UNIVERSITÀ DEGLI STUDI DI NAPOLI
"FEDERICO II"

Tesi di Dottorato

"New Prospective on Sentinel Animal Systems: Experiences in Southern Italy Polluted Areas"

Coordinatore	Candidato	Tutor
Prof. Giuseppe Cringoli	Dott. Alessandro Costagliola	Prof. Orlando Paciello

To My Daughter, Ginevra.

Go Forth and Pursue All Your Dreams

Table of Contents

Table of Contents

List of Abbreviations

ED	Endocrine Disruptors
OS	Organ System
PP	Pathological Process
ADI	Acceptable Daily Intake
ASL	Public National Sanitary System
CKD	Chronic Kidney Disease
GIS	Geographical Information System
PTS	Persistent Toxic Substances
ROS	Reactive Oxygen Species
SCF	Scientific Committee for Food
SIN	Site of National Interest for Remediation
VAM	Veterinary Activity Management
WHO	World Health Organization
PAHs	Hydrocarbons Polycyclic Aromatic
PCBs	Polychlorinated biphenyls
POPs	Persistent Organic Pollutants
IARC	International Agency For Research on Cancer
UNEP	United Nation Environment Programme
VMDB	(American) Veterinary Medical Database
CRIUV	Regional Centre for Veterinary Urban Hygiene
JECFA	Joint FAO/WHO Expert Committee on Food Additives
CReMoPAR	Regional Center for Monitoring Parasitic Infections

List of Figures

List of Figures

List of Figures

List of Tables

Abstract

The aim of the present work is to provide a picture of the current knowledge on Animal Biomonitoring Systems and highlight the link between environmental pollution and Public Health concerns. In this paper are showed the results of three studies performed during the PhD scholarship focused on environmental monitoring using domestic animals as sentinel animal systems.

Environmental biomonitoring through domestic animals appears to be more feasible and effective compared to other research models, because animals share with humans the same risks of exposure to pollutants; the growing need of an early detection of industrial pollutants in the environment, especially micropollutants which have adverse effects at very low concentrations, is at the basis of Public and Environment Health surveillance programs. Furthermore, it's important to disclose the presence of these compounds directly or through certain molecular biomarkers in living organisms rather than in the natural environment, where they are often present below the detection threshold.

Here we report the preliminary data of a project developed in Basilicata region (Southern Italy): Project S.E.BIO.VET. (Environmental Epidemiology Study and VETerinary BIOmonitoring in Basilicata) using sheep as animal sentinel to verify the impact of gas drilling on the environment. Furthermore we investigated the utility of necropsy on pets to evaluate environmental pollution by heavy metals, through a research project performed on the entire Campania Region, leaded by the Regional Referal Center of Veterinary Urban Hygene (CRIUV). Laslty, we provide new insights for the use of dairy cattle as early warning for environmental

pollution by nitrate and nitrite, highlighting new aspects of nitrate toxicosis which may occur both in animals and humans.

Results of this work confirmed that dog is a good sentinel animal system in urban and rural areas, and can be used for epidemiological and comparative pathology studies for tumors in humans. Dairy cattle are one of the most sensible specie to nitrate and nitrite toxicosis, compounds which can be released even in milk which can be a dangerous source of exposure for humans. In this study, the preliminary data of SEBIOVET project confirm the crucial role of small ruminants to monitor environmental pollution on large scale, to have indications on the presence of unknown polluted site, or to monitor already known contaminated areas.

GENERAL INTRODUCTION

I. Animal biomonitoring and micropollutants in public health

A pollutant is defined as a substance that interfere directly or indirectly with biological processes of animals, plants or the environment of an ecosystem (Tietenberg, T. 2006). The problem of its detection may acquire great importance in the case of micropollutants (i.e. heavy metals, dioxins, etc.), usually present in the environment at very low concentrations (Rombolà P. 2012). Furthermore, even if the environmental chemistry techniques may provide a direct measure of the concentration of a broad spectrum of pollutants into different environmental media (soil, water, air), such analytical methods are often laborious, expensive and not always suitable to return information on the effects of pollutants on the quality and health of the environment. The use of specific biomarkers for the assessment of Environmental quality is the basis of the definition of "Environmental biomonitoring" (Rombolà P. 2012). This term refers to a regular and systematic evaluation of the conditions of the environment through a set of scientific methods that use animal or plant species to measure the impact of polluting agents on the environment itself (Bragazza A. et al. 2000). In polluted ecosystems, toxic substances can alter the state of health of living organisms causing a stress syndrome which makes them more vulnerable to further environmental variations, and that can be suitably quantified through the use of appropriate "Bioindicators" (Rombolà P. 2012). For example, monitoring the concentration of toxins in animal tissues, or the rate at which

some diseases occur in animal populations, or changes in their physiological behavior, can be considered good tools to evaluate the health state of the environment (Grabarkiewicz DJ. et al. 2008). The most significant advantages of animal biomonitoring reather than instrumental methods of environmental chemistry, include:

- the possibility to obtain data on large scale (assuming that the presence of the animal species selected as bioindicator is ubiquitarious in the area of interest)
- the possibility to evaluate the effects of the synergic interaction of different pollutants on the organism;
- easiest way to detect some pollutants that are usually present at low dosage in the environment: levels of contaminants that bioaccumulate are often higher in organism tissues than those found in the substrate in which they were dispersed (water, air, sediment)

On the contrary, the use of living organisms as bio-indicators is also a source of critical aspects:

- it's possible only an "Indirect estimation" of pollutants concentration in the environment, according to the study of toxicity indices;
- the high variability of environmental conditions and different responses and adaptation of living organisms, are not easy to interpret and need a high competence.

However, environmental biomonitoring and classical instrumental methods do not represent alternative approaches to evaluate the health of an ecosystem, nor one method excludes the other; by contrast they can be

considered complementary instruments to be applied in epidemiologic surveillance programs. Biological monitoring of environment can be performed on large areas and throughout long period of investigation, and it can be considered an important baseline for further steps allowing more targeted and less expensive instrumental chemical analysis (Rombolà P. 2012).

II. Bioavailability and hazard of heavy metals contamination

Heavy metals are defined as metallic elements that have a relatively high density compared to water (Fergusson JE.1999). In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by these metals. Also, human exposure has risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic and technological applications (Bradl H 2002). Although heavy metals are naturally occurring elements that are found throughout the earth's crust, most environmental contamination and human exposure result from anthropogenic activities such as mining and smelting operations, industrial production and use, and domestic and agricultural use of metals and metal-containing compounds (He ZL 2005). In biological systems, heavy metals have been reported to affect cellular organelles and components such as cell membrane, mitochondrial, lysosome, endoplasmic reticulum, nuclei, and some enzymes involved in metabolism, detoxification, and damage repair (Wang S 2001).

Their toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals. Metal ions have been found to interact with cell components such as DNA and nuclear proteins, causing DNA damage and conformational changes that may lead to cell cycle modulation, carcinogenesis or apoptosis (Tchounwou PB, 2014). Several studies have demonstrated that reactive oxygen species (ROS) production and oxidative stress play a key role in the toxicity and carcinogenicity of metals such as arsenic (Yedjou CG 2006, 2007), cadmium (Tchounwou PB 2001), lead (Yedjou CG 2007), and mercury (Sutton D 2002). Because of their high degree of toxicity, these five elements rank among the priority metals that are of great public health significance. They are all systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. According to the United States Environmental Protection Agency (U.S. EPA), and the International Agency for Research on Cancer (IARC), these metals are also classified as either “known” or “probable” human carcinogens based on epidemiological and experimental studies showing an association between exposure and cancer incidence in humans and animals.

Heavy metal-induced toxicity and carcinogenicity involves many mechanistic aspects, some of which are not clearly elucidated or understood. However, each metal is known to have unique features and physic-chemical properties that confer to its specific toxicological mechanisms of action.

II.I. Lead

Lead is a naturally occurring bluish-gray metal present in small amounts in the earth's crust. Although lead occurs naturally in the environment, anthropogenic activities such as fossil fuels burning, mining, and manufacturing contribute to the release of high concentrations. In recent years, the industrial use of lead has been significantly reduced from paints and ceramic products, caulking, and pipe solder. In humans, exposure to lead occurs mainly via inhalation of lead-contaminated dust particles or aerosols, and ingestion of lead-contaminated food, water, and paints. Adults absorb 35 to 50% of lead through drinking water and the absorption rate for children may be greater than 50%. Lead absorption is influenced by factors such as age and physiological status. In the human body, the greatest percentage of lead is taken into the kidney, followed by the liver and the other soft tissues such as heart and brain, however, the lead in the skeleton represents the major body fraction (Flora SJS 2006).

One of the major mechanisms by which lead exerts its toxic effect is through biochemical processes that include lead's ability to inhibit or mimic the actions of calcium and to interact with proteins. Lead binds to sulfhydryl and amide groups of enzymes, altering their configuration and diminishing their activities. Lead may also compete with essential metallic cations for binding sites, inhibiting enzyme activity, or altering the transport of essential cations such as calcium (Flora SJ 2007). Many investigators have demonstrated that lead intoxication induces a cellular damage mediated by the formation of reactive oxygen species (ROS). Experimental studies have indicated that

lead is potentially carcinogenic, inducing renal tumors in rats and mice (Walkers MP 1995), and is therefore considered by the IARC as a probable human carcinogen. Lead exposure is also known to induce gene mutations and sister chromatid exchanges, morphological transformations in cultured rodent cells, and to enhance anchorage independence in diploid human fibroblasts (Hwua YS 1998). In vitro and in vivo studies indicated that lead compounds cause genetic damage through various indirect mechanisms that include inhibition of DNA synthesis and repair, oxidative damage, and interaction with DNA-binding proteins and tumor suppressor proteins.

II.II. Mercury

Mercury is a widespread environmental toxicant and pollutant which induces severe alterations in the body tissues and causes a wide range of adverse health effects (Bhan A. 2005). Both humans and animals are exposed to various chemical forms of mercury in the environment. These include elemental mercury vapor (Hg^0), inorganic mercurous (Hg^{+1}), mercuric (Hg^{+2}), and the organic mercury compounds. Because mercury is ubiquitous in the environment, humans, plants and animals are all unable to avoid exposure to some form of mercury (Holmes P. 2009). Mercury is utilized in the electrical industry (switches, thermostats, batteries), dentistry (dental amalgams), and numerous industrial processes including the production of caustic soda, in nuclear reactors, as antifungal agents for wood processing, as a solvent for reactive and precious metal, and as a preservative

of pharmaceutical products (Tchounwou PB 2003). Humans are exposed to all forms of mercury through accidents, environmental pollution, food contamination, dental care, preventive medical practices, industrial and agricultural operations, and occupational operations. Mercury enters water as a natural process of off-gassing from the earth's crust and also through industrial pollution (Dopp E 2004). Algae and bacteria methylate the mercury entering the waterways. Methyl mercury then makes its way through the food chain into fish, shellfish, and eventually into humans (Sanfeliu C 2003).

The molecular mechanisms of toxicity of mercury are based on its chemical activity and biological features which suggest that oxidative stress is involved in its toxicity (Valko M 2005). Through oxidative stress mercury has shown mechanisms of sulfhydryl reactivity. Once in the cell both Hg^{2+} and MeHg form covalent bonds with cysteine residues of proteins and deplete cellular antioxidants. Antioxidant enzymes serve as a line of cellular defense against mercury compounds. The interaction of mercury compounds suggests the production of oxidative damage through the accumulation of reactive oxygen species (ROS) which would normally be eliminated by cellular antioxidants. Hence, mercury has been shown to induce the formation of ROS known to cause DNA damage in cells, a process which can lead to the initiation of carcinogenic processes (Valko M 2006). The direct action of these free radicals on nucleic acids may generate genetic mutations. Although mercury-containing compounds are not mutagenic in bacterial assays, inorganic mercury has been shown to induce mutational events in eukaryotic cell lines at very low doses. These free radicals may

also induce conformational changes in proteins that are responsible for DNA repair, mitotic spindle, and chromosomal segregation. To combat these effects, cells have antioxidant mechanisms that work to correct and avoid the formation of ROS (free radicals) in excess. These antioxidant mechanisms involve low molecular weight compounds such as vitamins C and E, melatonin, glutathione, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase that protect the cells by chelating mercury and reducing its oxidative stress potential (Pinheiro MC 2008).

II.III. Arsenic

Arsenic is a ubiquitous element that is detected at low concentrations in virtually all environmental matrices. Environmental pollution by arsenic occurs as a result of natural phenomena such as volcanic eruptions and soil erosion, and anthropogenic activities. Several arsenic-containing compounds are produced industrially, and have been used to manufacture products with agricultural applications such as insecticides, herbicides, fungicides, algicides, sheep dips, wood preservatives, and dye-stuffs. They have also been used in veterinary medicine for the eradication of tapeworms in sheep and cattle (Tchounwou PB 1999). It is estimated that several million people are exposed to arsenic chronically throughout the world, especially in countries like Bangladesh, India, Chile, Uruguay, Mexico, Taiwan, where the ground water is contaminated with high concentrations of arsenic. Exposure to arsenic occurs via the oral route (ingestion),

inhalation, dermal contact, and the parenteral route to some extent (Tchounwou PB 1999). Diet, for most individuals, is the largest source of exposure, with an average intake of about 50 µg per day. Intake from air, water and soil are usually much smaller, but exposure from these media may become significant in areas of arsenic contamination.

