therefore, the purpose of this thesis work is to summarize the studies carried out throughout the PhD scholarship, which were based on development of new methodological approaches in the veterinary forensic field. As regard to injuries secondary to neglect, particular interest was given to post-mortem findings in emaciated dogs. Indeed, our study offers new insights in the differential involvement of the liver in the pathogenesis of starvation and cachexia. A quantitative assessment of the number of portal tracts in histological samples has proven to be a useful tool assisting the determination of the cause and mechanism of death in abused animals, not as a unique and definite determination, but as supporting evidence together with the other findings suggestive of starvation. In addition, as regards the estimation of age in puppies, we have conducted studies aimed at identifying new indirect methods to assess the age of puppies in association with other methods described in literature. A morphometric study of the kidney and an immunohistochemical study were then carried out in order to establish if there is a correlation between the number of glomeruli and age of puppies and to evaluate the positivity of the glomeruli to the anti-a-Actin smooth muscle antibody. Finally, as regard to forensic traumatology, we investigated the applicability of the forensic necropsy for the identification and monitoring of accidental and non-accidental injuries in dogs and cats when a body is recovered in outdoor crime scenes and no witness testimony is available. Together, these findings will provide useful tools to increase the knowledge in the veterinary forensic field.

1.1 Veterinary forensic medicine

The word **forensic** comes from the Latin word "*forensis"* meaning "relating to, used in, or suitable to a court of law" (Merriam Webster Dictionary). Thus, any science used for the purposes of the law is a **forensic science**. Veterinary forensic medicine can be defined as "*the applied use of veterinary medical knowledge, especially pathology, to the purpose of the law*". The increasingly widespread use of the term "forensic science" is a reminder that the subject is often multidisciplinary, with important contributions from specialists in such fields as toxicology, ballistics, entomology, and DNA technology (Cooper et al., 1998). In the last few decades the use of forensic sciences in the veterinary field has considerably increased, determined by a combination of factors, such as increased awareness of associations between crimes against animals and crimes against humans, and important improvements in laws that protect animals (Parry et al., 2020). The purpose of conducting a forensic examination is: (a) to discover and record any injury, disease, or abnormality, and (b) to interpret these findings in a manner that allows a Court of Law to understand the cause(s) and significance of any changes (Munro 2008). The duties of a veterinary forensic pathologist during a forensic necropsy are similar to those of a human medical examiner and include crime scene investigation, necropsy photography, reviewing medical history, necropsy of the cadaver, necropsy report writing, and courtroom testimony. Despite the similarities in duties and expectations, there is no unified system to support these activities on behalf of the veterinarian during an animal crime investigation (Brooks, 2018). The ultimate goal is to determine the cause of death of the animal, whether natural or violent, the manner of death, the time since death (post-mortem interval) and to examine and preserve any physical evidence that might produce information useful to identify and charging those guilty of a crime against animals.

1.2 Crime scene investigation

A forensic necropsy does not begin on the necropsy table; rather it begins at the crime scene. Knowledge of the scene may be crucial to interpreting findings at necropsy and determining the cause of death, making it preferred for a forensic veterinarian or pathologist to examine the scene for every case in which they assist (Gerdin, 2013). Unfortunately, at least in Italy, veterinary forensic pathologist hardly ever visit the crime scene (Brooks, 2018). Usually, the police or other investigators remove the cadaver from the crime scene and submit it for examination some time later. However, crime scene can be divided in primary crime scene, secondary crime scene and disposal site. The primary crime scene is the *place where the most of the crime act was committed* or, more in detail, "*the place where offender engaged in the majority of his or her attack or assault upon the victim or victims*" (Turvey, 1999; Savino et al. 2011). In contrast, the secondary crime scene *is "any place where there are evidence of interaction between criminal and victim outside the primary scene"* (Turvey,1999; Savino et al. 2005). Finally, disposal site is "*the place where the body of the victim is found"* (Savino et al. 2011). The information obtained from the crime scene analysis is important not only for correctly interpreting injuries observed during the forensic post-mortem examination but also to establish specific relationship between a suspect and the crime scene or victim (Gardner, 2011; Touroo and Fitch; 2016). As a matter of fact, *crime scene processing is the examination and evaluation of the scene for the express purpose of recovering physical evidence and documenting the scene's condition in situ, or as found* (Gardner, 2011). Indeed, in most cases, the law enforcement does not have specialist skills. Therefore, it is likely that important findings from a medical point of view are not detected, photographed and collected.

The final goal of crime scene analysis is to collect evidence in a pristine condition.

1.2.1 Evidence

Evidence is defined as “*anything that can prove or disprove a fact in contention*” (Gardner, 2011). In general, all evidence obtained from a crime scene can be divided in three categories: **testimonial**, **physical** and **situational**. Testimonial evidence can be defined as “*evidence based on the statements made by witnesses and suspects*”. Testimonial evidence should be supported by tangible facts. The physical evidence is “*any object that could be used to determine whether a crime has been committed or not, that could provide or disprove a link between a crime and victim or a link between a crime and a suspect*” (Touroo and Fitch,2016). Finally, the situational evidence is “*any potentially transitory element on the crime scene, such as weather conditions, sounds and temperatures*” (Touroo and Fitch, 2016). Finally, the evidence obtained during the evaluation of the crime scene should then be matched with the findings obtained from the forensic anatomopathological examination, histological analysis and collateral examinations.

According to Toouro and Fitch (2016), we report in textbox 1 the main steps of the crime scene analysis in veterinary forensic medicine.

Textbox 1. Main steps of the crime scene analysis

1. photographic documentation of the crime scene upon arrive
2. photographic documentation of the environments and living conditions of the animal
3. evaluation of the presence of animals other than victim, if present, photographic documentation of the animal condition, execution of a brief forensic clinical examinations, identification and preservation of the evidences and rapid transport to the hospital
4. photographic documentation of the victim using a photomacrographic scale (ABFO No. 2 Standard Reference Scale, i.e.)
5. external examination of the victim, photographic documentation of the evidence on the surface of the body, preservation of the evidences.
6. estimate of the time since death
7. support to law enforcement for the identification and preservation of medical and nonmedical evidences (anabolic steroids or dogfighting paraphernalia, i.e.)

1.3 Forensic necropsy

Necropsy, literally meaning "observation of the corpse", is carried out for clinical (diagnostic or "clinical" necropsy) as well as for medico-legal purposes (forensic necropsy). Diagnostic necropsy is performed to establish the nature of the disease that has caused the death of the animals in cases in which the ante-mortem diagnosis has failed. Furthermore, clinical necropsy can be carry out to epidemiological purposes, to check and verify the diagnoses formulated in life or to study specific disease even when the cause of death is known (Mason et al, 2007, Tariq et. al 2008, Kuker et al 2018, Brooks, 2018, Nwoha and Onoja; 2016). In contrast, the aims and objectives of forensic necropsy are very broad and include not only the determination of the cause of death, but also the establishment of the identity of the deceased, evaluatating the manner of death, estimating thr time since death (post-mortem interval) and examining and preserving any physical evidence observed during the necropsy (Brooks, 2018, Brooks, 2016, Touroo and Fitch; 2016). Although the forensic necropsy technique is similar to that performed during a diagnostic necropsy, there are important differences in the approach and pathologist's training (Brooks, 2018). Indeed, since the information obtained from the forensic necropsy is used for legal purposes, this procedure should be performed using previously established standard guidelines to avoid possible disputes during the process. Currently, the National protocols and the published international guidelines as well as the textbook of veterinary forensic pathology advocate a schematic, rigorous and multidisciplinary approach to the investigation of all cases of veterinary forensic interest (Brooks et al. 2016, Brooks, 2018). In particular, the necropsy should consist of 2 components, specifically an "outer" and an "inner" necropsy (Piegari et al. 2018). The outer necropsy is the external examination of the body, the inner necropsy is the internal examination of the body and starts with the skinning of the cadaver (Piegari et al. 2018). However, the "outer" necropsy should not begin before a careful examination of the modalities and the conditions in which the cadaver has been received (Brooks, 2018). In particular, in the event that the cadaver is delivered inside a package, a radiographic examination should be performed before removing its from the packaging (Brooks, 2018). This procedure is useful to detect visible radiographically evidence (such as metal) that might be contained within the packaging. After this procedure, packaging and any physical evidence should be collected and preserved for any further laboratory test. Once removed from the package, the body should be radiographed to assess the presence of bone fractures, metallic objects in the animal body or other radiographically visible evidence (Brooks, 2018;

Brooks et al. 2016). After radiographic examination, the body should be photographed from multiple angles using a photomacrographic scale (Brooks, 2018; Piegari et al., 2018). During this step, any evidence present on the surface of the body should be documented, photographed, and subsequently sampled before any further handling of the cadaver (Brooks, 2018). The pathologist should document species, breed, and sex of the animals, estimate the time since death and, if possible, estimate the age of the victim. (Brooks, 2018; Piegari et al., 2018). Subsequently, after a general evaluation of the body, a systematic evaluation of body parts of the animals should be performed. For this purpose, the head, mouth, mucous membranes, thorax, abdomen, perianal region, outer genitalia, hair coat, tail, as well as the front and hind limbs should be thoroughly examined . After this, a full skilling of the animals, inspection of the muscles and sub cutis as well as the opening of all body cavities (skull, chest, abdomen, pelvis) should be performed. Finally, all organs should be examined and dissected (Piegari et al. 2018). Each of these phases requires a careful and complete photographic documentation. Since necropsy is an unrepeatable procedure, the forensic photographic documentation should be accurate and detailed, but also produce a minimum delay in the execution time of the necropsy (Piegari et al. 2018). Forensic photography should be used to document both the presence of injuries (positive photograph) and the absence (negative photographs) of injuries (Dolinak et al. 2005). In addiction, during images acquisition, a photomacrographic scale (ABFO No. 2 Standard Reference Scale, i.e.) placed near the injuries should be used to provide a geometrical reference in the forensic photographic documentation of the evidences (Brooks, 2018; Piegari et al., 2018). At the end of necropsy, representative tissue samples should be collected and fixed in 10% neutral buffer formalin for further histopathological examinations. In human forensic pathology, the National Association of Medical Examiners recommends to perform the histological examination on all forensic cases where the cause of death is not determined after the forensic macroscopic examination (Peterson and Clark; 2006). The histological examination of tissues can in fact provide a wide range of additional information that can help the pathologist to better interpret the injuries found during the necropsy. It may help to exclude other possible causes of death, confirm, or deny the diagnosis formulated on the macroscopic examination or allow the detection of specific injuries that cannot be observed on the forensic macroscopic examination (such as myocarditis or neurodegenerative diseases) (Brooks, 2018). According to Piegari et al. (2018), we report in textbox 2 the main steps of the forensic necropsy in veterinary forensic pathology.

Textbox 2. Forensic necropsy protocol.

1. Victim identification procedures

2. Evaluation of thanatological aspects and estimation of the time elapsed since death

3. External examination of the body (state of nutrition, mucous membranes, body orifices, general conformation, superficial lesions, hair coat, external parasites, and teeth)

4. Skinning with evaluation of subcutis and muscles

5. Opening and evaluation of body cavities (skull, thorax, abdomen, and pelvis)

6. Extraction and general macroscopic evaluation of organs

7. Dissection of all organs

8. Specific evaluation of wounds or injuries

9. Complete photographic documentation of external appearance of the animals, body cavities, organs, and injuries

1.4 Animal law

Forensic veterinary medicine has its reason to exist thanks to the laws that protect animals and make those who commit crimes against them liable to penalties or sanctions. In Italy, the most significant animal protection laws include:

- **Legislative Decree 157/92** “Rules for the protection of homeothermal wild animals and for hunting purposes”.
- **Legislative Decree 281/91** “Low on pets and the prevention of straying”.
- **Legislative Decree 150/92** “Regulation of offences relating to the application in Italy of the Convention on the international trade of endangered animal and plant species”.
- **Legislative Decree 151/2007** “Penalty provisions for violating the provisions of the Regulations (EC) no. 1/20055”.
- **Legislative Decree 189/2004** “Provisions concerning the prohibition of animal abuse, as well as the use of animals in clandestine fighting or unauthorised competitions”.
- **Regulation (ec) 1523/2007** of the European Parliament and Council of 11 December 2007 banning the placing on the market and the

import to, or export from, the Community of cat and dog fur, as well as products containing such fur.

- **Ministerial Ordinance of 18 December 2008** and its subsequent amendments: “Guidelines on the prohibition of the use and keeping of poisoned baits”.
- **Article 727 of the Italian Criminal Code** punishes the abandonment of animals.

Among these, **Law 189 introduced in 2004**, is one of the most important in the field of the veterinary forensic medicine. This law changed the legal basis for the protection of animals, which until then had been governed only by Article 727 of the Criminal Code. With the amendment of this law, animal sentiment is now being harmed and no longer just the "human morality".

With Art. 1"Amendments to the Criminal Code" of the aforementioned Law, the legislator introduced (after title IX) the title IX bis, entitled "Crimes against animal sentiment" into the Criminal Code. Following this change, animal abuse by just one offence becomes a crime.

This change involves:

1) an aggravation of penalties (from fines to imprisonment);

2) the impossibility of overturning the offence by means of an oblation;

3) the lengthening of the limitation period;

4) the necessity of willful misconduct, also in the form of the so-called "potential" misconduct (negligent misconduct is excluded from the regulations, except for the offence referred to in Article 727 of the Criminal Code).

The articles introduced by this law are:

- **544 bis:** killing of animals;
- **544 ter:** animal abuse;
- **544 quater:** prohibited shows or events using animals;
- **544 quinquies:** prohibition of animal fighting.