Contamination with high levels of arsenic is of concern because arsenic can cause a number of human health effects. Several epidemiological studies have reported a strong association between arsenic exposure and increased risks of both carcinogenic and systemic health effects. Interest in the toxicity of arsenic has been heightened by recent reports of large populations in West Bengal, Bangladesh, Thailand, Inner Mongolia, Taiwan, China, Mexico, Argentina, Chile, Finland and Hungary that have been exposed to high concentrations of arsenic in their drinking water and are displaying various clinico-pathological conditions including cardiovascular and peripheral vascular disease, developmental anomalies, neurologic and neurobehavioural disorders, diabetes, hearing loss, portal fibrosis, hematologic disorders (anemia, leukopenia and eosinophilia) and tumors. Several studies have indicated that the toxicity of arsenic depends on the exposure dose, frequency and duration, the biological species, age, and gender, as well as on individual susceptibilities, genetic and nutritional factors. Most cases of human toxicity from arsenic have been associated with exposure to inorganic arsenic (Abernathy CO 1999).

Epidemiological investigations have indicated that long-term arsenic exposure results in promotion of carcinogenesis. Several hypotheses have been proposed to describe the mechanism of arsenic-induced carcinogenesis.

Zhao et al. (1997) reported that arsenic may act as a carcinogen by inducing DNA hypomethylation, which in turn facilitates aberrant gene expression. In another study, Trouba et al. (2000) concluded that long-term exposure to high levels of arsenic might make cells more susceptible to mitogenic stimulation and that alterations in mitogenic signaling proteins might contribute to the carcinogenic action of arsenic. Collectively, several recent studies have demonstrated that arsenic can interfere with cell signaling pathways (e.g., the p53 signaling pathway) that are frequently implicated in the promotion and progression of a variety of tumor types in experimental animal models, and of some human tumors (Vogt BL. 2001). However, the specific alterations in signal transduction pathways or the actual targets that contribute to the development of arsenic-induced tumors in humans following chronic consumption of arsenic remains uncertain.

II.IV. Cadmium

Cadmium is a heavy metal of considerable environmental and occupational concern. It is widely distributed in the earth's crust at an average concentration of about 0.1 mg/kg. The highest level of cadmium compounds in the environment is accumulated in sedimentary rocks, and marine phosphates contain about 15 mg cadmium/kg (Gesamp 1987). Cadmium is frequently used in various industrial activities. The major industrial applications of cadmium include the production of alloys, pigments, and batteries. Although the use of cadmium in batteries has shown considerable

growth in recent years, its commercial use has declined in developed countries in response to environmental concerns (Wilson DN 1988). The main routes of exposure to cadmium are via inhalation or cigarette smoke, and ingestion of food. Skin absorption is rare. Human exposure to cadmium is possible through a number of several sources including employment in primary metal industries, eating contaminated food, smoking cigarettes, and working in cadmium-contaminated work places, with smoking being a major contributor. Other sources of cadmium include emissions from industrial activities, including mining, smelting, and manufacturing of batteries, pigments, stabilizers, and alloys.

Although the mechanisms of cadmium toxicity are poorly understood, it has been speculated that cadmium causes damage to cells primarily through the generation of ROS, which causes single-strand DNA damage and disrupts the synthesis of nucleic acids and proteins (Stohs SJ 1995).

Cadmium compounds are classified as human carcinogens by several regulatory agencies. The International Agency for Research on Cancer and the U.S. National Toxicology Program have concluded that there is adequate evidence that cadmium is a human carcinogen. This designation as a human carcinogen is based primarily on repeated findings of an association between occupational cadmium exposure and lung cancer, as well as on very strong rodent data showing the pulmonary system as a target site (IARC 1993). Thus, the lung is the most definitively established site of human carcinogenesis from cadmium exposure. Other target tissues of cadmium carcinogenesis in animals include injection sites, adrenals, testes, and the hemopoietic system (IARC 1993).

III. Environmental monitoring: bioindicators, bioaccumulators and biomarkers

The indicator species of environmental pollution are divided in *bioindicators* and *bioaccumulators*; instead, it's at the molecular or cellular level that is mostly defined the concept of *biomarkers*. Bioindicator organisms are plants or animals such defined by their ability to highlight environmental changes. The response to environmental changes may occur in different ways: absence/decline or overgrowth of such specie in the ecosystem, behavioral changes or increased number of malformations and/or diseases. The effects of certain pollutants are then used, with an indirect approach, to trace the actual concentrations of these substances in the environment. Such approach is firmly based on the quality and sensitivity of the indicator specie to a given pollutant.

A bioaccumulator is a plant or animal that accumulates environmental contaminants in its tissues; in environmental monitoring a bioaccumulator can be used as an indicator of pollution by these compounds, especially where amounts of pollutant in the environment are too low to be easily detectable. Usually the toxic substance accumulates in the tissues without significantly changing the survival rate (Tolerance), at least within a certain range, beyond which toxic effects are observed. The bio-accumulators are, therefore, organisms capable of surviving in the presence of a contaminant absorbed by the environmental media (air, water, soil), accumulating it and allowing its determination and quantification. The chemicals monitored in bioaccumulation are usually micropollutants such as heavy metals (i.e. Zn,

Cd, Cu, Hg, Pb), pesticides, PCBs (polychlorinated biphenyls), PAHs (hydrocarbons Polycyclic aromatic) and radionuclides. An example of bioaccumulator organisms are mussels, used for the monitoring of coastal marine water quality, for their ability to accumulate heavy metals. Some species, such as bees (or lichens), behave both as bio-indicators and bio-accumulators.

Typically, biomarkers are defined as quantitative measures of changes in the biological system that respond to either (or both) exposure to, and/or doses of, xeonbiotic substances that lead to biological effects. Although not explicitly contained in most definitions, the use of the term “biomarker” or “biomarker response” is often restricted to cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism are indicative of xenobiotic exposure and/or its effect (Lam PK et al. 2003).

As a rule, the choice to study a bioindicator specie, a bioaccumulator or a biomarker among the main biomonitoring methods depends on the type of environment to be monitored and the type of evaluation of the pollutant (estimation of its effects or direct measurement) (Figure I).

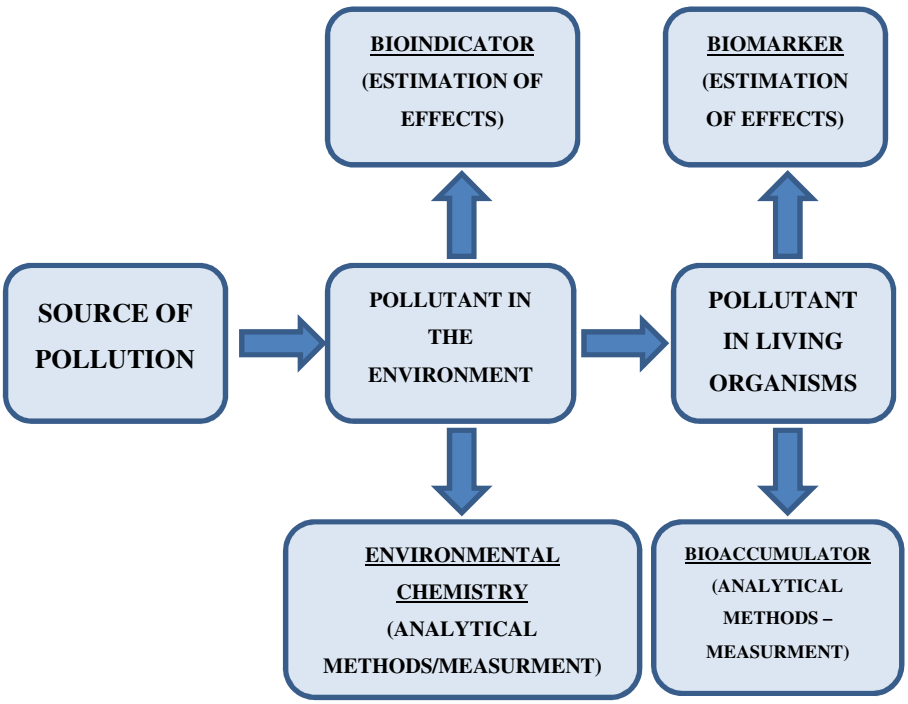


Fig. I. Main methods of biomonitoring (Rombolà P et al. 2012)

Ideal profiles of bioindicators and bioaccumulators used in environmental monitoring should include:

- good spatial coverage of the specie within the ecosystem (throughout all the seasons);
- life cycle and behavior which can increase the chance of exposure to pollutants;
- low mobility (though this consideration is to be done always in relation to the studied area);
- genetic uniformity;

- affordable assessment of the expected operating and maintenance costs of the analysis.

By the way, it's good to remind that the value of indicator species approach in environmental monitoring is low in the absence of other supporting data. But, careful choice of indicator species applied to well defined problems may be useful in detecting regional and site-specific contamination (US Environmental Protection Agency, 2012).

IV. Sentinel Animal Systems

The use of nonhuman organisms as early warning systems for human health risk is not new. In the early part of the 20th century, miners in Great Britain and the United States took caged canaries into coalmines in order to provide warning of the presence of toxic gases including carbon monoxide and methane. The concept of the "canary in the coal mine" giving warning of a human health hazard is based on several principles. First, canaries were found to be more sensitive than both humans and other animals such as mice to the toxic effects of carbon monoxide. Second, the birds were allowed to share the same air exposures as the humans. Third, the occurrence of carbon monoxide poisoning in a bird was quite recognizable to the miners, since sick birds would tend to fall off of their perches and appear visibly ill (Rabinowitz P et al. 2009).

The neurobehavioral symptoms displayed by some cats from Minamata Bay in Japan in the 1950s provide a good example of a serendipitous observation

that had significance for human health. In 1956, Minamata disease was described by two physicians in Japan, after observing an unusual number of patients with central nervous system disorders with unknown causes . An extensive three year study identified the causative agent to be organic mercury . Epidemiologic follow-up suggested that the cause of the outbreak was the release of mercury into the bay from a chloralkali production facility. This industrial pollution of the surrounding waters resulted in accumulation of mercury in fish and the consumption of these mercury-contaminated fish by local families, many of whom developed mercury poisoning that was most severe in infants and young children. Only after these tragic cases the authorities discovered the connection between the onset of disease in humans and the development several years earlier of neurological disease (called “dancing cat disease”) in local cats that were consuming large amounts of fish from the polluted harbor.

In 1960, the publication of *Silent Spring* firmly introduced the concept that animal populations, as well as humans, could have adverse health effects from exposure to environmental hazards (Carson, 1962). The implication of the book was that bird die offs related to the use of pesticides were a warning that these pollutants, including DDT and other organochlorine compounds, were causing widespread toxicity in the environment that could also be a threat to human health (Rabinowitz P et al. 2009). As Figure 2 shows, the same environmental toxins that cause chronic health problems in humans may, through parallel exposure routes, cause effects in animals as well. Alternatively, toxins may accumulate in food animals who then serve as a

source of exposure to humans, as in the case of fish contaminated with mercury or dioxins.

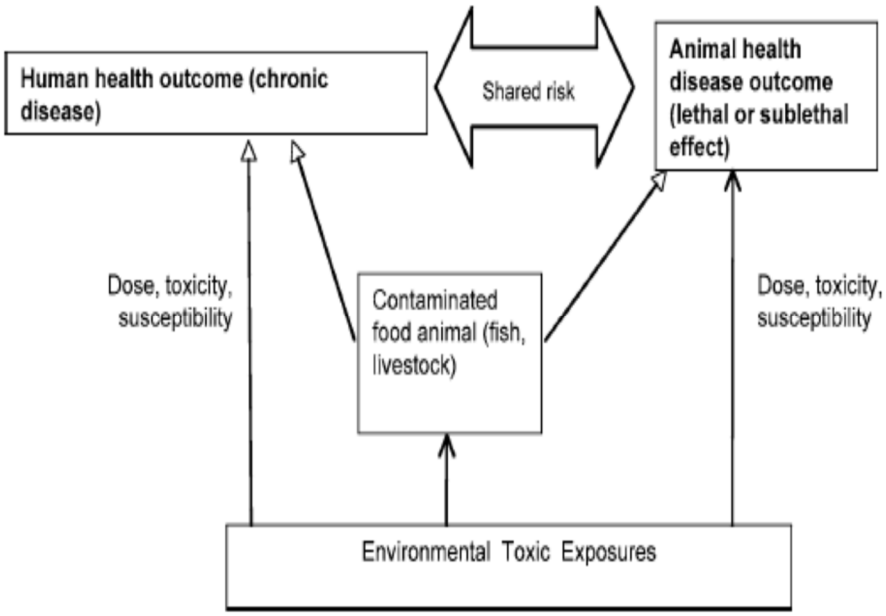


Fig. II. Relationship between environmental toxic exposures and health outcomes in humans and animals (Rabinowitz 2008)

Figure 3 depicts the same process for infectious hazards, which might result in symptomatic infection in nonhuman animal species as well as in humans. Therefore, both figures suggest that some nonhuman animal species might have some ‘‘shared risk’’ (with humans) of diseases due to environmental hazards.

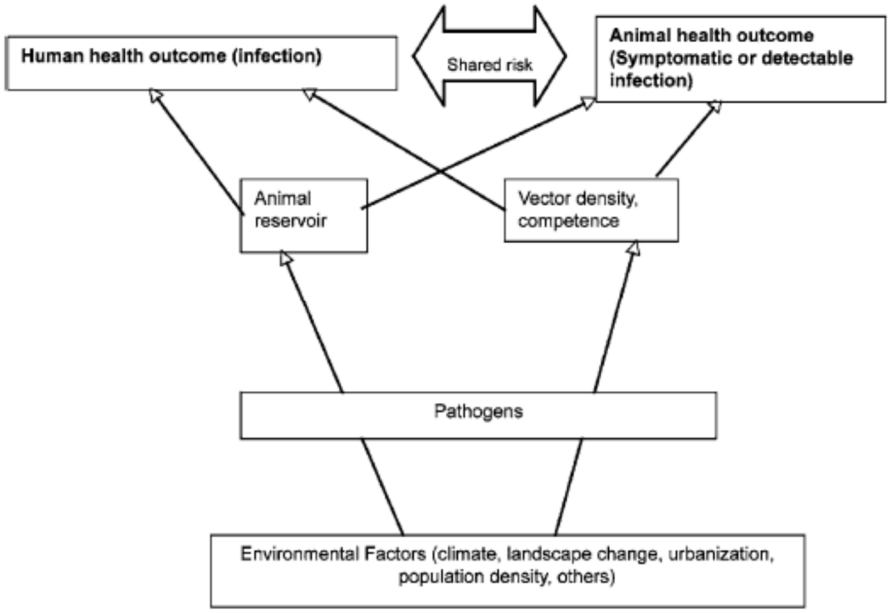


Fig.III. Relationship between environmental drivers of infectious disease and outcomes in humans and animals (Rabinowitz 2008)

Since both humans and animals may develop disease through exposure to environmental hazards, it has been suggested that animals, like the ‘‘canary in the coal mine’’ could serve as natural ‘‘sentinels’’ providing clues to humans about links between environmental hazards and human health risk (van der Schalie et al., 1999; Rabinowitz et al., 2005). As a 1991 National Academy of Sciences report outlined, animals possess a number of advantages for linking hazards and outcomes in the environment (Committee on Animals as Monitors of Environmental Hazards, 1991). Due to differences in body weight and metabolism from humans, animals may be

more susceptible than humans to particular hazards. Animals also tend to be less mobile, and are exposed at a higher level to a given environmental hazard, compared to humans living nearby who may be actively modifying exposures through clothing, buildings, and dietary choices. Furthermore, animals usually have shorter life spans than humans and therefore may exhibit a shorter latency for development of an environmentally induced condition; even in the setting of an infectious disease, they may exhibit a shorter incubation period to development of noticeable effects. Animals may also have increased or diminished susceptibility (relative to humans) to a particular hazard. There may be variations of susceptibility from species to species. For instance, horses may experience acute symptoms with anthrax infections, whereas pigs are relatively resistant. The same may be applicable for various forms of environmental contamination affecting each animal species vis-à-vis humans (Rabinowitz P et al. 2008).

Despite the apparent promise of using animal health outcome data to supplement other risk information about human environmental hazards, the application of Sentinel Animal Systems in environmental monitoring is not free from difficulties. One of the main limitations includes the results interpretation, which can be difficult and controversial, if not compared objectively and reasonably to human health hazard risks. A fish population killed by small concentrations of residual chlorine in water, for example, is of little value in terms of effects on humans. Exposure relevance is critical to comparisons between sentinel animals and humans. Exposure pathways important for sentinels may or may not have any relevance for human populations. If one believes that chemically induced cancer in a bottom

feeding catfish exposed to contaminated sediments is a concern, demonstration of potential human risk would be incomplete without identification of a plausible exposure route to the human population (van der Schalie 1999).

Moreover, the use of Sentinel Animal Systems is usually limited by the lack of standardized methods in researching programs, thus, on large scale, collection of data can be misleading and inadequate to set up a well structured and efficient database (Rombolà et al. 2012). Although certain focused databases exist (e.g., the registry of tumors in lower animals at George Washington University), the lack of a centralized database for ecological effects is a major deficiency in environmental monitoring programs. The standardization of information systems and electronic media would facilitate the coordination and collection of exposure and disease data from fish, wildlife, companion animals, and livestock. This kind of systematically organized information would facilitate data synthesis and analyses that could detect patterns of change through time, and would allow appropriate statistical analysis of results (National Research Council 1991).

V. Selection criteria of sentinel animals in environmental biomonitoring

Taking as starting point the scheme proposed by O'Brien et al. the selection criteria of sentinel animals are divided into:

Inherent Criteria

- **Sensitivity.** The proposed sentinel must be sensitive enough to be predictive of human exposure and its routes, and its reaction needs to be specific to the particular agent. Although some authors have demonstrated that a sentinel less sensitive than humans can be useful in pointing out an existing intoxication problem in humans, it would probably be of greater utility to choose a sentinel more sensitive to a particular toxin than its guarded human group. In this way, one might expect clinical signs in the sentinel before their appearance in man, fulfilling the aforementioned "early warning" function.
- **Physiological Characteristics.** Three key factors are necessary in regard to physiological characteristics. First, with regard to the toxicant in question, the sentinel needs to be similar enough to man physiologically to show comparable biological and pathological effects following exposure. Second, baseline parameters of the sentinel's physiology need to be known or have the potential to be feasibly determined so that "normal" characteristics can act as a standard to measure changes against. Third, the organism must accumulate the toxicant to levels that reflect environmental concentrations. Sentinel levels need to change in direct proportion to changes in the environment.
- **Longevity.** The sentinel should have a lifespan long enough to demonstrate the effects of exposure over time so that conclusions can

be drawn concerning the consequences of chronic exposure and concerning any variability of effects for different age groups.

- **Latent Periods.** The time span between initial exposure to a toxic agent and the appearance of biological effects or clinical signs should ideally be short, so that early warning of subsequent effects of chronic exposure in humans could be identified. In addition, a short latent period might allow better assessment of the length and course of the intoxication.

External Factors

- **Position in the Food Chain (Food Web).** Humans, under normal circumstances, are omnivores at the top trophic level of the food chain. In order to be comparable, a sentinel would ideally also be omnivorous and at the top of its food chain. Exceptions to this might be in cases where human exposure to a particular toxicant is primarily through a specific food source, such as meat or fish, in which case a strict carnivore or piscivore would be appropriate. Finally, an additional position for a sentinel would be as a food source for humans. This would give toxic levels in these organisms considerable public health implications because of the tendency of some toxicants to accumulate or biomagnify up food chains and because contaminated food has been a source of human toxic exposure in the past.
- **Migration.** Although the use of widely migratory mammals to monitor toxic contamination over vast areas such as oceans has been suggested, for an ideal sentinel species, migrations would be limited

or absent. A sedentary specie offers more precise data on environmental pollution of a well circumscribed area. This represents an important advantage compared to studies in human populations: one of the major obstacles interpreting results on the exposure of humans to environmental pollutants is linked to the long latency of many chronic conditions, persons may not be diagnosed with a particular disease before they have already left an environment that could have played a role in the causation. Adults may work 30–40 miles from their place of residence, and may pass through numerous different ‘‘environments’’ in the course of a single day.

- **Route of Toxic Exposure Similar to Humans.** Route of exposure is essentially a further specification of the idea that sentinels need to "share the same environment as man." Routes of intoxication must be identified and standardized to determine risk from environmental contamination and to predict biological and pathological consequences (because these can vary widely according to exposure route for a given toxicant).
- **Abundance and Distribution**. Sentinels need to be abundant enough to make statistically significant sampling logistically feasible. Moreover, it is important that the sentinel species chosen will not be adversely effected by the removal of individuals for sampling purposes. For this reason, the use of endangered species or species whose populations are depleted or unstable within a study area would not be appropriate. Some have suggested the use of "nuisance" species, whose thriving populations already need to be

managed on a continuing basis by trapping and removal. Such species could provide an abundant sampling source. In addition, the ideal sentinel should be widely distributed within the area to be assessed, so that levels in the organism are representative of the entire area of concern.

VI. Domestic Animals as Sentinels

Many of the sinanthropic species, or rather pets or food producing animals, sharing with man the same environments, may, for various reasons, be effective sentinels with more advantages than those already shown. Companion animals have been used as surrogates for the estimation of human exposure, especially in case of complex environments (such as home or work) in which it is difficult to make any estimation with conventional procedures. Furthermore, farm animals can represent a good model for environmental epidemiology studies, since the exposure evaluation is more simple than in humans, thanks to the standardization of certain conditions of life, including daily dietary, the limitation of movements out of the environment, and the absence of major confounding factors (Occupational exposure, smoking etc.) (Scaramozzino et al. 2010). Furthermore, the longevity of many domestic animals is often lower than human and the onset of the effects of possible age-related exposures to environmental pollutants (i.e. certain cancers in dogs) comes first compared to humans. Moreover, the procedures for collection of samples for laboratory tests are simplified, as

the removal of tissue from death animals or systematic checks at regular intervals for consumer protection (milk, eggs, meat etc.) are easier and cheaper compared to chemical analysis of matrix present in the environment. An additional factor encouraging the choice of domestic animals as sentinels is their potential density in the area of study: some of these species are in fact ubiquitous in many urban or rural areas, which is analogous to human populations.

Pet dogs have tremendous potential as sentinels for environmental health. This has been demonstrated in a number of studies with known environmental carcinogens (Backer CL. et al. 2001). For example, a study of pet dogs with mesotheliomas identified household asbestos exposure (e.g. from asbestos-related hobbies, such as auto mechanics; the use of flea powders containing talc; or clothing contaminated by occupational activities. as risk factors that might also increase the human risk of asbestos-related disease) (Glickman et al., 1983). Hayes et al. (1981) reported a dose-dependent association between household applications of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and malignant lymphomas in dogs. These findings were consistent with reports of a modest association between agricultural exposure to 2,4-D and an increased risk of non-Hodgkin's lymphoma in people (Hoar et al., 1986). Reif et al. (1992) conducted a case-control study of canine lung cancer and found, among short-muzzled dogs, an increased risk of lung cancer from exposure to environmental tobacco smoke. In summary, a number of studies have shown that pet dogs respond to at least some environmental contaminants, such as asbestos, herbicides, heavy metals and in an urban environment, respond similarly to the way

humans respond. Pet dogs have also been found to be good predictors of children's exposure to lead. Given that dogs and people can suffer similar illnesses when subjected to the same risk factors, it is likely that monitoring the health of pet dogs will help identify associations between exposure to environmental contaminants and the occurrence of disease. An emerging field of this concept is the so called "Comparative Oncology". One of the purposes of comparative oncology studies is the realization of cancer registries related to dogs (and other pets) in order to monitor areas at risk for environmental pollutants and validate any correlation between tumor incidence in the human population and the dog, as well as identifying possible sources of common exposure.

Many authors have demonstrated the suitability of herbivorous animals for environmental monitoring of contaminants that propagate via water or air. The study of the asbestos fibers in the lungs of cattle in Piemonte Region (Italy) was found useful for detecting the presence of asbestos exposure of human populations from serpentiniche rocks (Fornero E. et al. 2009). In Canada it has been confirmed the practice of using livestock cattle population as sentinel to estimate levels of contaminants originating from oil and gas industry emissions in the environment (Cheryl L. 2008) (Waldner CL 2008). In Italy, cattle and sheep were tested for monitoring level of pollution from b-exachlorecycloexane of Sacco River (Lazio) caused by industrial waste (Battisti A. 2007). In epidemiological studies the use of farm animals (cattle, sheep and buffalo) for biomonitoring environmental levels of dioxins and heavy metals is very frequent (Hirako M. 2008) (Somasundaram J 2005).

Another example of the utility of farm animals in environmental monitoring is the surveillance of birth malformation, to detect the possible action of environmental pollutants in teratogenic effects. About this last point, the difficulties are similar and perhaps greater than those encountered in the implementation of human and animals tumor registers. Breeders rarely report these cases and as a result, the veterinary service system often do not have the possibility to register and elaborate data that could be of interest in environmental monitoring.

Finally, to set up good surveillance programs a mention to the role of anagraphic registers of animals, in particular the canine and livestock registers, is mandatory. These records are required by law (Law 14 August 1991 n. 281, "Law on animals disease and prevention of stray dogs, "for the canine Registry and Presidential Decree 317/1996, 437/2000 and DPR to DM 31.01.2002 Livestock Registry) and represent important databases for the purposes mentioned above, and are of great value for statistical analysis and the management of the geographical component data (i.e. geo-referencing of farms) as it will be discussed later.

Lastly, data from domestic animals exposed to environmental contaminants in their habitats play a key role in the process of assessment of risk to human health, by providing an early warning in situations that require further investigation and allow to monitor the food chain and the effectiveness of environmental surveillance or reclaim programs (Van der SchalieWH 1999).