In particular, the articles of greatest interest for the forensic veterinary pathologist are 544 bis and 544 ter. Art. 544 ter states the following:

Par. 1 "*Anyone who, for cruelty or without necessity, causes injury to an animal or subjects it to torture, hard labour or behaviour, or to unbearable work due to its ethological characteristics, shall be punished with imprisonment from 3 months to 1 year, or given a fine ranging from €3,000 to €15,000*"

Par. 2 "*The same punishment is applicable to anyone who administers prohibited or narcotic substances to animals or subjects them to treatments that cause damage to their health*"

Par. 3 "*The penalty is increased by half if the facts referred to in the first paragraph results in the death of the animal*"

From this article, it can be deduced that the action perpetrated constitutes a crime when the conscience and will to cause damage to the animal is present. This damage, according to what has been mentioned above, may be of different types: physical injury, torture, hard labour or behaviour, or unbearable work due to its ethological characteristics, administration of drugs or prohibited treatments that cause damage to the health of the animal. The third paragraph of Article 544 provides for an aggravating circumstance, which materializes if the conduct referred to the first paragraph results in the death of the animal. Such an aggravating circumstance only exists if the death of the animal is an unintended consequence of the abuse. If this is not the case, the crime of killing animals is then committed as provided for in Article 544 bis of the Italian Criminal Code, pursuant to which:

"*Whoever, whether cruelly or without necessity, causes the death of an animal shall be punished by imprisonment from three to eighteen months*".

This low does not provide for:

- a distinction between owned or stray animal;

- the specific methods used to cause the death of the animal; both action and negligence, resulting in death, are punishable.

Finally, **article 727c.c**., reports the following:

Par. 1 "*Whoever abandons pets or animals that have acquired captive habits is liable to imprisonment for up to one year or a fine between €1,000 and €10,000.*"

Par. 2 "*The same punishment is applicable to anyone keeps animals in conditions incompatible with their nature and producing severe suffering*."

Here, we have two types of punishment: the abandonment of animals and their keeping in conditions that conflict with their nature and cause suffering. The concept of abandonment can be traced back to carelessness or neglect of the animal and not to cruelty to the animal or the infliction of gratuitous suffering, attitudes that are punished with the crime of animal abuse (art. 544 ter). Abandonment, in any case, is not just a matter of abandoning the animal, but must be understood in the more general intention of no longer taking care of it. Furthermore, as regard the keeping of animals in conditions incompatible with their nature, it is not to be understood as a necessarily intentional offence, as it can be committed through negligence alone. Therefore, the keeper of animals in conditions incompatible with their nature or in a state of abandonment is criminally liable even through negligence alone. With regard to "serious suffering", the Court of Cassation has specified that, “while it is undeniably true that the concept of the gravity of the suffering necessary to fulfill the conduct described in art. 727 of the Italian Criminal Code is indeed different from the concept of serious damage to the animal's health provided for in art. 544 ter of the Italian Criminal Code, it is nevertheless essential that the suffering to which poorly kept animals should be subjected reach a level such as to make the condition in which they are kept absolutely irreconcilable with the proper conditions for the animal to be in a situation of well-being”. This opinion should be expressed with reference to contingent situations, it being clear that a temporary situation of distress for the animal cannot be confused with the “contra legem” situation set forth in paragraph 2 of art. 727.

1.5 Animal abuse

In literature animal abuse is defined as "*socially unacceptable behavior that intentionally causes unnecessary pain, suffering, or distress to and/or the death of an animal*" (Ascione, 1993). No species of animal is immune to cruelty; however, it is domesticated and captivity animals that are dependent upon humans for survival because they require someone to feed, house, and care for them (McEwen, 2017). Cruelty and neglect cross all social and economic boundaries and is common in both rural and urban areas. Thus, intentional cruelty to animals is often correlated with other crimes, including violence against people. Animal cruelty is often committed by a person who feels powerless, unnoticed or under the control of others. The motive may be to shock, threaten, intimidate, or offend others. Some see harming an animal as a safe way to get revenge against—or threaten—someone who cares about that animal. Animal cruelty impacts human health in different ways, and it is an important sentinel for domestic violence and child abuse. Animal abuse raises important questions about the type of society we wish to live in (Arkow, 2015). Guidelines published by the Professional Conduct Department of the Royal College of Veterinary Surgeons in the United Kingdom (UK) state: "*when a veterinary surgeon is presented with an injured animal whose clinical signs cannot be attributed to the history* *provided by the client, s/he should include non-accidental injury in the differential diagnosis*”. The terms batterer and abuser will include those who emotionally, psychologically, physically, or sexually harm or abuse a human being or an animal. There are several classifications of animal abuse. They include but are not limited to physical abuse, emotional abuse, sexual abuse, neglect, organized abuse (animal fighting).

1.5.1. Neglect

Neglect is the most common form of animal abuse encountered. The term “neglect” is used to refer to the failure to provide for an animal’s needs. This includes adequate space, appropriate food, water, maintaining the animal’s hair coat and nails, and providing sanitary conditions. It also includes lack of veterinary treatment that can apply to both injuries and naturally occurring disease. Neglect can also apply to any situation that has a negative impact on the animal, such as embedded collars, short tie-outs, and heavy chains. Neglect is often a continuum of action or lack of action by the owner over a prolonged period of time (Merck, 2007). Often, acts of neglect result from a poor understanding or ignorance of the individual's needs, a lack of motivation, or poor judgment. In many cases of neglect, the body condition score of the cadaver is markedly reduced. This results in emaciation.

1.5.2. Physical abuse

Physical abuse is also known as non-accidental injuries (NAI) and it is what everyone think of when they hear of animal abuse. It is the act of causing direct harm to an animal by hitting or kicking an animal or inflicting other types of blunt force trauma, sharp force trauma such as the cutting or stabbing of an animal, shooting an animal with a gun or arrow, burning, drowning, suffocating, or other similar act (Byrd, 2020).

1.5.3. Emotional abuse

Emotional neglect is a failure to fulfill the animal's emotional needs. Unpleasant emotions in animals include fear, anxiety, separation anxiety, boredom, frustration, anger, and depression. Emotional abuse is easier to recognise in humans where persistent emotional maltreatment of the person by bullying, exploitation, verbal harassment or corruption leads to a fragile emotional state. In animals, persistent threatening behaviour or a failure to provide basic behavioural needs constitutes emotional abuse. Conditions that may be considered emotional abuse include:

1. *Keeping a social animal in isolation*
2. *Keeping an animal in a small space such that it cannot exhibit normal postures or activities*

3. *Housing an animal so that it cannot exercise or move about freely*
4. *Housing an animal in an environment devoid of mental stimulation appropriate for the age, sex, species, and traits of the animal*
5. *Maintaining an animal in an environment where it has no control or ability to avoid aversive conditions*
6. *Maintaining an animal in an environment in which it is repeatedly exposed to danger and has no escape from the unsafe conditions (McMillan, 2005; Merck, 2013)*

1.5.4. Sexual Abuse

Animal sexual abuse (**ASA**) is also known by the terms **zoophilia, bestiality, zoosadism**. "*Zoophilia refers to sexual preference for or sexual attraction to an animal or animals*" (Byrd, 2020). However, the term **animal sexual abuse** is preferred in the animal welfare field over the other terms; zoophilia, bestiality, etc. refer to the motives and behavior of the perpetrator not focusing on the harm inficletd to the animal. ASA includes the sexual molestation of animals by humans, including a wide range of behaviors, such as vaginal, anal, or oral penetration or oral-genital contact, penetration with an object, and injuring or killing an animal for sexual gratification (**zoosadism**) (Stern, 2016). Some activities may seem harmless, but some result in severe physical harm and may even cause death (Munro, 2008). ASA is an activity that may be perpetrated by men and women; however, some acts can be carried out only by men (Stern, 2016). Permanent or recurrent failure with women can induce zoophilia in men (Hvozdík, 2006). The range of animals (and birds) used by humans for sexual purposes is extensive and involves a wide variety of activities with both male and female animals by men and women. Prior to performing the forensic necropsy, a complete history should be provided to the pathologist, including the circumstances of the event, type of sexual contact suspected, use of weapons/objects, and information regarding the animal (Stern, 2016). But these type of informations are not always available. Cases of abuse with minimal or absent injuries are extremely difficult to recognise since animals are unable to communicate their abuse. During the post-mortem examination particular attention must be taken during the examination of the gastrointestinal tract, specifically the descending colon and rectum. If the abuser penetrated the animal with a foreign body, perforation of the rectum or colon can occur, and identification of the perforation site is essential. Similarly, the entire reproductive tract should be assessed in situ and then removed from the body. If anal or vaginal lesions are identified, it is important to document their location. Animal victims of sexual abuse may have a variety of injuries to the anus/rectum and genitalia, while others have no such injuries despite sexual contact. The most common lesions are vaginitis, vaginal prolapse, uterine hemorrhage, the presence of intrauterine, intracervical, or vaginal foreign objects, peritonitis, proctitis, rectal fistulas, and/or rectal prolapse (Stern,2016).

References

Arkow P. Recognizing and responding to cases of suspected animal cruelty, abuse, and neglect: what the veterinarian needs to know. *Vet Med (Auckl)*. 2015;6:349-359. Published 2015 Nov 5. doi:10.2147/VMRR.S87198

Ascione F.R. Children who are cruel to animals: a review of research and implications,for,developmental,psychopathology. *Anthrozoös*. 1993; 6(4): 226–47. 10.2752/089279393787002105.

Brooks, Jason W., ed. *Veterinary Forensic Pathology*, *Volume 1*. Springer, 2018.

Brownlie HW, Munro R. The Veterinary Forensic Necropsy: A Review of Procedures and Protocols. Vet Pathol. 2016 Sep;53(5):919-28. doi: 10.1177/0300985816655851. Epub 2016 Jul 1. PMID: 27371536.

Byrd, J.H., Norris, P., & Bradley-Siemens, N. (Eds.). (2020). Veterinary Forensic Medicine and Forensic Sciences (1st ed.). CRC Press. https://doi.org/10.4324/9781315121918

Cooper JE, Cooper ME. Forensic veterinary medicine: a rapidly evolving discipline. Forensic Sci Med Pathol. 2008;4(2):75-82. doi: 10.1007/s12024-008-9036-x. Epub 2008 May 20. PMID: 19291476.

Gerdin JA, McDonough SP.Forensic pathology of companion animal abuse and neglect. Vet Pathol. 2013;50:994–1006

H.M.C. Munro, M.V. Thrusfield ‘Battered pets’: sexual abuse Journal of Small Animal Practice, 42 (2001), pp. 333-337

Hvozdík A, Bugarský A, Kottferová J, Vargová M, Ondrasovicová O, Ondrasovic M, Sasáková N. Ethological, psychological and legal aspects of animal sexual abuse. Vet J. 2006 Sep;172(2):374-6. doi: 10.1016/j.tvjl.2005.05.008. Epub 2005 Jul 14. PMID: 16019241.

Imbschweiler I, Kummerfeld M, Gerhard M, Pfeiffer I, Wohlsein P. Animal sexual abuse in a female sheep. Vet J. 2009 Dec;182(3):481-3. doi: 10.1016/j.tvjl.2008.09.004. Epub 2008 Oct 10. PMID: 18848792.

References

McEwen B. Eternally Vulnerable: The Pathology of Abuse in Domestic Animals. *Acad Forensic Pathol*. 2017;7(3):353-369. doi:10.23907/2017.032

McMillan, F. D. ed. 2005. Emotional Maltreatment of Animals. *Mental Health and Well-Being of Animals*. Ames, IA: Wiley-Blackwell, pp.167–180.

Merck M. Veterinary forensics: animal cruelty investigations. Ames: Blackwell Publishing; 2007.

Munro R, Munro HMC.Animal abuse and unlaw-ful killing: forensic veterinary pathology. 1st ed. Edinburgh: Saunders; 2008.

Munro HM. Animal sexual abuse: a veterinary taboo? Vet J. 2006 Sep;172(2):195-7. doi: 10.1016/j.tvjl.2005.08.033. Epub 2005 Oct 24. PMID: 16246601.

Parry NMA, Stoll A. The rise of veterinary forensics. Forensic Sci Int. 2020 Jan; 306:110069. doi: 10.1016/j.forsciint.2019.110069. Epub 2019 Nov 20. PMID: 31830618.

Savino JO, Turvey BE, Baeza JJ, 2011. Rape Investigation Handbook. 2nd edition, MD: Academic Press

Stern, A. W., & Smith-Blackmore, M. (2016). Veterinary Forensic Pathology of Animal Sexual Abuse. Veterinary Pathology, 53(5), 1057–1066.

Touroo R, Fitch A. Identification, Collection, and Preservation of Veterinary Forensic Evidence: On Scene and During the Postmortem Examination. Vet Pathol. 2016 Sep;53(5):880-7. doi: 10.1177/0300985816641175. Epub 2016 Mar 30. PMID: 27030370.

Turvey, Brent E., and Criminal Profiling. "An introduction to behavioral evidence analysis." *Criminal Profiling: An Introduction to Behavioural Evidence Analysis* (2012).