The purpose of this thesis work is to summarize the studies carried out throughout the PhD scholarship, which were based on the use of animals as

sentinels, to assess the presence of environmental pollutants potentially dangerous to human health. Particular attention was paid to the use of domestic species as animal sentinel systems, specifically:

1. Dog as sentinel for environmental pollution in urban areas
2. Cattle as an early warning system for environmental pollution from nitrate compounds
3. Sheep as animal sentinel system to evaluate the impact of oil drilling on the environment

Thus we evaluated the utility of three different animal species validating the "*envorinmental necropsy*" as a tool for the study of environmental pollution.

Abernathy, C. O., Liu, Y. P., Longfellow, D., Aposhian, H. V., Beck, B., Fowler, B., ... & Waalkes, M. (1999). Arsenic: health effects, mechanisms of actions, and research issues. *Environmental health perspectives*, *107*(7), 593.

Battisti, A., & Sala, M. (2007). Environmental pollution in the zootechnical productions: the experience of the b-exachlorecycloexane contamination in Sacco River Valley. In *Proceedings IX National Congress SIDiLV* (pp. 23-24).

Bhan, A., & Sarkar, N. N. (2005). Mercury in the environment: effect on health and reproduction. *Rev Environ Health*, *20*(1), 39-56.

Bradl, H. (Ed.). (2005). *Heavy metals in the environment: origin, interaction and remediation* (Vol. 6). Academic Press.

Butler, C. A., & Timperley, M. H. (1996). Fertilised farmland as a source of cadmium in oysters. *Science of the total environment*, *181*(1), 31-44.

Carson, R. (1994). Silent spring. 1962.

Dopp, E., Hartmann, L. M., Florea, A. M., Von Recklinghausen, U., Pieper, R., Shokouhi, B., ... & Obe, G. (2004). Uptake of inorganic and organic derivatives of arsenic associated with induced cytotoxic and genotoxic effects in Chinese hamster ovary (CHO) cells. *Toxicology and applied pharmacology*, *201*(2), 156-165.

Fergusson, J. E. (1990). *Heavy elements: chemistry, environmental impact and health effects*. Pergamon.

Flora, S. J., Flora, G., & Saxena, G. (2006). Environmental occurrence, health effects and management of lead poisoning. *Lead chemistry, analytical aspects, environmental impacts and health effects*.

Flora, S. J. S., Saxena, G., Gautam, P., Kaur, P., & Gill, K. D. (2007). Response of lead-induced oxidative stress and alterations in biogenic amines in different rat brain regions to combined administration of DMSA and MiADMSA. *Chemico-biological interactions*, *170*(3), 209-220.

Fornero, E., Belluso, E., Capella, S., & Bellis, D. (2009). Environmental exposure to asbestos and other inorganic fibres using animal lung model. *Science of The Total Environment*, *407*(3), 1010-1018.

Gerdol, R., Bragazza, L., Marchesini, R., Alber, R., Bonetti, L., Lorenzoni, G., ... & Pison, S. (2000). Monitoring of heavy metal deposition in Northern Italy by moss analysis. *Environmental Pollution*, *108*(2), 201-208.

Glickman, L. T., Domanski, L. M., Maguire, T. G., Dubielzig, R. R., & Churg, A. (1983). Mesothelioma in pet dogs associated with exposure of their owners to asbestos. *Environmental Research*, *32*(3), 305-313.

Grabarkiewicz, J. D., & Davis, W. S. (2008). *An introduction to freshwater fishes as biological indicators*. US Environmental Protection Angecy, Office of Environmental.

Hayes, H. M., Tarone, R. E., Cantor, K. P., Jessen, C. R., McCurnin, D. M., & Richardson, R. C. (1991). Case-control study of canine malignant lymphoma: positive association with dog owner's use of 2, 4-dichlorophenoxyacetic acid herbicides. *Journal of the National Cancer Institute*, *83*(17), 1226-1231.

He, Z. L., Yang, X. E., & Stoffella, P. J. (2005). Trace elements in agroecosystems and impacts on the environment. *Journal of Trace elements in Medicine and Biology*, *19*(2), 125-140.

Hirako, M. (2008). Transfer and accumulation of persistent organochlorine compounds from bovine dams to newborn and suckling calves. *Journal of agricultural and food chemistry*, *56*(15), 6768-6774.

Hoar, S. K., Blair, A., Holmes, F. F., Boysen, C. D., Robel, R. J., Hoover, R., & Fraumeni, J. F. (1986). Agricultural herbicide use and risk of lymphoma and soft-tissue sarcoma. *Jama*, *256*(9), 1141-1147.

Holmes, P., James, K. A. F., & Levy, L. S. (2009). Is low-level environmental mercury exposure of concern to human health?. *Science of the total environment*, *408*(2), 171-182.

Hwua, Y. S., & Yang, J. L. (1998). Effect of 3-aminotriazole on anchorage independence and mutagenicity in cadmium-and lead-treated diploid human fibroblasts. *Carcinogenesis*, *19*(5), 881-888.

International Agency for Research on Cancer (IARC) (1993). Monographs – Cadmium. Lyon, France

Kumar, S., Yedjou, C. G., & Tchounwou, P. B. (2014). Arsenic trioxide induces oxidative stress, DNA damage, and mitochondrial pathway of apoptosis in human leukemia (HL-60) cells. *Journal of Experimental & Clinical Cancer Research*, *33*(1), 42.

Lam, P. K., & Gray, J. S. (2003). The use of biomarkers in environmental monitoring programmes. *Marine Pollution Bulletin*, *46*(2), 182-186.

National Research Council. (1991). *Animals as sentinels of environmental health hazards*. National Academies Press.

Pinheiro, M. C. N., Macchi, B. M., Vieira, J. L. F., Oikawa, T., Amoras, W. W., Guimarães, G. A., ... & do Nascimento, J. L. M. (2008). Mercury exposure and antioxidant defenses in women: a comparative study in the Amazon. *Environmental Research*, *107*(1), 53-59.

Rabinowitz, P. M., Gordon, Z., Holmes, R., Taylor, B., Wilcox, M., Chudnov, D., ... & Dein, F. J. (2005). Animals as sentinels of human environmental health hazards: an evidence-based analysis. *EcoHealth*, *2*(1), 26-37.

Rabinowitz, P. M., Odofin, L., & Dein, F. J. (2008). From "us vs. them" to "shared risk": can animals help link environmental factors to human health?. *EcoHealth*, *5*(2), 224-229.

Rabinowitz, P., Scotch, M., & Conti, L. (2009). Human and animal sentinels for shared health risks. *Veterinaria italiana*, *45*(1), 23.

Reif, J. S. (2011). Animal sentinels for environmental and public health. *Public Health Reports*, *126*(1 suppl), 50-57.

Rombolà, P., Battisti, S., & Scaramozzino, P. (2012). Biomonitoraggio animale e microinquinanti in sanità pubblica–Rassegna bibliografica. *Epidemiol Prev*, *36*(5 suppl 4), 1-52.

Sanfeliu, C., Sebastia, J., Cristofol, R., & Rodriguez-Farre, E. (2003). Neurotoxicity of organomercurial compounds Neurotoxicol Res 5: 283–305. *Find this article online*.

Scaramozzino, P., Rombolà, P., Battisti, S., Sala, M., Ferrari, G., & La Torre, G. (2012). Surveillance model for environmental contaminants through their monitoring in animal production. *Italian Journal of Public Health*, *7*(1).

Somasundaram, J., Krishanasamy, R., & Savthri, P. (2005). Biotransfer of heavy metals in Jersey cows. *The Indian Journal of Animal Sciences*, *75*(11).

Stohs, S. J., & Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free radical biology and medicine*, *18*(2), 321-336.

Sutton, D. J., Tchounwou, P. B., Ninashvili, N., & Shen, E. (2002). Mercury induces cytotoxicity and transcriptionally activates stress genes in human liver carcinoma (HepG2) cells. *International Journal of Molecular Sciences*, *3*(9), 965-984.

Tchounwou, P. B., Wilson, B., & Ishaque, A. (1999). Important considerations in the development of public health advisories for arsenic and arsenic-containing compounds in drinking water. *Rev Environ Health*, *14*(4), 211-229.

Tchounwou, P. B., Ishaque, A. B., & Schneider, J. (2001). Cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells (HepG2) exposed to cadmium chloride. *Molecular and Cellular Biochemistry*, *222*(1), 21-28.

Tchounwou, P. B., Ayensu, W. K., Ninashvili, N., & Sutton, D. (2003). Review: Environmental exposure to mercury and its toxicopathologic implications for public health. *Environmental toxicology*, *18*(3), 149-175.

Tietenberg, T. (2006). Economics of Pollution Control, Chapter 15 in Environmental and Natural Resource Economics, 7th Edition, Pearson, Boston.

Trouba, K. J., Wauson, E. M., & Vorce, R. L. (2000). Sodium arsenite-induced dysregulation of proteins involved in proliferative signaling. *Toxicology and applied pharmacology*, *164*(2), 161-170.

US Environmental Protection Agency (2012). Criteria for Choosing Indicator Species for Ecological Risk Assessments at Superfund Sites

Valko, M. M. H. C. M., Morris, H., & Cronin, M. T. D. (2005). Metals, toxicity and oxidative stress. *Current medicinal chemistry*, *12*(10), 1161-1208.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-biological interactions*, *160*(1), 1-40.

Van der Schalie, W. H., Gardner Jr, H. S., Bantle, J. A., De Rosa, C. T., Finch, R. A., Reif, J. S., ... & Stokes, W. S. (1999). Animals as sentinels of human health hazards of environmental chemicals. *Environmental health perspectives*, *107*(4), 309.

Vogt, B. L., & Rossman, T. G. (2001). Effects of arsenite on p53, p21 and cyclin D expression in normal human fibroblasts—a possible mechanism for arsenite's comutagenicity. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *478*(1), 159-168.

Waldner, C. L. (2008). Western Canada study of animal health effects associated with exposure to emissions from oil and natural gas field facilities. Study design and data collection I. Herd performance records and management. *Archives of environmental & occupational health*, *63*(4), 167-184

Wang, S., & Shi, X. (2001). Molecular mechanisms of metal toxicity and carcinogenesis. *Molecular and cellular biochemistry*, *222*(1), 3-9.

Wilson, D. N. (1988). Association Cadmium. *Cadmium-market trends and influences*, 9-16.

Yedjou, C. G., & Tchounwou, P. B. (2005, December). OXIDATIVE STRESS IN HUMAN LEUKEMIA (HL-60), HUMAN LIVER CARCINOMA (HepG2), AND HUMAN (JURKAT-T) CELLS EXPOSED TO ARSENIC TRIOXIDE. In *Metal ions in biology and medicine: proceedings of the... International Symposium on Metal Ions in Biology and Medicine held...= Les ions metalliques en biologie et en medecine:... Symposium international sur les ions metalliques.*. (Vol. 9, pp. 298-303). NIH Public Access

Yedjou, C. G., & Tchounwou, P. B. (2007). In-vitro cytotoxic and genotoxic effects of arsenic trioxide on human leukemia (HL-60) cells using the MTT and alkaline single cell gel electrophoresis (Comet) assays. *Molecular and cellular biochemistry*, *301*(1-2), 123-130.

Yedjou, C. G., & Tchounwou, P. B. (2007). N-acetyl-l-cysteine affords protection against lead-induced cytotoxicity and oxidative stress in human liver carcinoma (HepG2) cells. *International journal of environmental research and public health*, *4*(2), 132-137.

Zhao, C. Q., Young, M. R., Diwan, B. A., Coogan, T. P., & Waalkes, M. P. (1997). Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. *Proceedings of the National Academy of Sciences*, *94*(20), 10907-10912.

Chapter 1
The Role of Necropsy in Environmental Monitoring: the Regional Reference Center of Urban Veterinary Hygiene (CRIUV) experience

1.1 Introduction

For more than two decades, many zones in the provinces of Caserta (CE) and Naples (NA), in the northern part of the Campania Region (Italy), have been affected by extensive illegal dumping of mixed waste of urban as well as industrial origin, while waste has frequently been burnt in the open (Diletti et al., 2004, 2008; Esposito et al., 2014; Giovannini et al., 2014; Martuzzi and Mitis, 2007; Neugebauer et al., 2009; Rivezzi et al., 2013): a cocktail of environmental pressure factors potentially resulting in a release of organic as well as inorganic chemical contaminants.

The territory covered by the aforesaid provinces comprises 196 municipalities and is highly inhabited. The pressure on the environment from the prolonged and diffuse local practice of illegal waste disposal/treatment and other sources of environmental degradation (including mishandled legal landfills) – altogether entailing potential risks for human health – prompted the Italian authorities to acknowledge an emergency status (1994–2009) and declare 77 of those municipalities as SIN "site of national interest for remediation" (Figure 1.1) (Bianchi et al., 2004; Comba et al., 2006; Fazzo et al., 2008, 2011; Martuzzi et al., 2009).

During 2010, ARPA (Regional Agency for Environmental Protection) has identified and characterized the various authorize/unauthorized dumping sites in the provinces of Naples and Caserta, in order to locate the possible areas exposed to a higher waste related health risk.. As a first result, some municipalities along the coast and north of Naples have been shown to be characterized by high-risk impact areas (Figure 1.2).

Fig. 1.1. Illegal dumping in Regi Lagni (site of national interest for remediation) province of Caserta (A). Water buffalos found dead in the same area of figure A (B) (Eolopress 2012).