Chapter 1

Basic quantitative histological evaluation unravels different degrees of liver atrophy in cachectic and starved dogs

1.1. Introduction

Cases of neglect in dogs are among the most represented forensic cases submitted for post-mortem examination, according to current literature (Gerdin, 2016; Ottinger, 2014) and to the Department of Veterinary Pathology, Liverpool University over the last 10 years. Among fatal cases of neglect, starvation represents a commonly alleged cause of death, when an emaciated animal is found dead (or put to sleep on humane grounds) in suspicious circumstances. It is important to note that emaciation of the body is the common end point of multiple conditions, including the outcome of spontaneous disease (Gerdin, 2016). Starvation is a term used to describe the serious and fatal consequences of primary protein-energy malnutrition in which an otherwise healthy animal is able or willing to eat but unable to do so due to extrinsic restrictions to the availability of food. Differently from wildlife, where availability of food is susceptible to seasonal fluctuation, domestic animals such as dogs, rely completely on human carers/owners for nutritional support, especially if living in enclosed premises. On the other hand, cachexia is a term used to identify secondary protein-energy malnutrition mostly consequent to endogenous factors. In this part of the thesis, this term, which is alternatively restricted to describe the negative cytokine-mediate effect of tumours growth over body mass (Gerdin, 2013;

Kumar, 2015), is used to indicate any form of negative energy balance derived from the presence of concurrent chronic pathological processes, such as infections, severe organ degenerations and neoplasia, affecting the assimilation and utilization of nutrients. Despite both conditions are able to lead to similar degrees of loss of body mass of the emaciated dead body, starvation is characterized by prominent decrease of basal metabolic rate with mobilization of endogenous sources of lipids and proteins as source of energy from skeletal muscles, after complete exhaustion of fat stores (Gerdin, 2016; Mccue, 2010). In contrast, in cachexia the animal shows a voluntary reduction in caloric intake and/or an increase in metabolic demand with consequent increase in protein catabolism and a disproportionate loss of lean body mass compared to adipose tissue. The latter condition is often refractory to nutritional support alone, while starvation is not (Gerdin, 2016). From a legal perspective, it is of paramount importance that the pathologist describes in detail the condition of the carcase without omission of body compartments or organs since the absence of concurrent diseases in presence of emaciation in a deceased animal may support the diagnosis of starvation (Gerdin, 2016). In other words, death by starvation is a diagnosis by exclusion, supported by the complete absence of any other relevant and contributory concurrent disease. In dogs, specific findings, such as detection of abnormal gastric and intestinal content (foreign bodies, non-alimentary material), is indicative of preserved appetite and observed in ~50% of the animals dead by starvation, in a study (Gerdin, 2016). The latter finding, together with the presence of a chronic disease in cachectic animals, were the only distinctive differences between the two studied groups (Gerdin, 2016). In dogs, in both starvation and cachexia, several organs, in addition to muscles and adipose tissue, are described to undergo atrophy. These include liver, testicle, and skin; however, no quantitative assessment has been approached in dogs to investigate the difference to normal conditions and to explore if a difference exists between starvation and cachexia. It has been demonstrated that liver undergoes atrophy in golden hamster models of starvation (Tongiani, 1971), while microscopical hepatic changes during cachexia have not been studied extensively (Petruzzelli, 2016), and results are often conflicting. In cancer cachexia, for example, liver inflammation is reported more often than atrophy (Petruzzelli, 2016), and in some cases increased activity and function of the organ is reported (Porporato, 2016). We hypothesised that in emaciated dogs, the degree of liver atrophy in starvation cases is higher than in cachexia cases, and that this can be investigated microscopically, regardless the degree of body decomposition.

1.2. Materials and Methods

Necropsy reports of dog who undergone complete post-mortem examination (gross and histopathology) from January 2014 to April 2018 were retrieved from the archive of the Department of Veterinary Pathology and Public Health, Faculty of Veterinary Science, University of Liverpool. Inclusion criteria were as follows:

- Control Dogs Group: Non emaciated dogs (WSAVA Body score > or = 2/6 evaluated clinically in case the animal was found alive and euthanised due to life-quality issues; Normal aspect of the cadaver (adequate muscle bulk and presence of adipose tissue); Anamnestic and pathological confirmation of acute deaths.
- Emaciated dogs Group: (WSAVA Body score <2/6 evaluated clinically in case the animal was found alive and euthanised due to life-quality issues; Generalised emaciated aspect of the cadaver (Evident bony prominences in particular scapular spine, ribs and iliac crest, muscle atrophy and reduction/loss of adipose tissue). A final post-mortem diagnosis documenting either neoplastic, chronic inflammatory or degenerative condition.
- Starved dogs (WSAVA Body score <2/6 evaluated clinically in case the animal was found alive and euthanised due to life-quality issues;

Emaciated aspect of the cadaver with a final post-mortem diagnosis of starvation based on absence of any chronic pathological conditions, in presence of muscle atrophy and reduction/loss of adipose tissue, and additional corroborating evidence (e.g. presence of foreign material within the gastrointestinal tract).

Based on the expected adult weight for each canine breed, dogs were divided in 3 groups: small (1-10kg), medium (10-30kg) and large (above 30kg).

All cases underwent a complete post mortem examination which was defined as gross examination of all the organs in situ, removed and subsequently opened, followed by histological investigation of the main organs as per forensic protocol of Liverpool Department: pituitary gland, thyroid and parathyroid glands, adrenal glands, skin (sample from the flank), skeletal muscles (quadriceps and triceps, left and right, diaphragm), brain (frontal cortex, hippocampus, cerebellum, medulla oblongata), nerves (sciatic and brachial plexus, right and left), heart (right and left ventricles and interventricular septum), spleen, lymph nodes (mesenteric), bone marrow, stomach, small intestine (duodenum, jejunum, ileum), liver, pancreas, omentum, mesentery, kidneys, urinary bladder, retrobulbar adipose tissue, ovaries or testis (when present). Liver histology was assessed on samples taken from the left lateral lobe and stained with Haemoatoxylin

Eosin. The degree of tissue autolysis varied according to the post mortal interval, which was unknown for most of the bodies, from minimal post mortal artefacts (in reasonably fresh cadavers) to marked autolysis (in cadavers presented after prolonged post-mortem interval). Post-mortem liver histological changes were scored as follows: “Mild” (1): minimal changes including loss of red blood cell outline and detachment of vascular endothelium in sinusoid vessels. Normal cytoplasm and nuclei of hepatocytes visible with identifiable portal tracts; “Moderate” (2): loss of cohesion between hepatocytes, homogenisation of cytoplasm with loss of details, but with nuclei and cellular outline still visible and identifiable portal tract; “Severe” (3): loss of nuclear details of the hepatocytes with only hepatic cordons discernible but still identifiable portal tracts. Cases where the histology of the liver was considered completely compromised (e.g. organ identification was not possible, or portal tracts not discernible) were excluded from the study. A quantitative histomorphological study of the liver section was performed on 20 randomly chosen, not overlapping microscopic fields at low power magnification (10x, 21mm diameter field of view, ~346 mm2), referred as “Low Power Fields” (LPFs) using a brightfield microscope (Nikon Eclipse 80i). Portal tracts were identified by the presence of a bile duct and associated vascular structures (vein and arteries) with supporting connective tissue and were counted manually in each single LPF and the number subsequently averaged per animal. The number of portal tracts was used as an indirect measure of atrophy with the rationale that the number of portal tracts increases per surface area, as a consequence of single cell reduction in size with consequent reduction of the lobule size. Congestion, degeneration, and fibrosis were also assessed as present or absent.

1.2.1. Statistical Analysis

Comparisons between groups (control vs cachexia vs starvation) were made through Mann-Whitney U Test. When comparing more than two groups, Bonferroni correction was applied. Significance was set as $P < 0.05$.

1.3. Results

The study included a total of 46 animals: 23 Starved, 11 Cachectic and 12 control dogs. Dogs in control group died due to the following conditions: gunshot wounds, thoracic traumas or skull fractures, acute cardiovascular failure, fatal acute bacterial infection, or strangulation. Among these 2 were euthanatized on humane grounds while the remaining 10 died suddenly. Dogs in cachectic group suffered the following chronic conditions:

neoplasia (including urothelial carcinoma, hepatoid gland carcinoma, haemangiosarcoma, soft tissue sarcoma, ceruminous gland carcinoma, and mammary carcinoma), chronic bacterial infections or degenerative conditions (degenerative disk disease). Among these 5 were euthanatized on humane grounds while the remaining 6 died naturally. Among the starved group, only 2/23 animals were euthanatized. Regarding the age, 13/46 dogs were of unknown age. Among these 10 (6 in Starvation, 2 in Cachectic and Control groups) were tentatively classified as adult, 1 young (pertaining to Starvation group) and 2 undetermined (both in Starvation group). The median age of dogs with known age included in the study was 5.2 years. Among dogs with known age, control dogs had an age range between 1 to 7 years (average 3), while Cachectic and Starved 3 to 15 (average 11) and 1 to 8 (average 2.8) respectively. Within the total number of animals, 19 were female, 27 were male. Among the total of 46, Small, medium and large breed dogs were 9, 25 and 12 respectively. Small breeds included 3 Chihuahua, 2 Yorkshire terriers and a single subject for Jack Russel, puggle, poodle and terrier cross breeds. Medium breeds included 12 Staffordshire bull terriers, 3 English bull terriers, and a single subject for American bulldog cross, springer spaniel, collie, American bulldog, Weimaraner, Dalmatian, Cockapoo and border terrier breeds. A single subject was a non identfied cross breed. Large breeds included 3 Bull mastiffs, 2 German shepherds, 2 Labradors and a single subject for pit bull type, rottweiler, Dogue de Bordeaux, Great Dane and English Bulldog breeds. Liver histological sections were classified according to the degree of autolysis/post-mortem as follows: 17 cases exhibited Grade 1 (mild) post-mortem changes, 20 Grade 2 (moderate) and 9 Grade 3 (severe). These were distributed among the categories as follows: control (2 mild, 6 moderate, 4 severe), cachexia (5 mild, 5 moderate, 1 severe) and starvation (10 mild,9 moderate, 4 severe). Congestion was detected in 17/46 cases 6 in control and cachexia cases, and 5 in Starvation cases); Degeneration was identified in 3 cachectic cases and in two cases of both control and starvation groups. Fibrosis was only detected in one case of the control groups. Periportal tracts were identifiable in all degrees (mild, moderate, severe) of post-mortem changes (Fig 1-3); in particular the bile ducts within the portal tracts were always more preserved than the parenchyma and served as a solid hallmark for the identification of the portal tract. There was no difference in the number of portal tracts per field between degrees of autolysis or no correlation between portal tract numbers and age, sex, or breed group. There was no statistical association between the number of portal tracts per field and the presence of congestion, degeneration or fibroisis. Mean values of the number of periportal spaces was 7 (median 6.4; SD 2.6) for starvation cases (Fig.4), 4,3 (Median 4.1; SD 1.13) for cachectic cases and 2,6 (Median 2.5; SD 0.6) for non-emaciated control cases (Fig.5). The number of portal tracts in control dogs was significantly lower compared to both cachectic (P<0.01) and starved dogs (P<0.01). The number of portal tracts was significantly lower in cachectic dogs compared with starved dog (P<0.01), indicating a higher degree of atrophy in starved dogs compared to cachectic. Considering Starved and Cachectic dogs together (Emaciated), these exhibited significantly more portal tracts per field (median: 5.2; average: 5.5; SD 3) compared to controls. Differences among groups are shown in Fig.6.

GROUPS	MEAN VALUES OF THE NUMBER OF PERIPORTAL SPACES
STARVED DOGS	7
CACHECTIC DOGS	4,3
CONTROL DOGS	2,6

Table 1. Mean values of the number of periportal spaces in assessed groups.

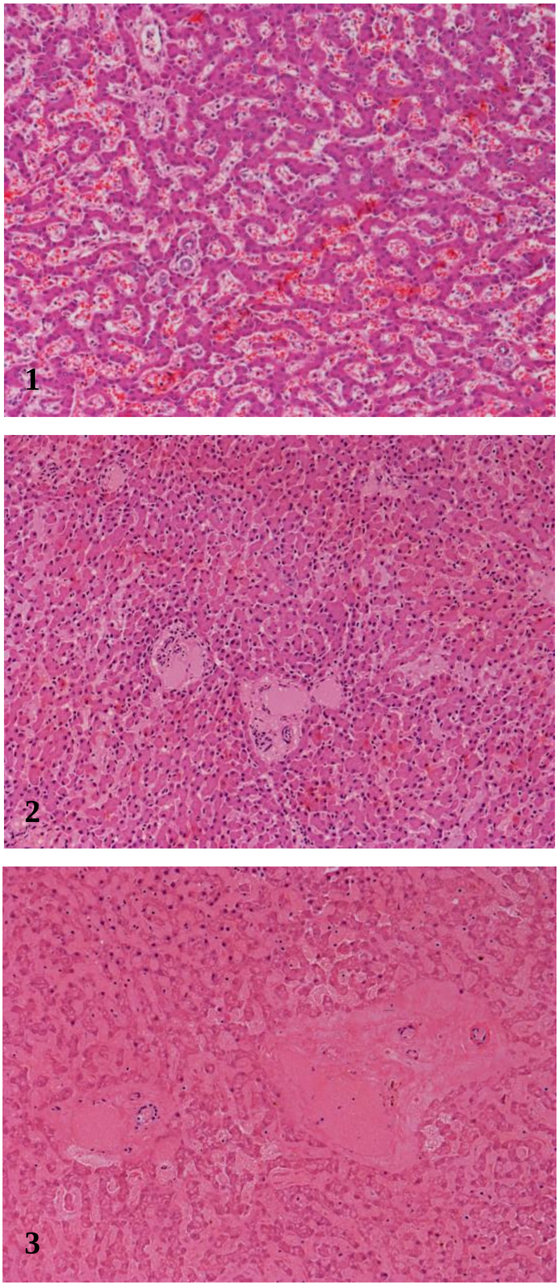

Fig. 1-2-3. Dog, Liver: Different post-mortem changes in liver object of the study; 1. Example of Score 1 (mild) post-mortem changes. (10x) 2. Example of Score 2 (moderate) post-mortem changes (20x). 3. Example of Score 3 (marked) post-mortem changes. Hematoxylin and Eosin (40x).

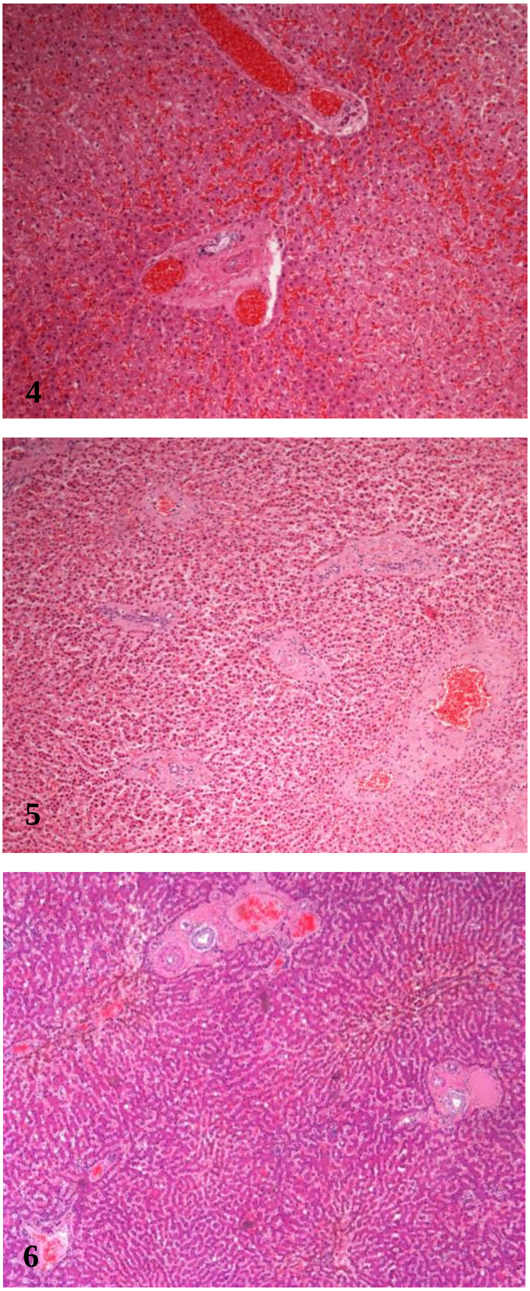

Fig. 4-6: Dog, Liver; Examples of identification of portal tracts in different groups. Fig.4: example of portal tracts (arrows) identified in histological section of a dog from control group. Hematoxylin and Eosin (40x). Fig. 5: Dog, Liver; example of portal tracts (arrows) identified in histological section of a dog from starvation group (40x). Fig. 6: Dog, Liver; example of portal tracts (arrows) identified in histological section of a dog from cachexia group (40x).

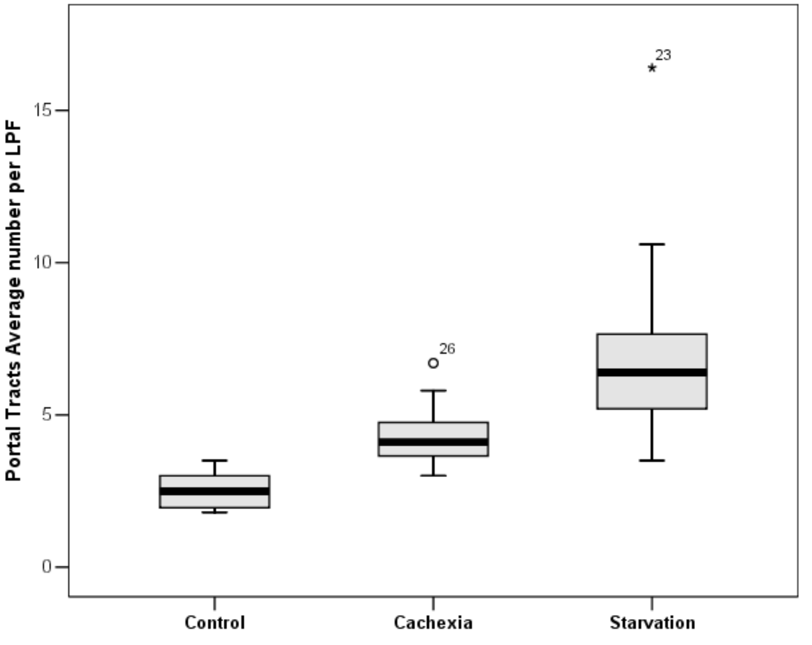

Fig.6: Differences in average numbers of portal tracts per low power field (LPF) among groups. There is a significant difference between Starvation group compared to Cachexia and Control group.

1.4. Discussion

Differential diagnosis of starvation or cachexia should be considered whenever an emaciated body is presented to the post-mortem room. It is the veterinary pathologist job to determine if the reason for an emaciated body condition is a chronic disease, or starvation due to neglect. This has serious implications, as can, in most of the cases, help the decision of a Court of Law, in relation to a criminal case. Cachectic dogs are more likely to have an empty stomach than starved dogs (Jeusette, 2010); This finding suggests that during cachexia, caused by a chronic disease, the animal shows a significant loss of appetite and a voluntary reduction in caloric intake. In starved cases foreign bodies within the stomach or intestine are commonly found in our experience and in published data (Gerdin, 2016). The presence of material, including food and organic and inorganic substances in the stomach demonstrates the presence of perimortem appetite. These two conditions are characterised by different metabolic scenarios, and different organs, including liver, respond and adapt in appropriate ways. Our results are in line with the results of Gerdin and colleagues (Jeusette, 2010) who qualitatively observed macroscopical subjective atrophy of liver in emaciated dogs grossly. This is confirmed histologically in our study grouping Starved and Emaciated dogs, which resulted significantly more atrophic than controls. In our study however, due to the use of a quantitative histological tool, starved dogs resulted in a higher count of periportal tracts per LPF compared to cachectic dogs suggesting that the degree of liver atrophy is objectively higher in starvation compared to cachexia and controls. Starvation and cachexia normally lead to an emaciated animal, however the pathogenesis leading to the loss of body condition as previously discussed is different and liver metabolic activity in these two conditions is different as well. In starvation the animal preserves the appetite, or even exhibit an increase appetite. Liver in starvation has been observed in animal models to undergo atrophy due to reduction in cell size after the 4th day of starvation (Tongiani, 1971), and this is likely due to the generalised "energy saving" program which affects almost all the body organs (Gerdin, 2016). In cancer cachexia, liver atrophy is not reported in humans as being a primary change, in contrary liver mass has been observed to paradoxically increase in cancer cachexia patients (Porporato, 2016). During tumour growth liver is recruited to support the high energy demand of the neoplasm, through gluconeogenesis from the lactate derived from glycolysis. Liver is a metabolically active organ in cancer cachexia, as also demonstrated in a rat model (Dumas, 2011), where mitochondria were observed nutrients-demanding compared to controls, due to the pronounced oxidative activity of these cells. It is however plausible that, in the last stages of cachexia, the organ suffers from the generalised negative energy balance, possibly undergoing a terminal and genuine atrophic process. This atrophy however seems not to reach the degree observed in starvation, at least according to our results. It is also true that animals suffering from severe underlying medical conditions probably reach single or multiple organ failures before the terminal and widespread exhaustion of energy and resources as expected in fatal starvation, otherwise healthy. The histomorphological study highlighted how during starvation, the liver maintains the sub-gross architectural structure composed triads easily identified at the angles of the hepatic lobules, but, as a result of the severe atrophy, the diameter of the hepatocytes is severely reduced, leading to the shortening of the distance between portal tracts. This was interestingly not influenced by the post-mortem changes, as the structure of the portal tract was in all cases recognisable under the microscope, and no differences were seen in number of portal tracts between the three categories of post-mortem changes. This observation is of pivotal importance considering that in the routine of forensic pathology most of the cases are in advanced post-mortem conditions. Kimura and colleagues (Kimura, 1994) investigated histological post-mortem changes in a rat model and identified a shrinkage of subcapsular hepatocytes at 72h and named this as “atrophy”. We believe that since this happened post-mortem the name atrophy is potentially non-correct, however this “shrinkage” did only interest a small area of the liver and did not likely affect the reduction in size of the lobules in the internal portion of the organ, site where the evaluation was performed. Other authors did not find this change in a rat model (Huwait, 2014). It is also interesting to note that the presence of congestion in histological sections, seems not to affect substantially the number of portal tract observable in HPF. No significance was found between lobule size and presence of fibrosis and degeneration, however the degree of such changes in our population was never marked, and we can hypothesise that severe fibrosis or degeneration may have a potential influence in the lobular size, therefore careful judgement should be used. Other quantitative approaches have been adopted to assess liver atrophy, including cell or nuclear size (Bollo, 1999), however the system adopted in the present study appears less time consuming. This approach may be also more robust in regard to possible single hepatocyte shrinkage as described by Kimura and colleagues, since it relies on supercellular lobular anatomical entity, the size of which is affected by parenchymal and stromal components together. A system based on routine stain and brightfield microscope is favoured in the normal diagnostic pathology lab condition, allowing a more rapid quantitative determination as supporting evidence to the other findings in a forensic case. A limiting factor of the present study is represented by the variability of post-mortem changes affecting the sections. This is unfortunately a non-controllable parameter, linked with the wide range of preservation of tissues found in forensic cases. Despite we demonstrated that identification of portal tracts, using morphological features of bile ducts seems not to be affected by the post-mortem changes, it is not possible to completely exclude that in sections with higher degree of post-mortem changes (which has been excluded from the study as above the grade 3), the investigator may fail to identify some portal tracts. It is important to underline that, in this instance, the portal tracts count would be biased in a way that less portal tract would be identified, failing to support a potential true starvation case; in this view, this type of methodological error is far preferred in a forensic scenario, than a situation where a methodological error can support a diagnosis linked with a criminal act “beyond any reasonable doubt”.

1.5. Conclusions

The present study offers new insights in the differential involvement of the liver in the pathogenesis of starvation and cachexia. A quantitative assessment of the number of portal tracts in histological samples can prove to be a useful tool assisting the determination of the cause and mechanism of death in abused animals, not as a unique and definite determination, but as supporting evidence together with the other findings suggestive of starvation. In particular, the suitability of the system for specimens that underwent a degree of autolysis is extremely useful in practical circumstances, where cadavers are commonly retrieved in isolated placed, long after the animals' death.

Bollo E, Bassano B, Peracino V, Biolatti B. Effect of Emaciation on Liver Histology of Alpine Chamois During Winter. *J Wildl Dis*. 1999 Nov 1;35:770–773.

Dumas J-F, Goupille C, Julienne CM, et al. Efficiency of oxidative phosphorylation in liver mitochondria is decreased in a rat model of peritoneal carcinosis. *J Hepatol*. 2010/09/19. 2011 Feb;54:320–327.

Gerdin JA, McDonough SP. Forensic Pathology of Companion Animal Abuse and Neglect. *Vet Pathol*. 2013;50:994–1006.

Gerdin JA, McDonough SP, Reisman R, Scarlett J. Circumstances, Descriptive Characteristics, and Pathologic Findings in Dogs Suspected of Starving. *Vet Pathol*. 2016;53:1087–1094.

Huwait E. Histological Postmortem Study of Rat Liver to Estimate the Time since Death = دراسة هستولوجية لكبد الفئران لتحديد الوقت بعد الوفاة. *J Egypt Acadmic Soc Environ Dev (D-Environmental Stud)*. 2014 Jan 1;15:21–31.

Jeusette I, Greco D, Aquino F, et al. Effect of breed on body composition and comparison between various methods to estimate body composition in dogs. *Res Vet Sci*. 2010;88:227–232.

Kimura M, Abe M. Histology of postmortem changes in rat livers to ascertain hour of death. *Int J Tissue React*. 1994 Feb 1;16:139–150.

Mccue M. Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. *Comp Biochem Physiol A Mol Integr Physiol*. 2010 May 1;156:1–18.

Ottinger T, Rasmusson B, Segerstad CHA, et al. Forensic veterinary pathology, today's situation and perspectives. *Vet Rec*. 2014 Nov

8;175:459 LP – 459.

Petruzzelli M, Wagner EF. Mechanisms of metabolic dysfunction in cancer-associated cachexia. *Genes Dev*. 2016;30:489–501.

Porporato PE. Understanding cachexia as a cancer metabolism syndrome. *Oncogenesis*. 2016;5:1–10.

Kumar, Vinay, Abul K. Abbas, Nelson Fausto, Stanley L. Robbins, and Ramzi S. Cotran. *Robbins and Cotran Pathologic Basis of Disease*. Philadelphia: Elsevier Saunders; 2015.

Tongiani R. Hepatocyte classes during liver atrophy due to starvation in the golden hamster. *Z Zellforsch Mikrosk Anat*. 1971;122:467–478.

Chapter 2

Morphometrical and immunohistochemical examination of kidney as an indirect parameter to assess age in puppies in veterinary forensic pathology

1.1. Introduction

Estimation of age represents a central focus of veterinary forensic pathology. It has recently assumed significant relevance for correct age determininationin puppies illegally imported to Italy as well as to other European countries. As a matter of fact, in some circumstance, puppies younger than 8 weeks are separated from their mothers and littermates and transported illegally within the European Community when they are too young to be moved. Because of these reasons, veterinarians are increasingly demanded to precisely estimate the age of suspected illegally imported puppies. In veterinary literature, the main methods to evaluate the age of puppies are:

- the visual examination of the dentition, specifically completeness of dental eruption and extent of tooth wear.
- the skeletal age, specifically the radiographic appearance and formation of ossification centers.

However, both these methods are affected by environmental factors, such as nutritional, hormonal, and pathological changes. Moreover, these methods are based on subjective observations by the operator. In contrast, the kidney is characterized by a specific postnatal development in which this organ continues to mature both from a functional and anatomical point of view. To be specific, glomeruli continue to mature in the nephrogenic zone. For this reason, it has been hypothesized that due to the different post natal stages of renal development the kidneys might appear histologically different during distinct periods after birth.

In human glomerulogenesis, fetal mesangial and capillary endothelial cells change their immunohistochemical expression of these specific proteins with maturation: CD31, CD34, laminin, a-Smooth Muscle actin and vimentin (Naruse, 2000). Therefore, we hypothesized that the kidney histological and immunohistochemical examinations can be used as an indirect parameter for age determination in puppies' cadavers.