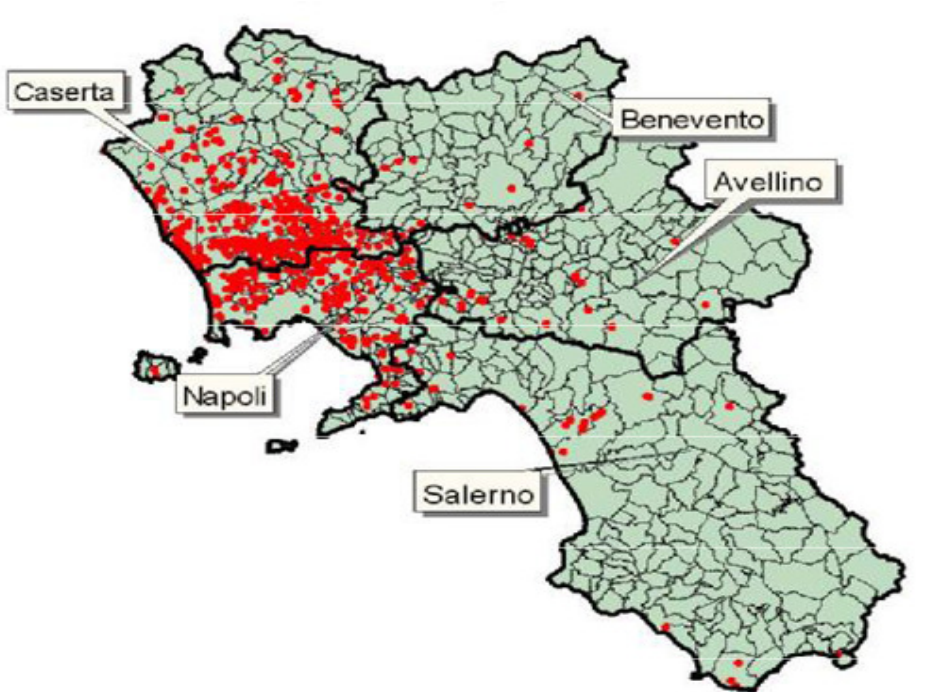


Fig. 1.2. Unuthorized dumping sites in Campania Region (ARPAC 2010).

As described by the same authors (De Felip et al. 2014), the results of epidemiological studies carried out in the area provide hints to potentially

associate increases in cancer mortality, development of chronic diseases and congenital malformations with environmental degradation and socioeconomic deprivation. However, due to the complexity and uncertainties of the exposure scenario, the assessment of a causal link between the pressure of environmental risk factors and its impact on communities' health was acknowledged to require further studies (De Felip et al. 2014). In this scenario, as it's said in an editorial paper of the scientific journal "Nature", the Campania Region is an excellent area for epidemiological investigation studies and environmental biomonitoring activities (Nature Publishing Group 2014).

In the light of the above, the Campania Region allocated funds for extensive biomonitoring community studies whose aim was to evaluate health risk of exposure of communities living nearby authorized and unauthorized dumping sites. In the final report of the working group ex D.M. 24.07.2012 "*Epidemiological situation of the Campania region and in particular of the provinces of Caserta and Naples (excluding cities), with reference to the incidence of mortality from oncological diseases*" is highlighted that the main risks for human health are associated to the exposure to PCDDs, PCDFs, and DL-PCBs which are persistent organic pollutants (POPs), a subgroup of persistent toxic substances (PTS) of the Chemicals Programme of the United Nation Environment Programme (UNEP) (http://www.chem.unep.ch/), which also includes a number of heavy metals. As written in the report, heavy metals were found to be the main cause of environmental pollution in several sites of the Campania region (Table 1.1).

Tab 1.1. Polluted (or potentially polluted) sites in the provinces of Naples and Caserta.

Site	District	Province	Activity	Polluted matrix	Kind of contaminants
Area located in Via Masarda	Pomigliano d'Arco	NA	Waste disposal in excavation	Soil	PCB, Heavy Metals
Land Eurocostruzioni Metalliche	Marcianise	CE	Waste disposal on soil	Undergroun d water	Heavy Metals
Loc. Via Giardino (I) - (Ecoleader)	Villa Literno	CE	Waste disposal on soil	Soil / Undergound water	PAHs
Loc. Torretta Tre Ponti Asse FFSS Nola Interporto	Marigliano	NA	Waste disposal on soil	Soil	Heavy Metals
Lotto 62 ASI Ma S.r.L	Nola	NA	Waste disposal on soil	Soil	Heavy Metals
Cava Loc. Difesa (I)-Frazione Polvica	Roccarainola	NA	Waste disposal in cave	Soil / Undergound water	Heavy Metals, PAHs
Loc.Saudine Frazione Tredici	Caserta	CE	Waste disposal in excavation	ongoing investigatio ns	ongoing investigations
Gorgone Canal	Acerra	NA	Waste disposal in surface water	ongoing investigatio ns	ongoing investigations
Cava Loc. Casella Pisani	Naples	NA	Waste disposal in cave	Soil / Undergound water	PCB, Heavy Metals, PAHs, Dioxins
Cava Loc. Spadari	Naples	NA	Waste disposal in cave	ongoing investigatio ns	ongoing investigations

Loc. Via Viulo (I) - Parco Nazionale del Vesuvio	Ercolano	NA	Waste disposal on soil	Soil	Heavy Metals, PAHs
"Istituto Santa Croce"	Castellammare di Stabia	NA	Waste disposal on soil	ongoing investigations	ongoing investigations
Nuovo Liceo Scientifico	Terzigno	NA	Waste disposal on soil	ongoing investigations	ongoing investigations
Loc. Palmentata I	Marcianise	CE	Area potentially contaminated by dioxin	Soil	Dioxins and Furans
Loc. Palmentata II	Marcianise	CE	Area potentially contaminated by dioxin	Soil	Dioxins and Furans
Loc. SP 131 – Zona ASI	Caivanno	NA	Area potentially contaminated by dioxin	Soil	Dioxins and Furans
Loc. Via Lagniuolo	Marigliano	NA	Area potentially contaminated by dioxin	Soil	Dioxins and Furans
Loc. San Vitaliano	San Vitaliano	NA	Area potentially contaminated by dioxin	Soil	Dioxins and Furans
Sito di Stoccaggio Loc. Frascale	Capua	CE	Waste storage site	Underground water	Fluorides
Sito Stoccaggio provvisorio località Foro Boario	Maddaloni	CE	Temporary waste storage site	ongoing investigations	ongoing investigations
Sito di Stoccaggio Provvisorio Loc. Ferandelle	S.Maria La Fossa	CE	Temporary waste storage site	Underground water	Heavy Metals

Sito di Stoccaggio Loc. Pozzo bianco	S.Maria La Fossa	CE	Temporary waste storage site	ongoing investigations	ongoing investigations
Sito di Stoccaggio Loc. Lo Spesso	Villa Literno	CE	Waste storage site	Underground water	Heavy Metals
Sito di Stoccaggio Loc. Pascarola	Caivanno	NA	Waste storage site	ongoing investigations	ongoing investigations
Sito di Stoccaggio Loc. Giuliani	Giugliano in Campania	NA	Waste storage site	Underground water	PAHs , Heavy Metals
Sito di Stoccaggio Ecoballe Fibe S.p.A Pontericcio	Giugliano in Campania	NA	Waste storage site	ongoing investigations	ongoing investigations
Stoccaggio Ecoballe Loc. Taverna del Re	Giugliano in Campania	NA	Waste storage site	Soil	Heavy Metals
Sito Stoccaggio Loc. Boscofangone	Marigliano	NA	Waste storage site	ongoing investigations	ongoing investigations
Sito di Stoccaggio Loc. Nespole della Monica	Terzigno	NA	Waste storage site	ongoing investigations	ongoing investigations
Ex Discarica Comunale Loc Ravone	Ailano	CE	Waste storage site	Soil / Undergound water	Heavy Metals
Ex Disc. Comunale Loc. Iungere	Alife	CE	Waste storage site	Soil / Und. water	Heavy Metals, PAHs
Discarica Comunale Loc. Fraolise	Alvignano	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Loc. Caprareccia	Baia e Latina	CE	Waste storage site	ongoing investigations	ongoing investigations

Ex Discarica comunale Loc. Monticello di Giano V.	Camigliano	CE	Waste storage site	Soil / Undergound water	Heavy Metals
Ex Discarica Comunale Loc. Santo Ianni	Capriati al Volturno	CE	Waste storage site	Undergound water	Heavy Metals
Discarica Ecologica Meridionale	Caserta	CE	Waste storage site	Undergound water	Heavy Metals, PAHs
Discarica ACSA CE 3	Caserta	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Lo Uttaro	Caserta	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Loc. Bortolotto	Castel Volturno	CE	Waste storage site	Undergound water	Heavy Metals, PAHs
Ex Discarica Sogeri	Castel Volturno	CE	Waste storage site	Undergound water	Heavy Metals
Ex Discarica Comunale Cappella	Dragoni	CE	Waste storage site	ongoing investigations	ongoing investigations
Ex Discarica Comunale Valle delle Conche	Gallo Matese	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Loc. Marotta	Giano Vetusto	CE	Waste storage site	Soil / Undergound water	Heavy Metals, Pesticides
Ex Discarica Comunale Loc. Petrito	Gioia Sannitica	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs
Discarica Loc. Monacelle (Bosco Alto)	Grazzanise	CE	Waste storage site	ongoing investigations	ongoing investigations

Ex Discarica Comunale Sava	Letino	CE	Waste storage site	ongoing investigations	ongoing investigations
Ex Discarica Comunale Cesco Cupo	Liberi	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Loc. S. Veneranda	Marcianise	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs
Ex Discarica Comunale Fecicce - Monticello	Mignano Monte Lungo	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs
Discarica Comunale Loc. S.Giorgio	Orta di Atella	CE	Waste storage site	Soil / Undergound water	Heavy Metals
Ex Discarica Comunale Loc. Pelatello	Pietramelara	CE	Waste storage site	Undergound water	Heavy Metals
Discarica Comunale Loc. S.Felice	Pietravairano	CE	Waste storage site	Undergound water	Heavy Metals
Ex Discarica Comunale Loc. Cauciano	Pignataro Maggiore	CE	Waste storage site	ongoing investigations	ongoing investigations
Ex Discarica Via dell'Officina	Prata Sannita	CE	Waste storage site	ongoing investigations	ongoing investigations
Ex Discarica Comunale Starzelle	Pratella	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Loc. Marconi	Roccaromana	CE	Waste storage site	Undergound water	Heavy Metals

Discarica Loc. Grotte	S. Gregorio Matese	CE	Waste storage site	Undergound water	Heavy Metals, PAHs
Discarica Comunale Ferraro I	S. Maria La Fossa	CE	Waste storage site	Soil / Undergound water	Heavy Metals
Discarica Comunale Ferraro II	S. Maria La Fossa	CE	Waste storage site	Soil / Undergound water	Heavy Metals
Discarica Comunale Parco Saurino 1	S. Maria La Fossa	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs
Discarica Comunale Parco Saurino 2	S. Maria La Fossa	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs
Discarica Comunale Loc. Casone	S. Tammaro	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Maruzzella (I) e (II)	S. Tammaro	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs
Discarica A.B. & F.	Sessa Aurunca	CE	Waste storage site	ongoing investigations	ongoing investigations
Ex Discarica Comunale Loc. Cantina	Teano	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Loc. S. Giuseppe o Pizzo Monte	Vairano	CE	Waste storage site	Undergound water	Heavy Metals, PAHs
Ex Discarica Comunale Ariole	Valle Agricola	CE	Waste storage site	ongoing investigations	ongoing investigations

Discarica Cuponi di Sagliano	Villa Literno	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs, Dioxins, Furanes, Phenols
Discarica Masseria Annunziata	Villa Literno	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs, Dioxins, Furanes, Phenols
Discarica Comunale Loc. Renella	Falciano del Massico	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs, Dioxins, Furanes, Phenols
Discarica Comunale Loc. Polledrara	Cellole	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs, Dioxins, Furanes, Phenols
Ex Discarica Comunale (La Pescara)	Cellole	CE	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs, Dioxins, Furanes, Phenols
Discarica Migliore Carolina	S. Marco Evangelista	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Loc. Cetrangolo	S. Marco Evangelista	CE	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Loc. S. Maria la Nova	Afragola	NA	Waste storage site	Soil / Undergound water	Heavy Metals, PAHs
Discarica Comunale Cava Pallarito	Barano d'Ischia	NA	Waste storage site	ongoing investigations	ongoing investigations

Ex Discarica Comunale Loc. S. Arcangelo	Caivano	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Comunale Cava Baino "Ex cava Pozzillo"	Casamicciola Terme	NA	Waste storage site	Soil	Heavy Metals
Discarica Comunale Loc. Punta Caruso	Forio	NA	Waste storage site	Underground water	Heavy Metals
Discarica Masseria del Pozzo - Schiavi	Giugliano in Campania	NA	Waste storage site	Underground water	Heavy Metals, PAHs
Discarica Ex Resit (Cava Z, Cava X)	Giugliano in Campania	NA	Waste storage site	Underground water	Heavy Metals, PAHs
CAVA Bianco	Giugliano in Campania	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Loc. Chiaiano(Ex Cava del Poligono)	Napoli	NA	Waste storage site	Soil	Heavy Metals
Ex DI.FRA.BI	Napoli	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Loc. Senga	Napoli	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Ex CITET	Napoli	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Loc. Piazzola di Nola	Nola	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Iovino Loc. Balle	Palma Campania	NA	Waste storage site	ongoing investigations	ongoing investigations

Discarica in loc. Carcavone	Pollena Trocchia	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Gambardella	Quarto	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Privata AMMENDOLA E FORMISANO	Ercolano	NA	Waste storage site	Underground water	Heavy Metals, PAHs
Ex Discarica Comunale Loc. Cava Pallarito (II)	Serrara Fontana	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Loc. Bosco (Fungaia)	Somma Vesuviana	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Sa.ri.srl	Terzigno	NA	Waste storage site	Underground water	Heavy Metals, PAHs
Cava Pozzelle I	Terzigno	NA	Waste storage site	ongoing investigations	ongoing investigations
Ex discarica - ex porcilaia	Torre del Greco	NA	Waste storage site	ongoing investigations	ongoing investigations
Discarica Cava Riconta	Villaricca	NA	Waste storage site	Underground water	Heavy Metals

The Integrated Regional Plan of official controls on food, feed, animal health and welfare of Campania Region enforces the execution of necropsies on at least 5% of the animals died in the region. To meet this and other needs in the field of public health, in 2010 was established the Regional Centre for Veterinary Urban Hygiene (CRIUV) first example in Italy of integration and

synergy among the national health system (ASL), Zooprophylactic Institute and University. Among the many tasks of the Centre activities there are:

- development of risk exposure evaluation models using synanthropic animal species and, consequently, the development of strategies applicable in Public Health surveillance in the Campania region;
- monitoring the efficacy of strategies developed by the Centre itself by the regional offices of Veterinary Public Health System;
- support to local services in cases of emergency events in the field of prevention of stray dogs, for clinical and surgical services and highly specialized diagnostic procedures, as well as the application of scientific research in Veterinary Urban Hygiene field.