1.2. Materials and Methods

Study design

To this aim, puppies' cadavers were divided in 4 groups defined by age:

- Group A included 12 cadavers with an age between 0 and 15 days,
- Group B included 6 cadavers with an age between 15 and 45 days,
- Group C included 6 cadavers with an age between 45 and 75 days
- Group D included 6 cadavers with an age between 75 and 105 days.

The inclusion criteria were as follow: 1) age (between 0 and 105 days), 2) size (small breed dogs) and 3) absence of renal diseases.

Macroscopic and Histological examination

The forensic necropsies were performed in all cadavers in the necropsy room of the Department of Veterinary Medicine and Animal Production of the University of Naples "Federico II" with a standard forensic necropsy protocol (Piegari et al. 2018). Cadavers showed different degree of post-mortem autolysis. For each case, kidney samples were collected for histopathologic examination; samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 microns, and stained with hematoxylin and eosin (HE). A histological and morphometrical study was performed in order to establish if there is a correlation between the number of glomeruli and age of puppies. The degree of tissue autolysis varied according to the post mortal interval, which was unknown for most of the cadavers, from minimal post mortal artefacts (in reasonably fresh carcases) to marked autolysis (in cadavers presented after prolonged post-mortem interval). Post-mortem kidney histological changes were scored as follows: “Mild” (1): minimal changes, normal cytoplasm, and nuclei of cells visible with identifiable glomeruli; “Moderate” (2): loss of cells details, but with nuclei and cellular outline still visible and identifiable glomeruli; “Severe” (3: loss of nuclear details of the cells with only Bowman’s capsule still identifiable. Cases where the histology of the kidney was severely compromised were excluded from the study. A quantitative histomorphological study of the kidney was performed on 10 randomly chosen, not overlapping microscopic fields at high power magnification (40x), referred as “High Power Fields” (HPFs) using a standard light microscope. Glomeruli were counted manually in each single HPF by two pathologist (ID and GP) and the number subsequently averaged per animal. The SPSS 20.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. The Kruskal Wallis test, a nonparametric test, was used to assess differences of number of glomeruli among groups. P-values < 0.05 were considered statistically significant.

Immunnohistochemiscal examination

Immunohistochemical staining for the evaluation of A-SMA was performed using a horseradish peroxidase (HRP) method (De Biase, 2019). Briefly, 4-μm-thick sections of kidney were mounted on a positively charged glass slides (Bio-Optica, Milan, Italy). Antigen retrieval pretreatments were performed using a heat-induced epitope retrieval (HIER) citrate buffer pH 6.0 (Bio-Optica, Milan, Italy) for 20 min at 98°C. Following, endogenous peroxidase (EP) activity was quenched with 3% hydrogen peroxide (H2O2) in methanol, and sections were blocked with a protein block (MACH1, Biocare Medical LLC, Concord, California, USA) for 30 min each. Slides were sequentially incubated overnight at 4°C with primary antibody diluted in phosphate-buffered saline (PBS) (0.01 M PBS, pH 7.2). Primary antibodies included purified rat anti-mouse CD31 diluted 1:200 (Mec 13.3), monoclonal mouse anti-human Smooth Muscle Actin, (1A4, DAKO), monoclonal mouse anti-vimentin (V9, DAKO). Antibody deposition was visualized using the 3,3′-diaminobenzidine (DAB) chromogen diluted in DAB substrate buffer, and the slides were counterstained with hematoxylin. Between all incubation steps, slides were washed two times (5 min each) in PBS. In the corresponding negative control sections, the primary antibody was either omitted or replaced with a 1:20 dilution of rabbit serum (Code

011-000-120, Jackson Immuno Research, West Grove, PA, USA) according to the most recent and relevant guidelines.

1.3. Results

Histological examination

Kidney histological sections were classified based on the degree of post-mortem autolysis as follows: 5 cases exhibited Grade 1 (mild) post-mortem changes, 25 Grade 2 (moderate) and 10 Grade 3 (severe). Glomeruli were identifiable in all degrees (mild, moderate, severe) of post-mortem changes. Mean values of the number of glomeruli were 11 for animals of Group A (Fig.1), 6 for animals of Group B (Fig. 2), 4 for animals of Group C (Fig. 3) and 2 for animals of Group D (Fig.4). As regard of the statystical analysis, Kruskal wallis Test allowed us to observe statistically significant differences among assessed groups ($p<0.001$).

GROUPS	MEAN VALUES OF THE NUMBER OF GLOMERULI
GROUP A	11
GROUP B	6
GROUP C	4
GROUP D	2

Table 1. Mean values of the number of glomeruli in assessed groups.

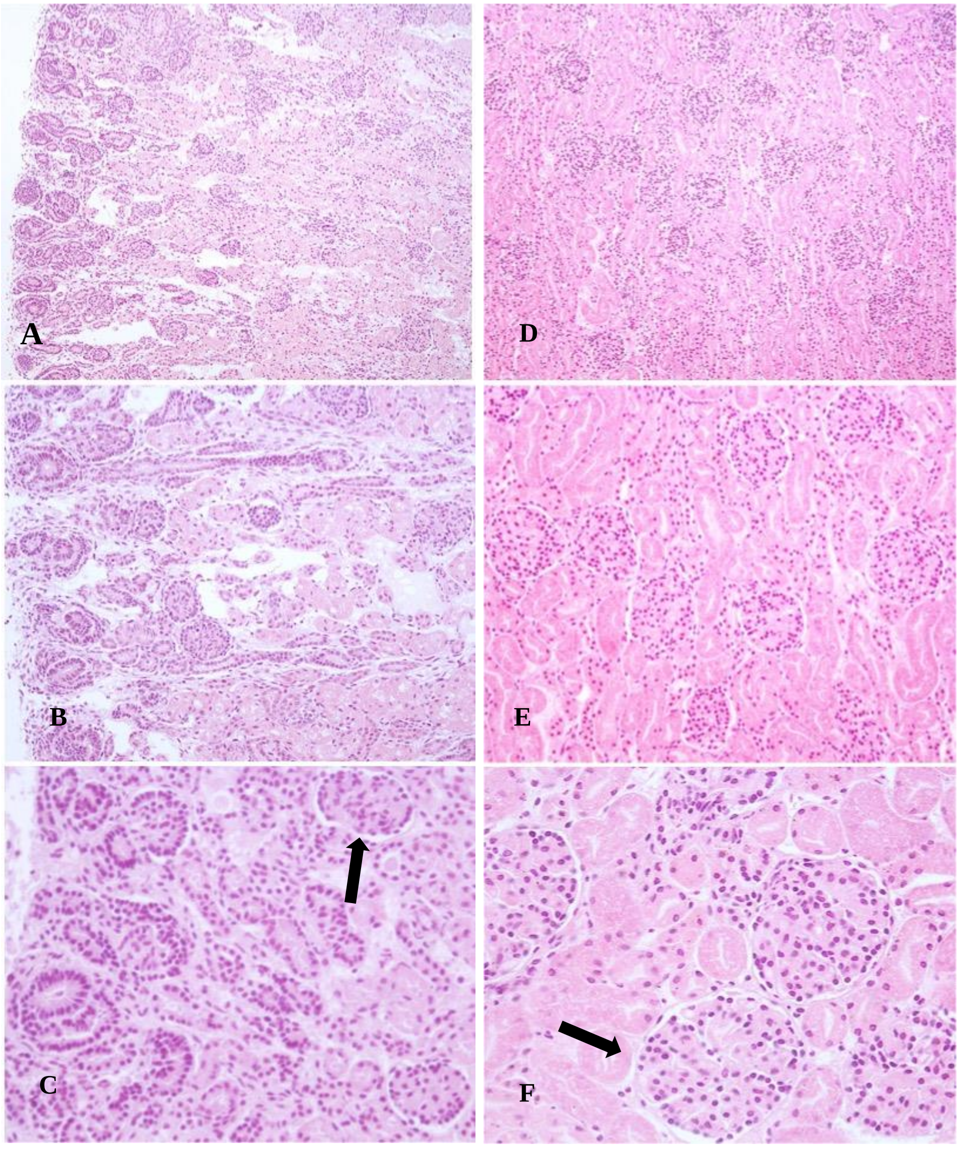

Fig.1: Morphometrical study of the kidney; (A, B, C) example of glomeruli (arrow) identified in histological section of a dog from Group A B (D, E, F). H&E. (Figures A and D: original magnification 10x; figures B and E: original magnification 20x; figures C and F: original magnification 40x).

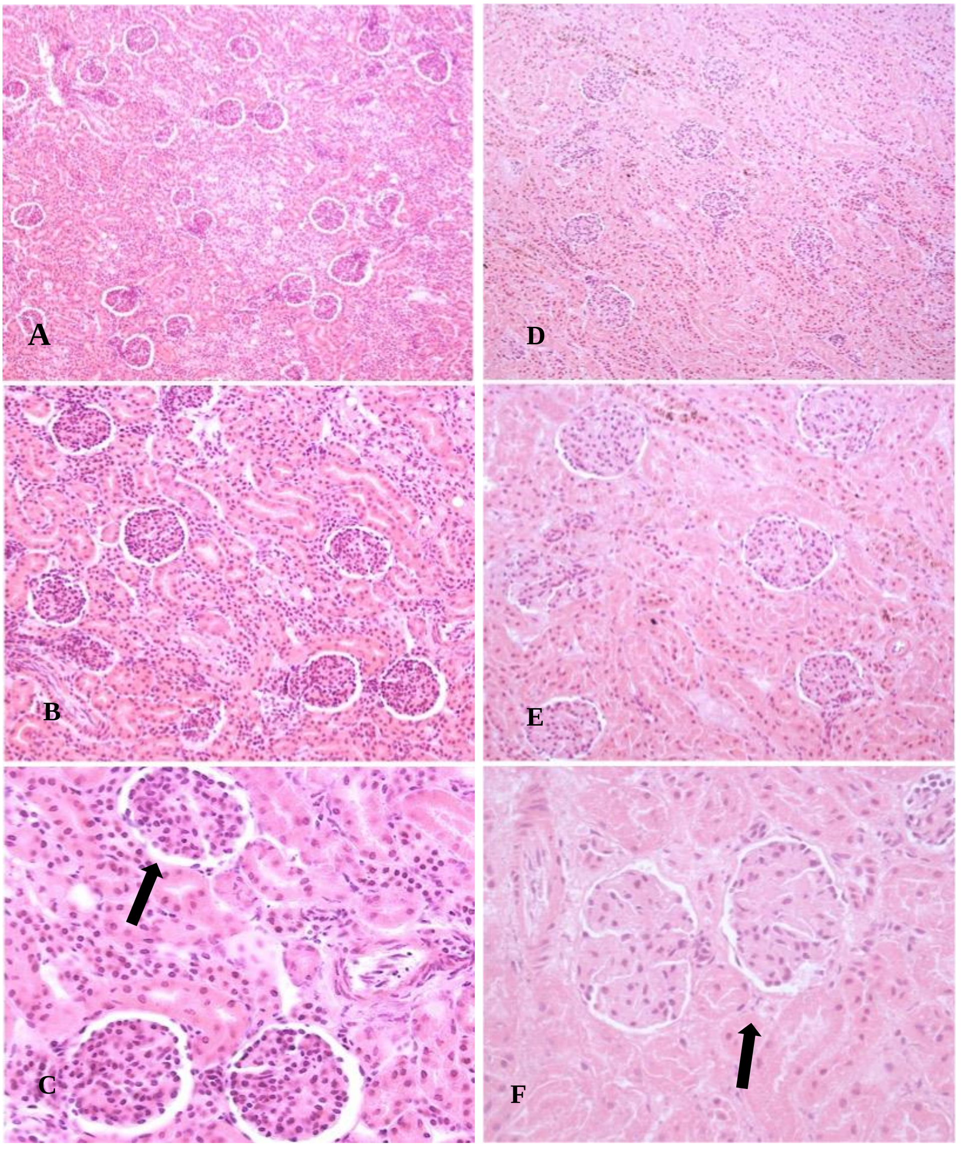

Fig.2: Morphometrical study of the kidney; (A, B, C) example of glomeruli (arrow) identified in histological section of a dog from Group C and Group D (D, E, F). H&E. (Figures A and D: original magnification 10x; figures B and E: original magnification 20x; figures C and F: original magnification 40x).

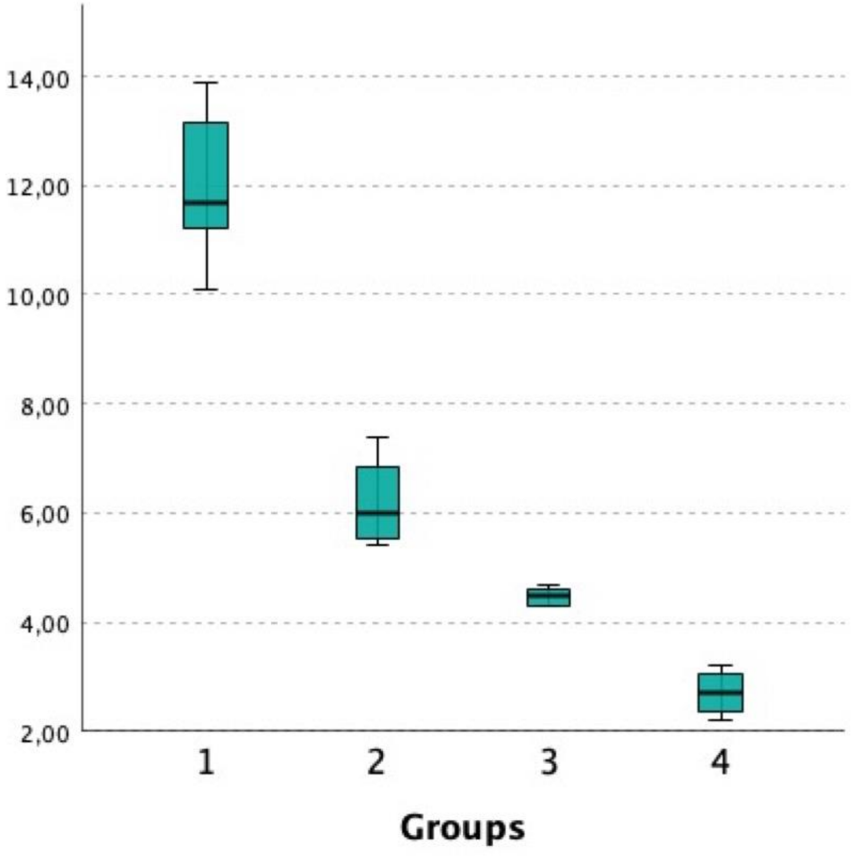

Fig.3: Differences in average number of glomeruli per high power field (LPF) among groups. Kruskal wallis Test allowed us to observe statistically significant differences among assessed groups (p<0.001).

Immunnohistochemiscal examination

Immunohistochemical staining for the evaluation of a-SMA allowed us to observe differences in the localization of immunopositive cells among assessed groups. Immunopositivity to a SMA was observed in endothelial cells of blood vessels of all 4 assessed groups.

In all animals in group A, aggregated mesenchymal cells at the root of immature glomeruli showed a strong immunopositivity to a-SMA, while a reduction of immunopositivity of mesenchymal cells was detected in group B. In addition, group C animals showed loss of immunopositivity of mesenchymal cells and the progressive migration of positive cells towards the periphery of glomeruli with a strong immunopositivity of the vascular pole. Finally, in group D animals, we observed a complete disaperreance of immunopositivity to α-SMA in the glomerulus.

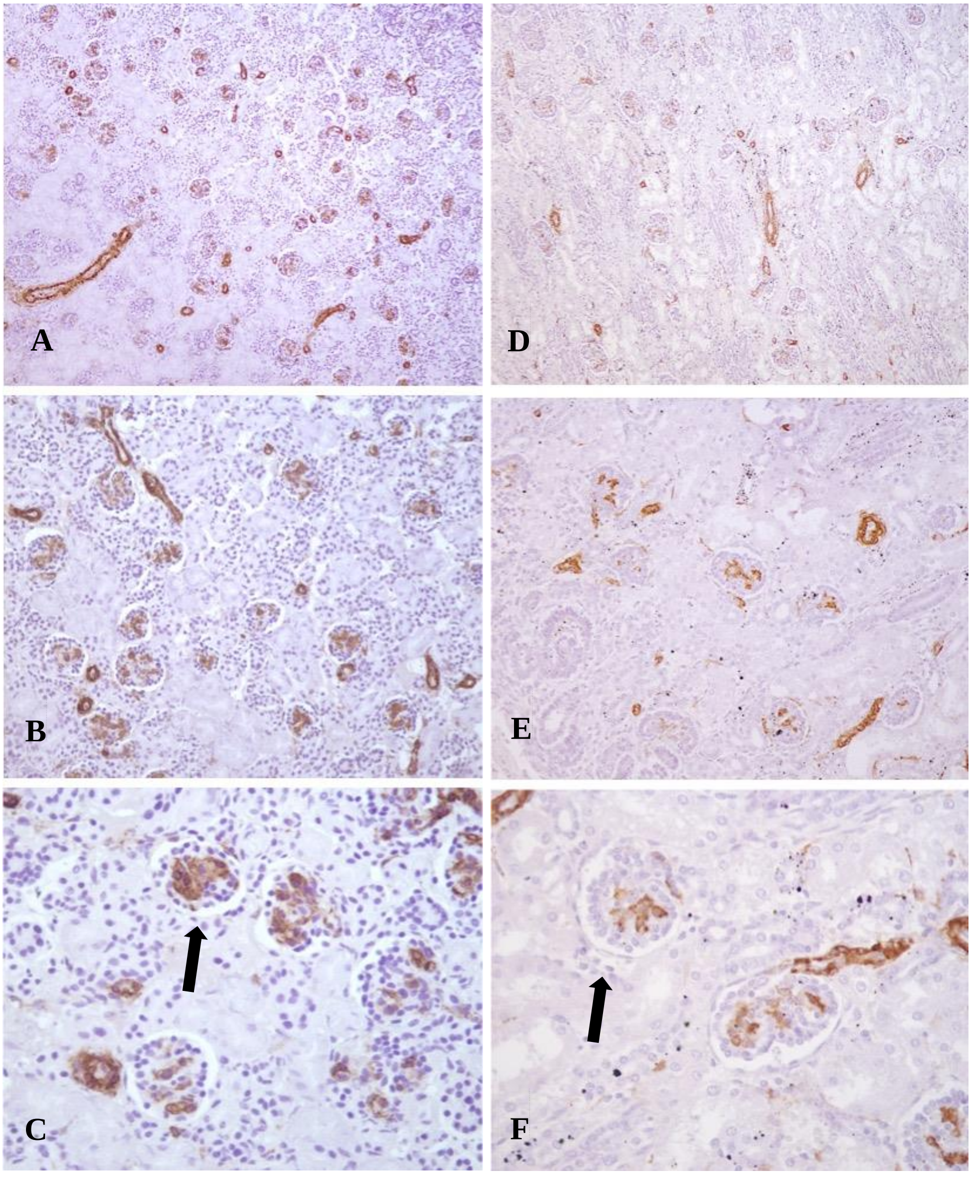

Fig.4: Immunohistochemical study of the kidney; (A, B, C) example of positivity to a-SMA in histological kidney section of a dog from Group A and Group B (D, E, F). (Figures A and D: original magnification 10x; figures B and E: original magnification 20x; figures C and F: original magnification 40x).

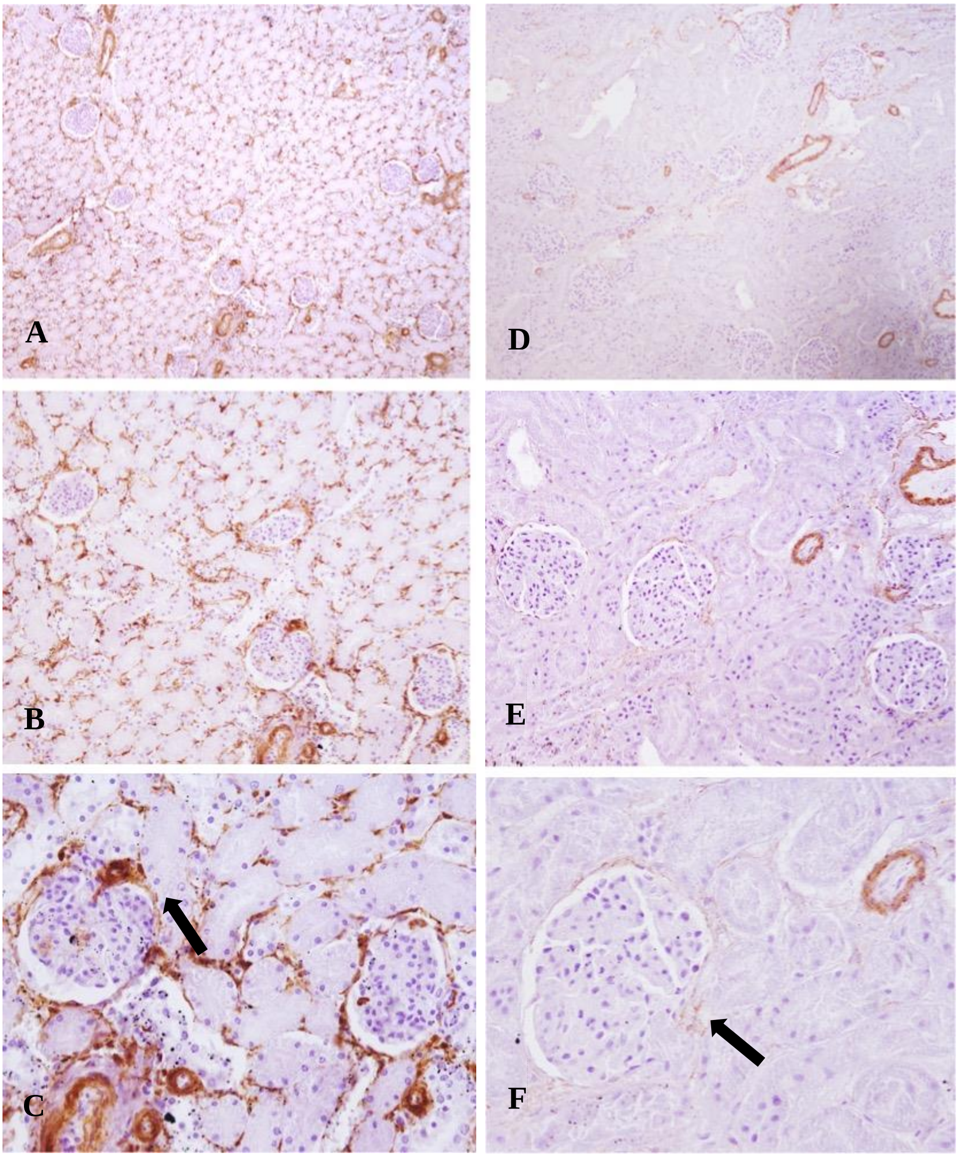

Fig.5: Immunohistochemical study of the kidney; (A, B, C) example of positivity to a-SMA in histological kidney section of a dog from Group C and Group D (D, E, F). (Figures A and D: original magnification 10x; figures B and E: original magnification 20x; figures C and F: original magnification 40x).

1.4. Discussion

The morphometrical analisys of the kidney allowed us to observe a provressive reduction in mean number of glomeruli per field with age. Furthermore, statistical analysis showed statistically significant differences among assessed groups. The correlation between age of puppies and the number of glomeruli could be explained by the progressive reduction of nephorgenic zone and the maturation of glomeruli. Indeed, the nephrogenic zone persists for 15 days in dogs, but the growth and maturation of the kidney continues long after nephrogenesis has ceased (Eisenbrandt, 1979). After 2 weeks of age the size and degree of maturation is dependent upon cortical depth. Glomeruli of the outer cortex are progressively bigger in size leading to the shortening of the distance between them. However, at 75 days after birth the subcapsular tubular zone is well-developed, renal corpuscles are mature and there is less difference in size between corpuscles of the outer and inner cortex. The histological features of the kidney are similar to those of adult dogs (Eisenbrandt, 1979). Therefore, our results suggest that the morphometrical study of the kidney can be a useful tool to estimate age of puppies in association with the visual examination of the dentition and the radiographic appearance and formation of ossification centers.

The immunohistochemical study on the kidney was conducted in order to evaluate if there is a phenotypic transformation of glomerular cells with glomerulogenesis. α-SMA is one of the six isoforms of the intra-cellular microfilament actin. The presence of α-SMA expression has been interpreted as a marker for smooth muscle derived tissue (Gonlusen, 2001). We observed a strong immunopositivity to α-SMA in all animals with an age range between 0 and 15 days. (Group A animals). A reduction of immunopositivity of mesenchymal cells was detected in group B (15 to 15 days) while group C (45 to 85 days) animals showed loss of immunopositivity of mesenchymal cells and the progressive migration of positive cells towards the periphery of glomeruli with a strong immunopositivity of the vascular pole. Lastly, in group D animals (85 to 105 days), we observed a complete disaperreance of immunopositivity to α-SMA in the glomerulus. Our results are in apparent agreements with those reported in humans by Gonlusen who demostrated that mesangial cell in fetal and infant kidney are immunopositive to α-SMA but they are negative in adult kidney. Similar studies were conduceted on fetal rats by Carey et al. and showed similar results. Once glomerular development is completed, and the glomerulus is mature, α-SMA expression disappear completely from the giomeruli. These findings suggest that α-SMA may be a useful marker in the study of the growth and development of maturing glomeruli. Also, it is believed that vasculogenesis of the glomerulus is accomplished by the migration of extraglomerular endothelial cells into the cleft of S-shaped glomeruli. In turn, these endothelial cells are followed by mesenchymal cells that eventually differentiate into mesangial cells (Ekblom, 1981). It is possible that during this active period of glomerulogenesis, cells expressing α-SMA are extraglomerular mesenchymal/endothelial cells with phenotypic characteristics of smooth muscle that migrate into the glomerular anlage to form the glomerular capillary bed. Regardless of the cell type involved, these observations clearly show that the expression of intraglomerular α-SMA is developmentally regulated and that α-SMA expression ceases when glomerular development is completed (Carey, 1992).

1.5. Conclusion

Our findings suggest a potential use of kidney morphometrical and immunohistochemical examination as an indirect parameter to assess the age of puppies in illegal imported animals in association with other methods described in literature. Further studies are needed to increase the immunohistochemical markers in order to better estimate the age of puppies.

References

Carey AV, Carey RM, Gomez RA. Expression of alpha-smooth muscle actin in the developing kidney vasculature. Hypertension. 1992 Feb;19(2 Suppl):II168-75. doi: 10.1161/01.hyp.19.2_suppl.ii168. PMID: 1735573.

De Biase D, Esposito F, De Martino M, Pirozzi C, Luciano A, Palma G, Raso GM, Iovane V, Marzocco S, Fusco A, Paciello O. Characterization of inflammatory infiltrate of ulcerative dermatitis in C57BL/6NCrl-Tg(HMGA1P6)1Pg mice. Lab Anim. 2019 Oct;53(5):447-458. doi: 10.1177/0023677218815718. Epub 2018 Dec 6. PMID: 30522404.

Eisenbrandt DL, Phemister RD. Postnatal development of the canine kidney: quantitative and qualitative morphology. Am J Anat. 1979 Feb;154(2):179-93. doi: 10.1002/aja.1001540205. PMID: 760491.

Ekblom P. Determination and differentiation of the nephron. Med Biol. 1981 Jun;59(3):139-60. PMID: 7311633.