Since 2011, the CRIUV supplies a biomonitoring service to Campania Region, through necropsies of synanthropic animals to obtain data on causes of death, and study the prevalence and frequency of certain diseases and possible associations between environmental quality and public health. Particular attention is given to cancer and chronic degenerative diseases, which can be both closely related to exposure to environmental pollutants. The epidemiological investigation has focused mainly on dog which, in urban areas, can be considered a good sentinel for many environmental pollutants. Given the situation previously described in the provinces of Naples and Caserta, and considering that heavy metals are one of the main environmental contaminants of public concern, it is on these compounds that the attention has been focused for a possible association between disease development and environmental pollution.

The aim of the study was to obtain, through necropsy activities, data on the effects of exposure of synanthropic animals to environmental pollutants (in particular to Heavy Metals) in Campania region (Italy), and evaluate a possible association with development of tumors or chronic diseases and the spread of zoonoses. Furthermore, through data analysis, verify the possible risk for human health and, in such a case, to program public health intervention plans.

1.2 Materials and Methods

From January 2011 to December 2016, 1473 necropsies were performed on synanthropic, wild and captive animals lived and died in Campania Region (Southern Italy). In particular, necropsies were performed on the following species (Table 1.2.1):

Tab 1.2.1. Animal species and number of necropsies performed during the CRIUV surveillance program.

Animal specie	**Number of necropsies**
Dog (*Canis lupus familiaris*)	**978**
Cat (*Felis catus*)	228
Water Buffalo (*Bubalus bubalis*)	75
Pig (*Sus scrofa domesticus*)	49
Horse (*Equus ferus caballus*)	26
Fox (*Vulpes vulpes*)	17
Cow (*Bos taurus*)	16
Goat (*Capra hircus*)	11

Rabbit (*Oryctolagus cuniculus*)	6
Sheep (*Ovis aries*)	6
Donkey (*Equus asinus*)	5
Guinea pig (*Cavia porcellus*)	5
Zebra (*Equus zebra*)	4
Nile lechwe (*Kobus megaceros*)	4
Alligator (*Alligator mississippiensis*)	4
Deer (*Cervus elaphus*)	3
Leopard (*Panthera pardus*)	3
Turtle (*Geochelone pardalis*)	3
Fallow deer (*Dama dama*)	3
Rhesus macaque (*Macaca mulatta*)	3
Badger (*Meles meles*)	2
Python (Python regius)	2
Tiger (*Panthera tigris*)	2
Wolf (*Canis lupus*)	2
Chimpanzee (*Pan troglodytes*)	2
Camel (*Camelus bactrianus*)	1
Hare (*Lepus europaeus*)	1
Tapir (*Tapirus terrestris*)	1
Hedgehog (*Erinaceus europaeus*)	1
Elephant (*Elephant maximus*)	1
Bison (*Bison bison*)	1
Kangaroo (*Macropus rufus*)	1
Sugar glider (*Petaurus breviceps*)	1
Barbary sheep (*Ammotragus lervia*)	1
Iguana (*Iguana iguana*)	1
Otter (*Lutra lutra*)	1
Panther (*Panthera leo*)	1
Chinchilla (*Chinchilla lanigera*)	1

Necropsies were performed at the necropsy room of the Department of Veterinary Medicine and Animal Production of the University of Naples "Federico II", at the necropsy room of the disposal and processing animals by-products Proteg S.p.A. firm, at the necropsy room of The Regional Center for Monitoring Parasitic Infections (CReMoPAR) and, when required, on field. On the total of 1473 necropsies performed, for reasons that previously has been widely discussed, only dogs (n.978) were considered in this study of environmental biomonitoring. Necropsies included (in order):

- Identification of the cadaver
- Collection (when possible) of anamnestic data
- Evaluation of thanathological aspects and estimation of time elapsed since death
- External examination of cadaver (state of nutrition, mucous membranes, body orifices, general conformation, superficial lesions, hair coat, external parasites, teeth)
- In case of "*forensic*" necropsies, after external examination, the hair coat was completely removed and a total body radiograph study (and, if required, a CT-scan examination of the cadaver, or part of) were performed
- Skin of cadaver
- Opening and evaluation of body cavities (abdomen, thorax and pelvic cavity)
- Extraction and macroscopic evaluation of abdominal, thoracic and pelvic organs

- If required, opening of skull and spinal cord to evaluate Central Nervous System organs

All phases of the necropsy were appropriately recorded on a designated necropsy report form (provided as supplementary material) and, for every single case, a photographic archive was created to support what has been accurately reported. The necropsy continued, depending on the additional required diagnostic tests and the state of conservation of the carcass, with sampling of organs and tissues for histopathological and toxicological examination to obtain a final cause of death.

The cause of death was unambiguously specified for each record and then all of the cases were grouped as previously described by Fleming JM et al. 2011 according to the scheme proposed by the American Veterinary Medical Database (VMBD) initiated in 1964 by the (American) National Cancer Institute. Each cause of death was classified by organ system (OS), and separately by pathophysiologic process (PP). The 10 OS categories were cardiovascular, dermatologic, endocrine, gastrointestinal, hematopoietic, hepatic, musculoskeletal, neurologic, respiratory, and urogenital. The 6 PP categories were degenerative, infectious, inflammatory (includes immune-mediated and cases where the observed inflammatory changes could not be attributed to any infectious agent or specific cause – (Eleni C. et al 2014), neoplastic, traumatic, and other. Cases of acute poisoning (i.e. anticoagulant rodenticides poisoning) were excluded from the study as, according to Italian Ordinanza Ministeriale 18 Dicembre 2008, they are of Experimental Zooprophylactic Institute competence; in cases of carcasses in advanced state of putrefaction or when it was not possible (even by histopathology,

microbiology or toxicology) to deliver a plausible cause of death, dogs were classified as “undetermined”.

In addition to diagnostic data, dogs were categorized as purebred (whether or not the named breed was recognized by the American Kennel Club) or crossbred, and then scored for age and grouped in the following 4 categories by a slight modification of the scheme reported by Eleni C. et al in 2014:

- 1 (0-1 year)
- 2 (2-5 years)
- 3 (6-10 years)
- 4 (>10 years)

Results of necropsies, including macroscopic and microscopic findings, as well as toxicological and microbiological results, were then transferred and archived through the Veterinary Activity Management (VAM) software.

The scientific techniques used in this activity were already validated and accredited by the scientific community and were not characterized by any experimental procedures on live animals.

*1.2.1 Histopathology**

In cases of dogs death from malignant tumors or chronic/degenerative diseases, in forensic cases, or in order to confirm or exclude specific causes of death, samples from all the major organs were collected and processed for histopathology. Furthermore, even when tumor was not associated with the cause of death, a sample from the neoplasm was collected to characterize the nature of the tumor. Samples were formalin fixed, paraffin embedded and

stained with haematoxylin/eosin for morphological evaluation of lesions (Jubb Kennedy and Palmer's 2015). Tumors were classified according to WHO histological classification of tumors in domestic animals (WHO Tumors of domestic animals, 2002).

*Histopathological examination was performed only in well preserved and unfrozen carcasses.

*1.2.2 Toxicology***

Samples of liver and kidney were collected from animals died from malignant neoplasm or chronic/degenerative diseases, identified and stored at -20°C until chemical analysis. Tissues were thawed, homogenized and then aliquots of each sample (0.50 ± 0.01 g) were digested in 4.0 ml of 70% nitric acid (Carlo Erba), 1.5 ml of 30% hydrogen peroxide (Baker Analyzed) and 3.5 ml of ultrapure water for atomic absorption spectroscopy (Best Chemicals) in a microwave digestion system (Milestone, FKW) under high pressure and temperature at 190°C.

Digested samples were analyzed for quantitative determination of heavy metals. Lead and cadmium were determined by atomic absorption spectrophotometer equipped with graphite furnace atomizer (AAS-GF) with Zeeman effect (Analyst 800, Perkin-Elmer). Mercury and Arsenic were determined by hydride-generation atomic absorption spectrophotometer (CV-AAS, 3110, Perkin-Elmer).

Matrix modifiers, monobasic ammonium phosphate and magnesium nitrate (1% Mg) were purchased from Perkin Elmer (US Massachusetts). Standard solutions of lead, cadmium, arcenic and mercury, prepared by dilution of

multi-elemental standard solutions of 1000 mg L−1, were purchased from Merck (Darmstadt, Germany) and working standard solutions of all trace elements were prepared by diluting stock solutions with ultrapure water. Quantification was performed by external standardization, without correction for recovery percentage. Calibration curves were obtained analysing standard solutions of each trace element. All concentration was expressed as mg kg^{-1} of wet weight. Results represent mean value of at least three independent analyses and were processed statistically using the analysis of variance (ANOVA).

**Toxicological analysis for Heavy Metals were performed starting from January 2014, and only in well preserved carcasses.

1.2.3 Geographical Information System (G.I.S.)

All data were georeferenced (at the county and street level) and the spatial distribution of dogs studied was compared with the legal and illegal dumping site of Campania Region.

1.2.4 Statistical analysis

The proportion of each cause of death and its 95% binomial exact confidence interval (95% CI) were calculated. The $\chi 2$ test was used to compare proportions of causes of death in dogs of different classes of age. Two-sided tests were conducted and a p-value of less than 0.05 was considered

statistically significant. The statistical analysis was performed using SOFASTAT® softwere (ver. 1.4.6).

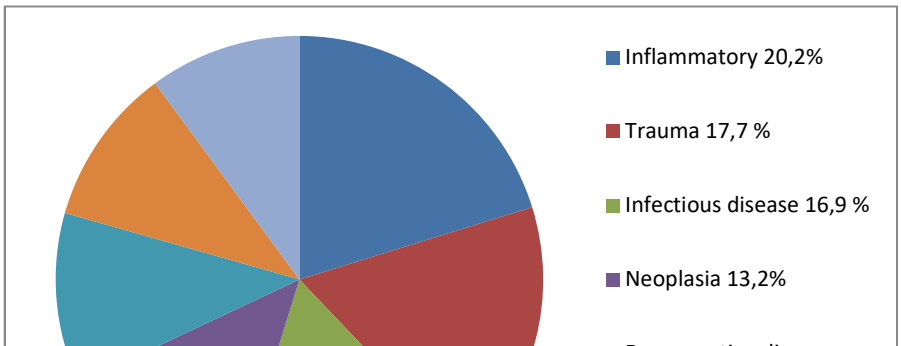
1.3 Results

The study included 978 dogs whose characteristics are summarized in Table 1.3.1

Tab 1.3.1. Characteristics of dogs in the studied population

Variable	N° of dogs (%)
Sex	
Male	466 (47.6%)
Female	512 (52.4%)
Age classes	
1 (0-1year)	270 (27.6%)
2 (2-5 years)	143 (14.7%)
3 (6-10 years)	289 (29.5%)
4 (>10 years)	276 (28.2%)
Breed	
Crossbred	733 (74.9%)
Purebred	245 (25.1%)

The majority of dogs were female (52.4%), while the distribution for age was quite heterogeneous, with group 3 and 4 being the more representative (29.5 – 28.2% respectively) and group 2 the less (14.7%). On 101 dogs (10.1%) the cause of death was “undetermined” due to the advanced state of putrefaction or the absence of significant lesions, even when minimum



standards tests, i.e. bacteriological and histological tests, were performed (Figure 1.3.1). No differences were detected in the frequency of causes of death between sexes.