Gonlusen G, Ergin M, Paydaş S, Tunali N. The expression of cytoskeletal proteins (alpha-SMA, vimentin, desmin) in kidney tissue: a comparison of fetal, normal kidneys, and glomerulonephritis. Int Urol Nephrol. 2001;33(2):299-305. doi: 10.1023/a:1015226426000. PMID: 12092643.

Modina SC. Age assessment in puppies: Coming to terms with forensic requests, Forensic Sci Int., 297:8-15. 2019.

Naruse K. An immunohistochemical study of developing glomeruli in human fetal kidneys. Kidney Int., 57(5):1836-46. 2000.

Piegari G, Iovane V, Carletti V, Fico R, Costagliola A, De Biase D, Prisco F, Paciello O. Assessment of Google Glass for Photographic

Documentation in Veterinary Forensic Pathology: Usability Study. JMIR Mhealth Uhealth. 2018 Sep 21;6(9):e180. doi: 10.2196/mhealth.9975. PMID: 30249586; PMCID: PMC6231880.

Chapter 3

Accidental and non-accidental injuries in companion animals in Campania Region

1.1. Introduction

In literature animal abuse is defined as “socially unacceptable behavior that intentionally causes unnecessary pain, suffering, or distress to and/or the death of an animal” (Ascione, 1993). There are several classifications of animal abuse. They include, but are not limited to, physical abuse, emotional abuse, sexual abuse, and neglect. Physical abuse is also known as non-accidental injury (NAI) which is an injury that is purposefully inflicted upon an animal. Animal abuse is any bodily injury that is intentionally inflicted on a vulnerable animal; it is the act of causing direct harm to an animal by inflicting a blunt force trauma, sharp force trauma, shooting, burning, drowning, suffocating, or another similar act (Byrd, 2020). In contrast, accidental injuries (AI) refer to traumatic lesions secondary to causal events, such as falls and collision with a high-speed object (motor vehicle accidents i.e.). The characterization of lesions following accidental and non accidental injuries has been extensively investigated in clinical practice to better treat affected animals. In contrast, only few reports have focused on post-mortem findings. In addiction, no studies have applied the post-mortem forensic examination in monitoring traumatic injuries in both urban and rural contexts. In light of these observations, the aim of this study is to evaluate the application of post-mortem examination in the identification and monitoring of accidental and non-accidental lesions in dogs and cats in Campania region.

1.2. Materials and Methods

Study design

To this aim, we performed a forensic post-mortem examination of every cadaver of dog and cat who was referred to the Unit of Pathology of the Department of Veterinary Medicine and Animal Production, University of Naples between January 2020 and September 2021. Before the forensic necropsy, all cadavers underwent to total body radiographic and CT studies. Radiographs were obtained using digital CR system (Agfa CR-X30, Agfa Italia, Milan, Italy). CT studies were performed using a helical 16-slice scanner (GE Lightspeed, General Electric Italia, Milan, Italy). The forensic necropsies were performed in all cadavers in the necropsy room with a standard forensic necropsy protocol. In addition, tissue samples were collected for histopathologic examination; samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 microns and stained with hematoxylin and eosin (HE) for morphological evaluation of lesions. The joint evaluation of imaging, forensic macroscopic and microscopic examinations of the cadaver allowed us to assess the presence/absence of traumatic injuries. The study population was recruted using the following inclusion criteria: 1) presence of trauma 2) absence of informations about the clinical history or witness testimony 3) outdoor crime scenes. Variables recorded were species, sex, age, and the localization of the injuries. Furthermore, we recorded whether lesions were observed in the skull, thoracic, abdominal, or inguinal regions or if the trauma involved multiple regions. Based on the visualization of dentition and toothwear animals were divided in four categories defined by age: puppies/kittens, juvenile, adult and senior. On the bases of these variables, the injuries were classified as accidental (AI) or non-accidental (NAI). Descriptive statistical analysis was performed using **SPSS® (ver. 13).**

1.3. Results

A total of 450 post-mortem examination were performed on dogs and cats over the period of the study. Of those, 160 were performed on cadavers recovered in outdoor crime scenes with an unknown medical history. 80 cadavers were exluded from the study because they did not meet our inclusion criteria or death occurred for either neoplastic, chronic inflammatory or degenerative condition. The remaining 60 animals met the inclusion criteria and were included in the study. Of the 60 cadavers, 14 were dogs (23%) and 46 were cats (77%). Dogs' population included 6 males (43%) and 8 females (57%); cats population included 24 males (52%) and 22 females (48%).

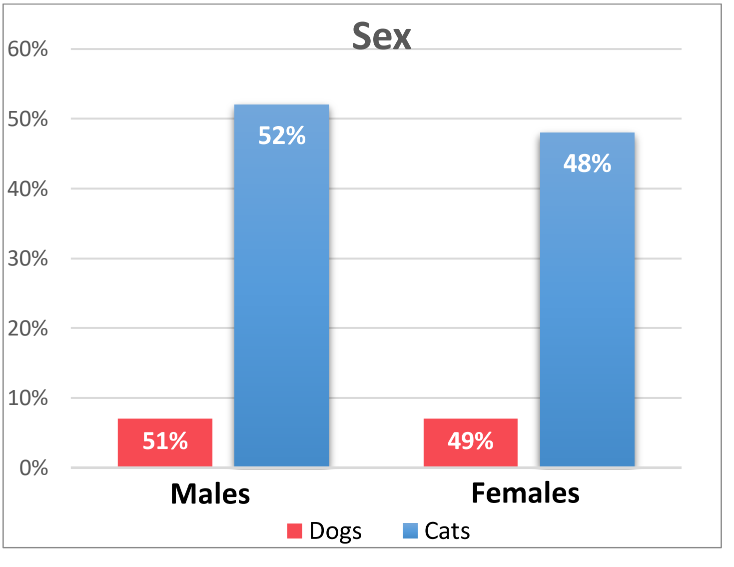

Figure 1. Sex of the dogs and cats examined.

With regards to cats, 4 were kittens (9%), 8 were juvenile (17%), 29 were adults (63%) and 5 were seniors (11%). 5 dogs were puppies (36%), 7 were adults (50%) and 2 were seniors (14%).

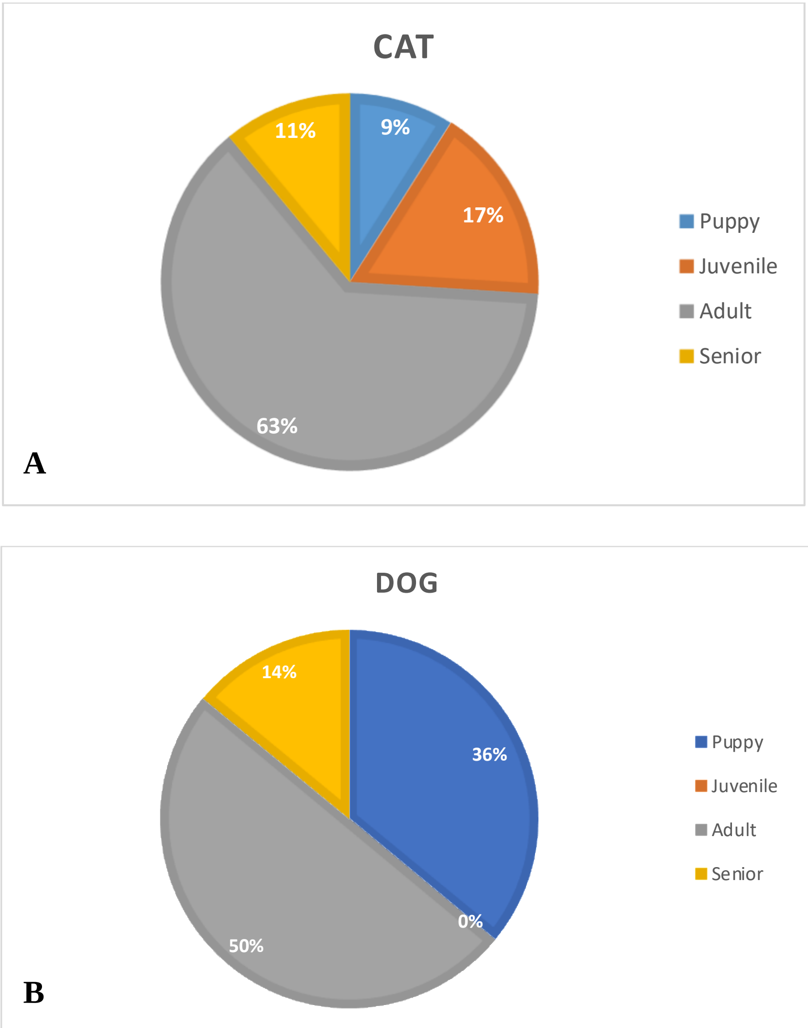

Figure 1. Frequencies of age categories of dogs and cats examined.

In cats, imaging and post-mortem examinations allowed us to observe 30% of lesions localized in the head, 22% in the torax region, 11% in the inguinal region and no injury localized only in the abdominal region. Injuries localized in different regions of the body were seen in the 37% of cases.

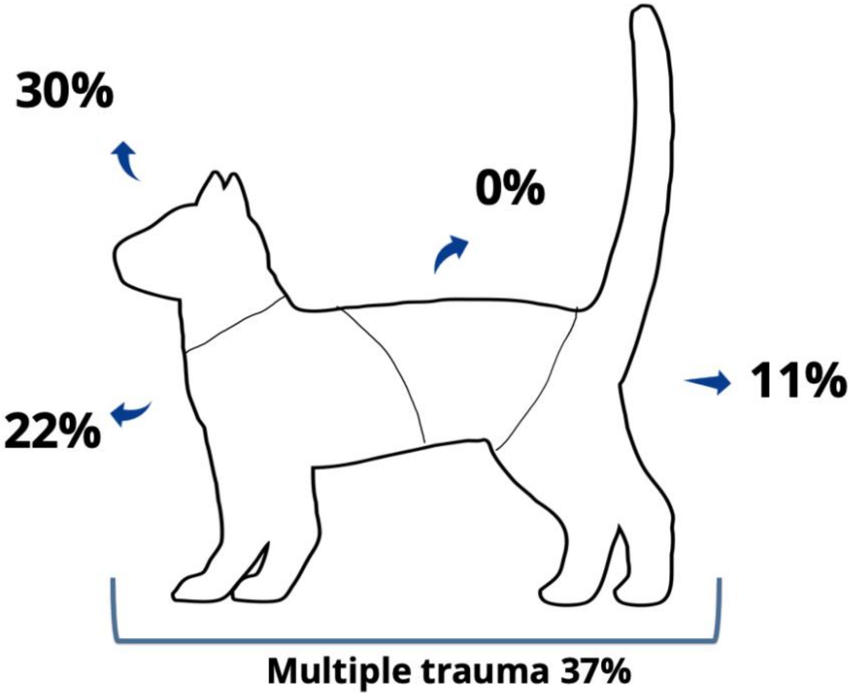

Figure. 3 Distribution of injuries in cats.

In dogs, imaging and post-mortem examination allowed us to observe that the areas most commonly injured were the torax region (43%) followed by the head (14%), the abdominal region (7%) and the inguinal region (7%). Multiple injuries were observed in 29% of cases.

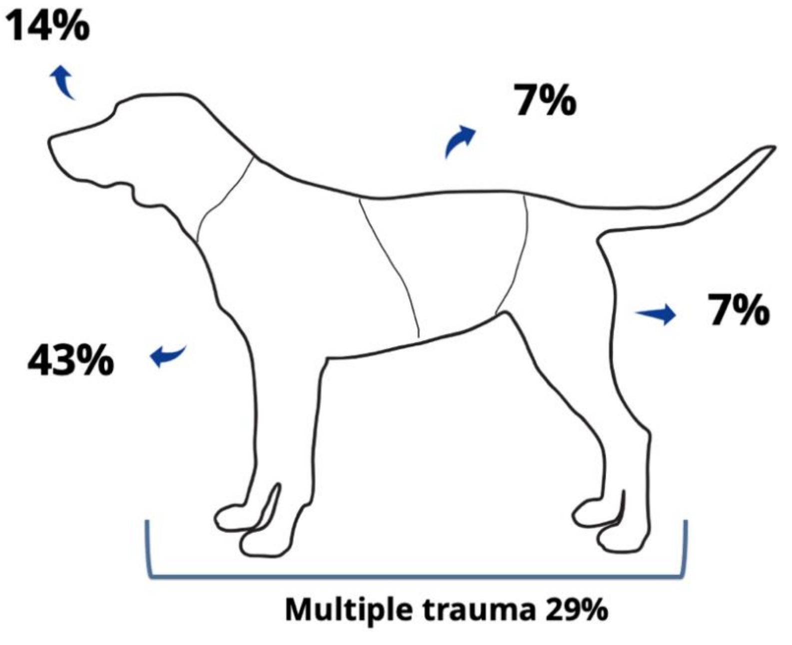

Figure 4. Distribution of injuries in dogs.

Based on the localization and type of lesion, it was possible to discriminate between non-accidental and accidental lesions. The frequency of non-accidental lesions was 57% in the dog and 43,5% in the cat. Of the 43,5% of cats with a diagnosis of non-accidental injury 11 were males (55%) and 9 were females (45%). Dogs were 4 males (50%) and 4 females (50%).

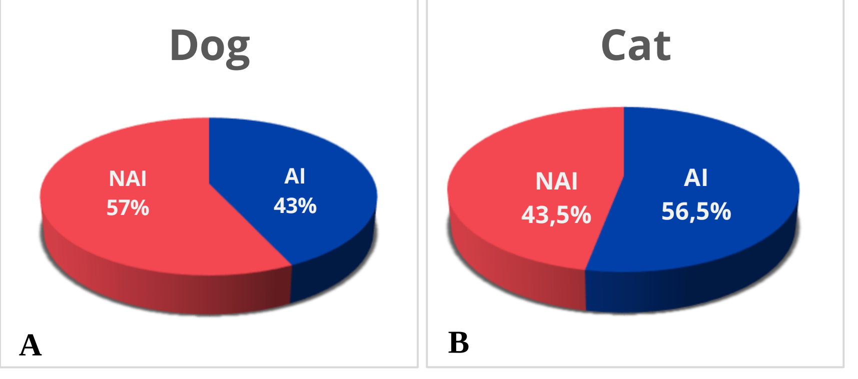

Figure 5. Frequencies of accidental and non-accidental lesions in dogs (A) and cats (B).

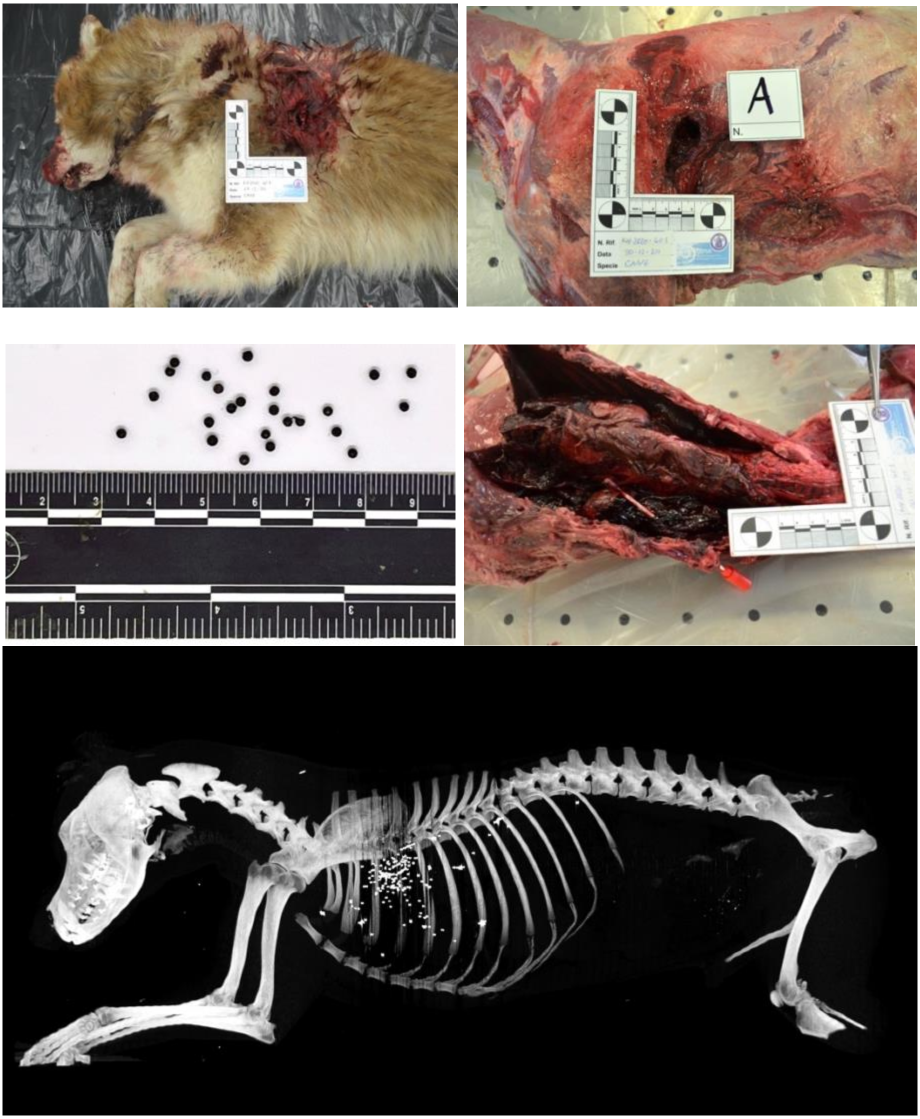

Figure 6. Example of NAI in a dog. Adult, male dog presenting the entry hole of a gunshoot in the left torax that caused laceration of intercostal muscles and rupture of thoracic aorta. Death occured for hypovolemic shock.

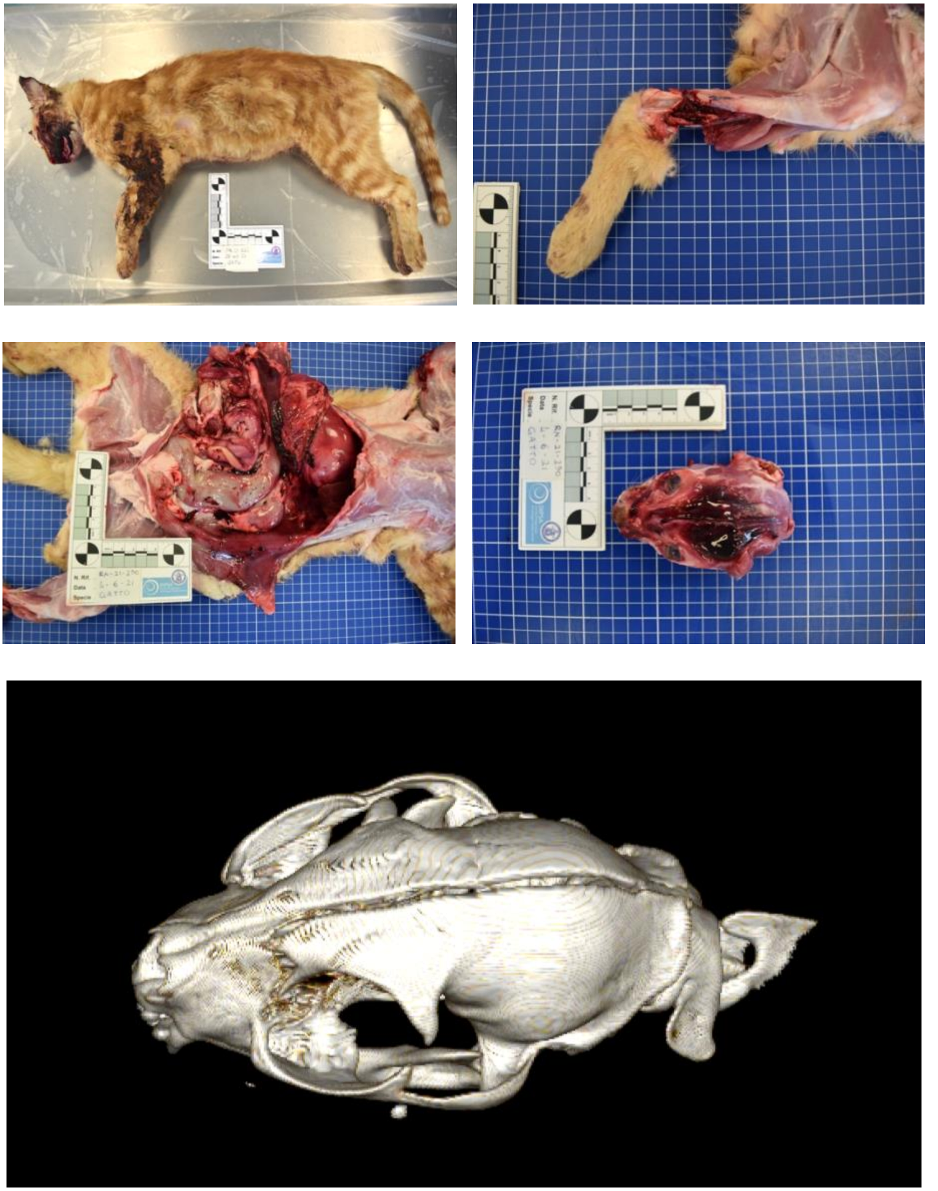

Figure 7. Example of AI in a cat; adult, male cat presenting a complete ftacture of the tibia, peri-mortme laceration of the spleen and head contusion and fracture of the skull.

1.4. Discussion

A total of 60 post-mortem examinations were conducted in this study on animals who met our inclusion criteria. In contrast to previous studies (Luke, 1997; Araújo, 2021), cats were the most affected species (77%, 46 cases of 60) followed by dogs (23%, 14 cases of 60). The main reason of this finding could be that in Campania region most of the stray population is represented by cats. Since our inclusion criteria encompassed cadavers recovered in outdoor crime scenes with no anamnestic data or witness testimony, the majority of the assessed animals were ownerless cats. Furthermore, it is known that cats are more independent than dogs (Araújo, 2021), and spend more time outside on the streets. In contrast, dogs are almost completely dependent on humans for primary needs, such as food, water, etc. throughout their lifetime. Moreover, as stated by Loockwood et al., dog's owners usually search for missing and possibly injured dogs if they do not return home and when the animal is found alive they will likely take it to the veterinarian. No significant difference was observed regarding sex in dogs and cats as previously reported by other authors (Araújo, 2021; Intarapanich, 2016). Other studies reported that male animals can be more prone to wandering on the street in attempt to find an oestrous female and are therefore more likely to be abused or involved in accidental scenarios

(Kolata, 1974; Childs, 1986). In our study 63% of cats and 50% of dogs were adults. Several studies reported instead that young animals are more likely to be abused or involved in motor vehicle accidents; young animals can be less manageble and more immature and therefore provoke aggressive behaviors from abusers. They also tend to explore more new environments and are more likely to be involved in road accident (Rochlitz, 2003; Munro, 2001; Intarapanich, 2016; Araújo, 2021). With regard to injuries location, the main affected region were head in cats (30%) and thorax in dogs (43%). We also observed a high percentage of multiple injuries that involved more than one region of the body in both assessed animals. However, we observed a higher frequency of multiple injuries in cats (37%) compared to dogs (29%). The different localization of these lesions in dogs and cats could be explained by anatomical differences between the examined species. In particular, dogs included in our study were all large breed and much bigger if compared to cats. Indeed, the area over which the kinetic energy is released can affects the degree of injury; the smaller the area over which the energy is placed, the greater the disruption of the affected tissues (Knight, 2004; Ressel, 2016). The joint evaluation of imaging, forensic macroscopic and microscopic examinations of the cadaver allowed us to observe in dogs a frequency of non-accidental lesions of 57% and of 43,5% in cats. A higher frequency of non-accidental trauma in the dog compared to the cat can be explained by the different ethology of the species examined. Dogs are highly social animals and submissive animals, more dependent on human interaction if compared to cats, and maintain these behaviors even when they are the victims of abuse (Intarapanich, 2016; Araújo, 2021); because of these reasons abusers will likely choose them over cats.

In conclusion, this study highlights the usefulness of forensic post-mortem examination of a cadaver in identifying and monitoring accidental and non-accidental lesions in cats and dogs when a body is recovered in outdoor crime scenes and no witness testimony is available.

References

Araújo D, Lima C, Mesquita JR, Amorim I, Ochôa C. Characterization of Suspected Crimes against Companion Animals in Portugal. Animals (Basel). 2021 Sep 20;11(9):2744. doi: 10.3390/ani11092744. PMID: 34573710; PMCID: PMC8464926.

Ascione F.R. Children who are cruel to animals: a review of research and implications,for,developmental,psychopathology. *Anthrozoös*. 1993; 6(4): 226–47. 10.2752/089279393787002105.

Byrd, J.H., Norris, P., & Bradley-Siemens, N. (Eds.). (2020). Veterinary Forensic Medicine and Forensic Sciences (1st ed.). CRC Press. https://doi.org/10.4324/9781315121918

Childs JE, Ross L. Urban cats: characteristics and estimation of mortality due to motor vehicles. Am J Vet Res 1986; 47: 1643– 8.

de Siqueira A, Cassiano FC, de Albuquerque Landi MF, Marlet EF, Maiorka PC. Non-accidental injuries found in necropsies of domestic cats: a review of 191 cases. J Feline Med Surg. 2012 Oct;14(10):723-8. doi: 10.1177/1098612X12451374. Epub 2012 Jun 13. PMID: 22694958.

Intarapanich NP, McCobb EC, Reisman RW, Rozanski EA, Intarapanich PP. Characterization and Comparison of Injuries Caused by Accidental and Non-accidental Blunt Force Trauma in Dogs and Cats. J Forensic Sci. 2016 Jul;61(4):993-9. doi: 10.1111/1556-4029.13074. Epub 2016 Mar 3. PMID: 27364279.

Knight, B, Saukko, P. Knight's Forensic Pathology. 3rd ed. Boca Raton, FL: CRC Press; 2004.

Kolata RJ, Kraut NH, Johnston DE. Patterns of trauma in urban dogs and cats: a study of 1,000 cases. J Am Vet Med Assoc 1974; 164: 499– 502.

Kolata RJ. Trauma in dogs and cats: an overview. Vet Clin North Am Small Anim Pract. 1980 Aug;10(3):515-22. doi: 10.1016/s0195-5616(80)50051-3. PMID: 6970441.

Luke, C.; Arluke, A. Physical Cruelty Toward Animals in Massachusetts, 1975–1996. Soc. Anim. 1997, 5, 195–204.

Marlet, E.F.; Maiorka, P.C. Análise retrospectiva de casos de maus tratos contra cães e gatos na cidade de São Paulo. Braz. J. Vet.
Res. Anim. Sci. 2010, 47, 385–394.

Munro HM, Thrusfield MV. 'Battered pets': non-accidental physical injuries found in dogs and cats. J Small Anim Pract. 2001 Jun;42(6):279-90. doi: 10.1111/j.1748-5827.2001.tb02041.x. PMID: 11440397.

Ressel L, Hetzel U, Ricci E. Blunt Force Trauma in Veterinary Forensic Pathology. Veterinary Pathology. 2016;53(5):941-961.

Rochlitz I. Study of factors that may predispose domestic cats to road traffic accidents: part 1. Vet Rec 2003; 153: 549– 53.

Rochlitz I. Clinical study of cats injured and killed in road traffic accidents in Cambridgeshire. J Small Anim Pract 2004; 45: 390– 4.