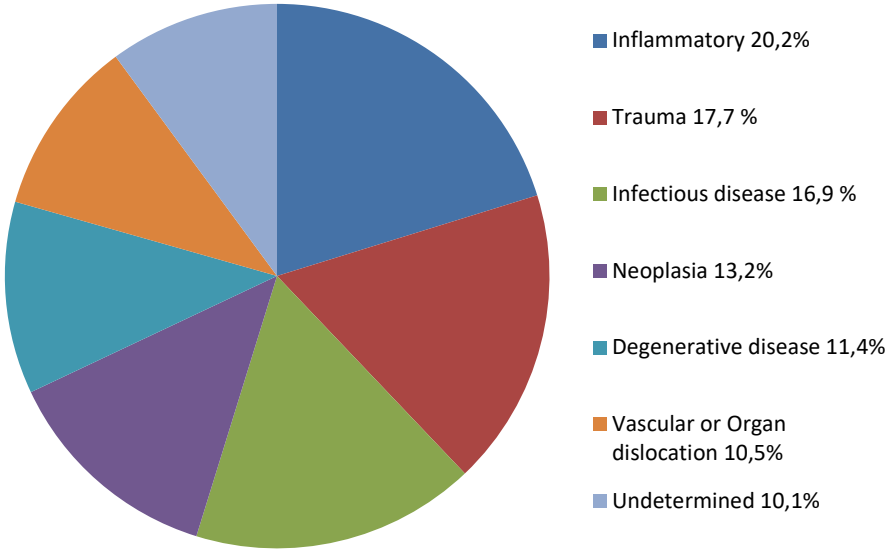


Fig 1.3.1. Main causes of death in dogs (N. 978) of Campania Region, examined from 2011 to 2016

The overall main cause of death was "inflammatory" (N.197 – 20,2%) with respiratory, gastrointestinal, and urogenital as the most frequently affected systems (Table 1.3.2).

Tab 1.3.2 Specific causes of death within each macro-category

Macro-Category/ Specific causes	**(%)**	**Macro-Category/** Specific causes	**(%)**
Inflammation	**Tot n. 197**	**Neoplasia**	**Tot n. 129**
Acute Pneumonia (with different kind of exudate)	42,4%	Multicentric Lymphoma	25,5%
Hemorrhagic Enteritis	28,7%	Haemangiosarcoma	18,6%
Nephritis (acute or chronic)	28,7%	Colangiocarcinoma/Hepatic	10,8%
Others	<1%	Osteosarcoma	8,5%
Trauma	**Tot n. 173**	Others	32,3%
Car accident	88,7%	**Degenerative/chronic diseases**	**Tot n. 111**
Generic trauma	9,4%	Chronic Nephropaties	51,3%
Post-surgery haemorrhages	<2%	Severe Hepatosis	35,1%
Infectious diseases	**Tot n. 165**	Severe Endocardiosis	13,5%
Parvovirosis	47,2%	**Vascular / Organ Dislocation**	**Tot n. 102**
Gram+/- Septicemia	11,3%	Cardiomyopathy	44,1%
Leishmaniasia	3,9%	Gastric Torsion/ Intussusception	35,2%
Others (1 Case TBC)	35,7%	CID	6,4%
		Others	<15%

For all dogs, Figure 1.3.2 shows the percentage of death attributable to each OS and PP category, grouped for age classes; results are summarized in table 1.3.2.

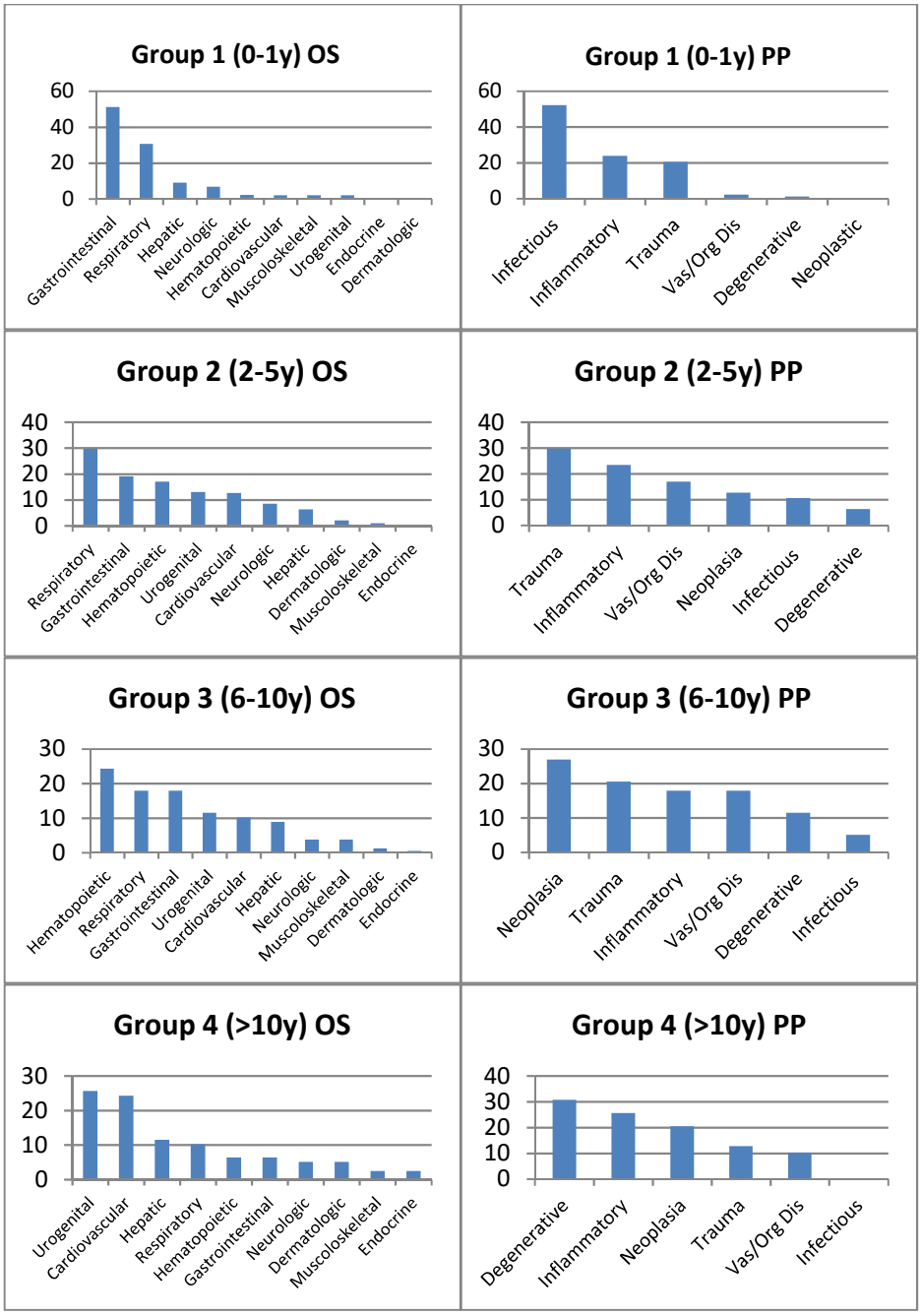


Fig. 1.3.2 Proportion of deaths attributable to each organ system (OS) and pathophysiologic process (PP) category for each of the studied group

Inflammation and infectious disease affecting the respiratory and gastrointestinal systems were the main cause of death (OS and PP) in puppies and young dogs, whilst hematopoietic, cardiovascular and urogenital systems were the most involved in adult and aged dogs (Figure 1.3.3)Cau. Among the infectious diseases, the most frequently diagnosed as cause of death was parvovirosis (47,2%); *Leishmaniasis* was the cause of death in 3,9% of dogs belonging to infectious-disease macrocategory. In one case tha cause of death was tuberculosis supported by *M. avium*.

Regarding PPs, trauma is a frequent cause of death for dogs within age groups 1 and 2 and declines steeply thereafter. Degenerative processes contribute to the increasing mortality rate of older dogs and has an ascending index throughout the 4 groups, and neoplasia as a cause of death peaks in group 3, and then declines as a contributing cause among dogs in the oldest age group (Figure 1.3.4)

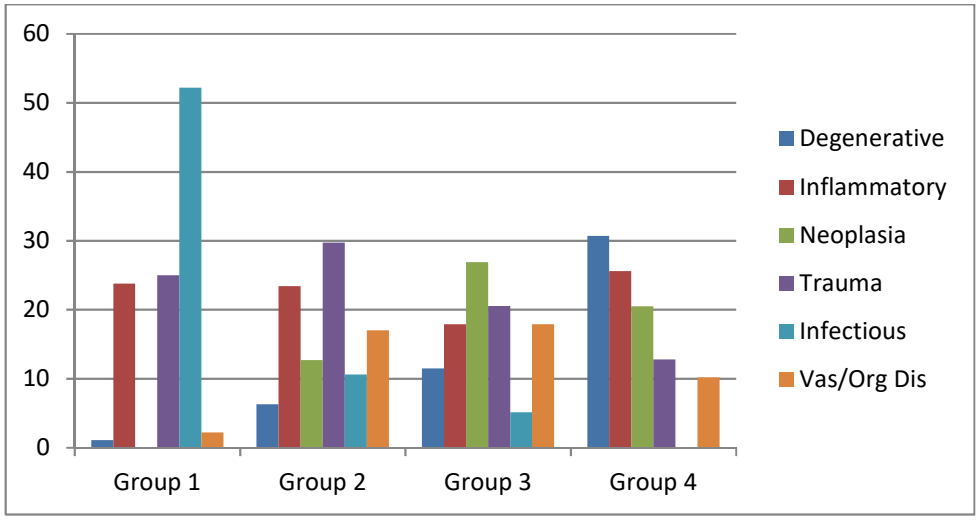


Fig 1.3.3 Causes of death categorized per PP and group

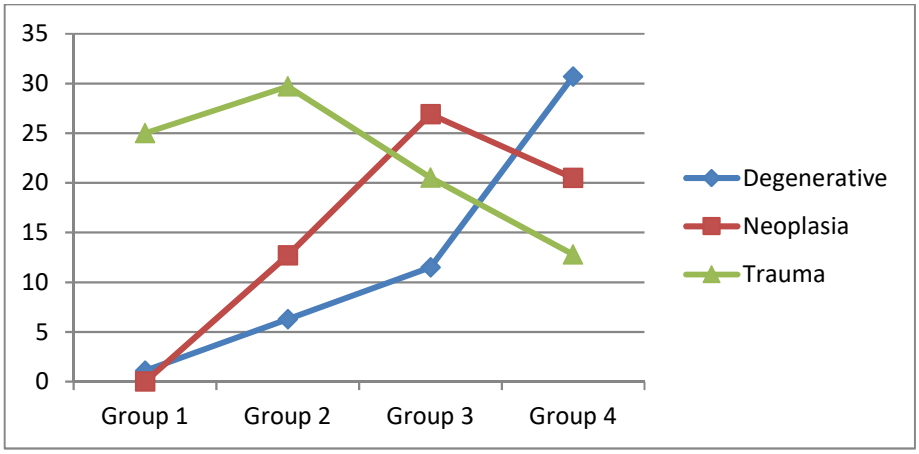
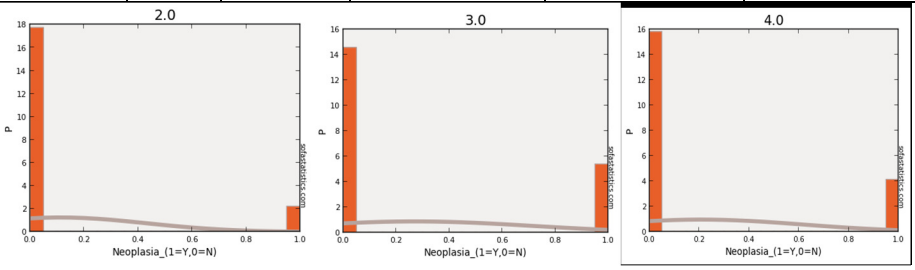


Fig 1.3.4 Frequency variances of each cause of death within each group of dogs

Statistical analysis, as expected, showed correlation between age of dogs and frequencies of infectious disease, degenerations and malignant neoplasm as causes of death (Figure 1.3.5 - 10).

Group	N.	Mean	Cl 95%	Std Deviation	p
1	270	0.0	0.000 - 0.000	0.000	-
2	143	0.112	0.060 - 0.164	0.316	< 0.001
3	289	0.27	0.219 - 0.321	0.445	< 0.001
4	275	0.207	0.159 - 0.255	0.406	< 0.001



		Age_Group									
		1		2		3		4		TOTAL	
		Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp
Neoplasia	N	270	228.3	127	120.9	211	244.3	218	232.5	826	826.0
	Y	0	41.7	16	22.1	78	44.7	57	42.5	151	151.0
	TOTAL	270	270.0	143	143.0	289	289.0	275	275.0	977	977.0

Fig 1.3.5 Results of ANOVA test of Neoplasia as cause of death for each Age Group (p< 0.001)

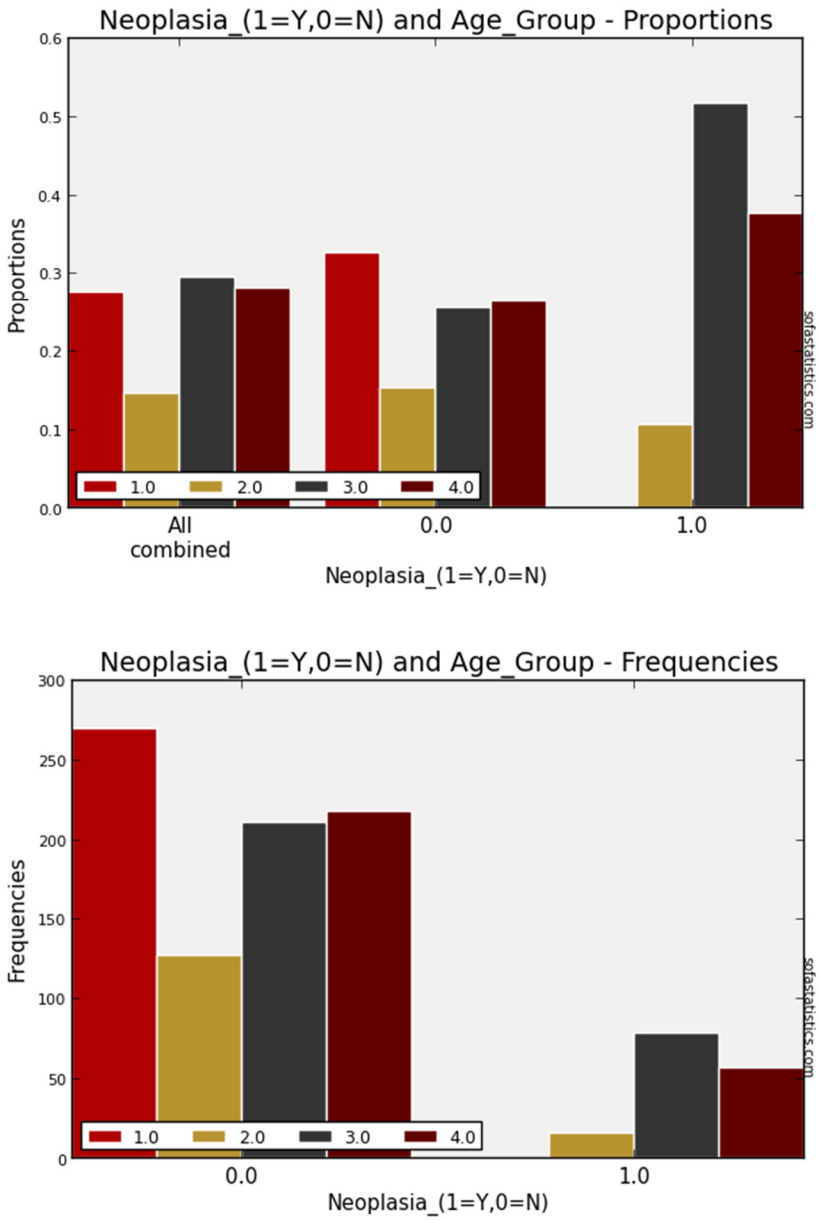
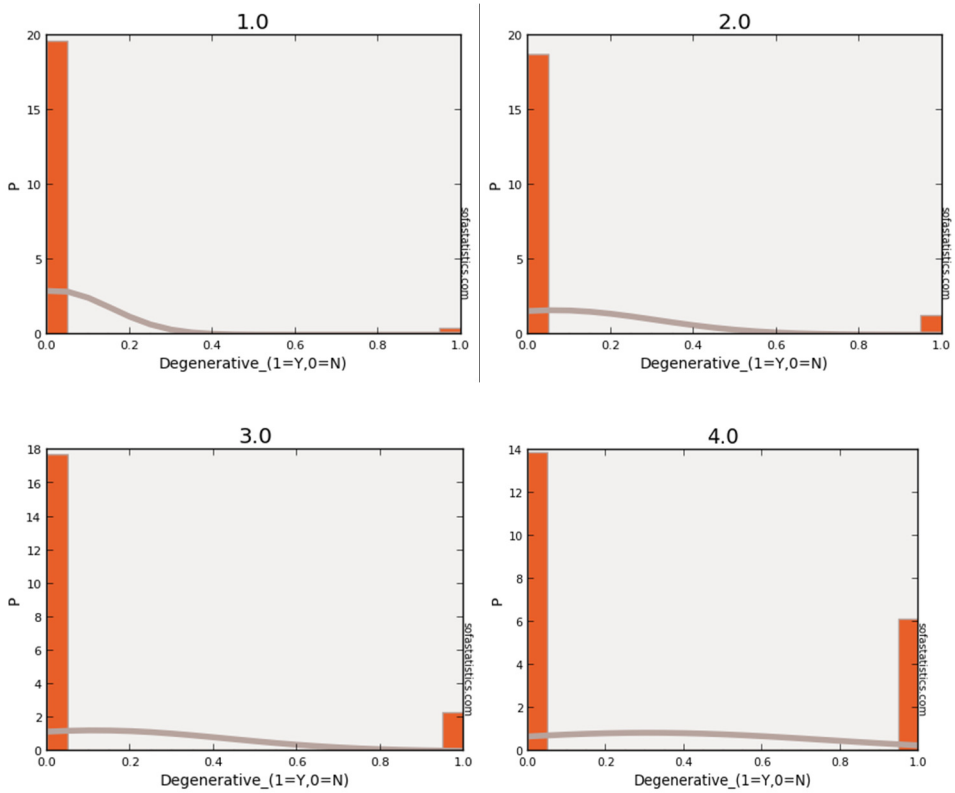


Fig 1.3.6. Results of Pearson's Chi Square Test of Association Between "Neoplasia (1=Yes,0=No)" and "Age_Group" ($p < 0.001$).

Group	N.	Mean	Cl 95%	St Deviation	p
1	270	0.019	0.002 - 0.035	0.135	< 0.001
2	143	0.063	0.023 - 0.103	0.244	< 0.001
3	289	0.114	0.077 - 0.151	0.319	< 0.001
4	275	0.305	0.251 - 0.360	0.461	< 0.001



		Age_Group									
		1.0		2.0		3.0		4.0		TOTAL	
		Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp
Degenerative_(1=Y,0=N)	0.0	265	233.8	134	123.8	256	250.2	191	238.1	846	846.0
	1.0	5	36.2	9	19.2	33	38.8	84	36.9	131	131.0
	TOTAL	270	270.0	143	143.0	289	289.0	275	275.0	977	977.0

Fig 1.3.7. Results of ANOVA test for Degenerative diseases as cause of death (1=Y,0=N) for Age_Group.

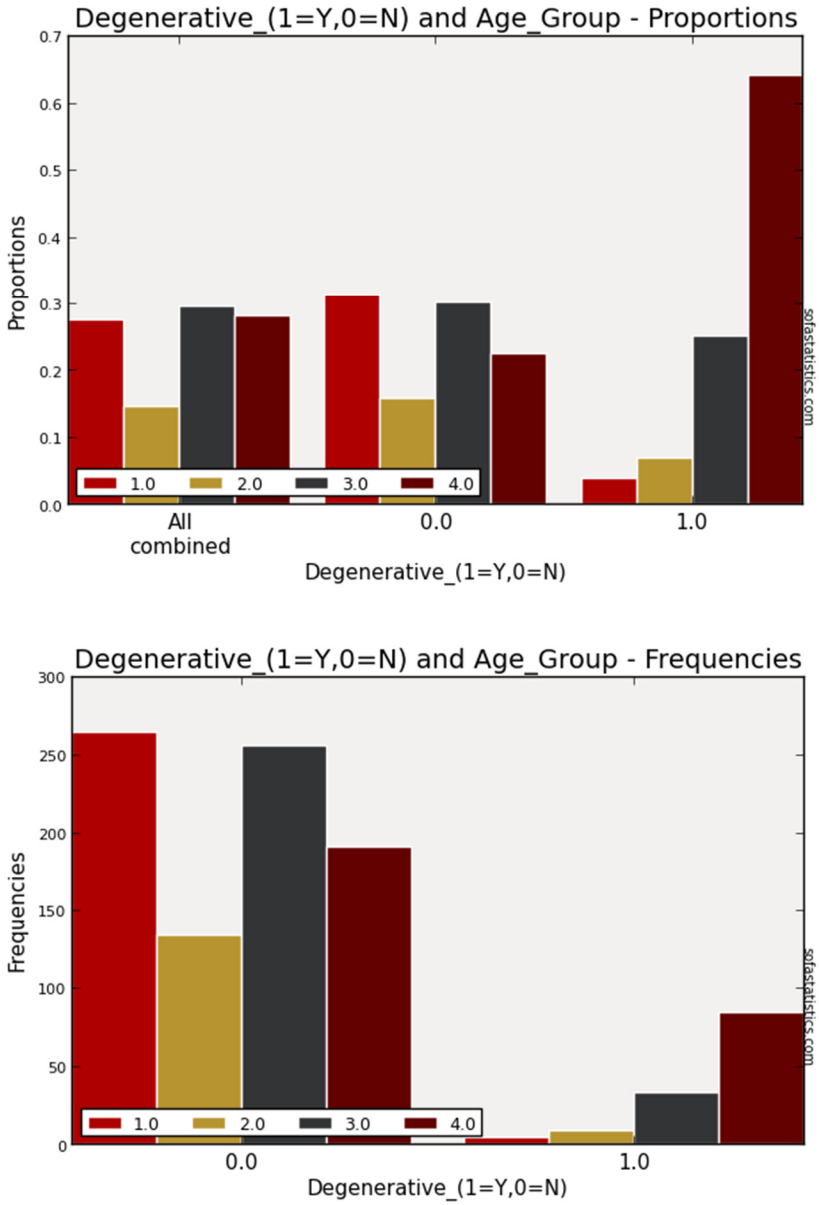
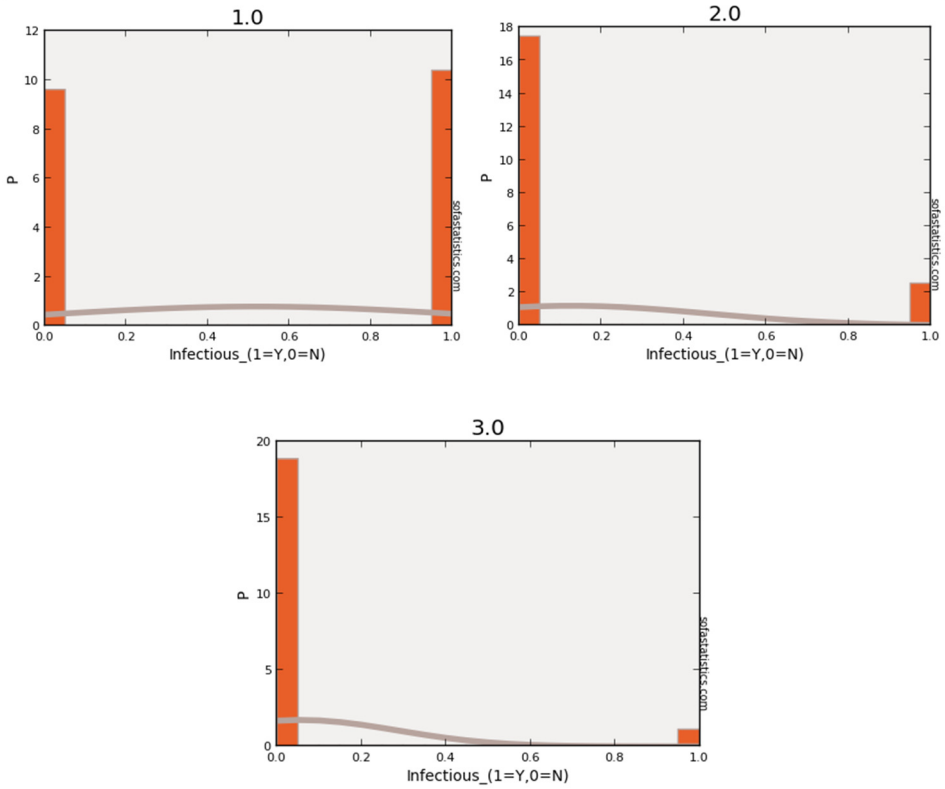


Figure 1.3.8. Results of Pearson's Chi Square Test of Association Between "Degdnerative diseases (1=Y,0=N)" and "Age_Group" (p< 0.001).

Group	N.	Mean	Cl 95%	St Deviation	p
1	270	0.519	0.459 - 0.578	0.501	< 0.001
2	143	0.126	0.071 - 0.180	0.333	< 0.001
3	289	0.055	0.029 - 0.082	0.229	< 0.001
4	275	0.0	0.000 - 0.000	0.000	-



		Age_Group									
		1.0		2.0		3.0		4.0		TOTAL	
		Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp
Infectious_(1=Y,0=N)	0.0	130	221.9	125	117.5	273	237.5	275	226.0	803	803.0
	1.0	140	48.1	18	25.5	16	51.5	0	49.0	174	174.0
	TOTAL	270	270.0	143	143.0	289	289.0	275	275.0	977	977.0

Fig 1.3.9. Results of ANOVA test for infectious diseases (1=Yes; 0=No) for Age group

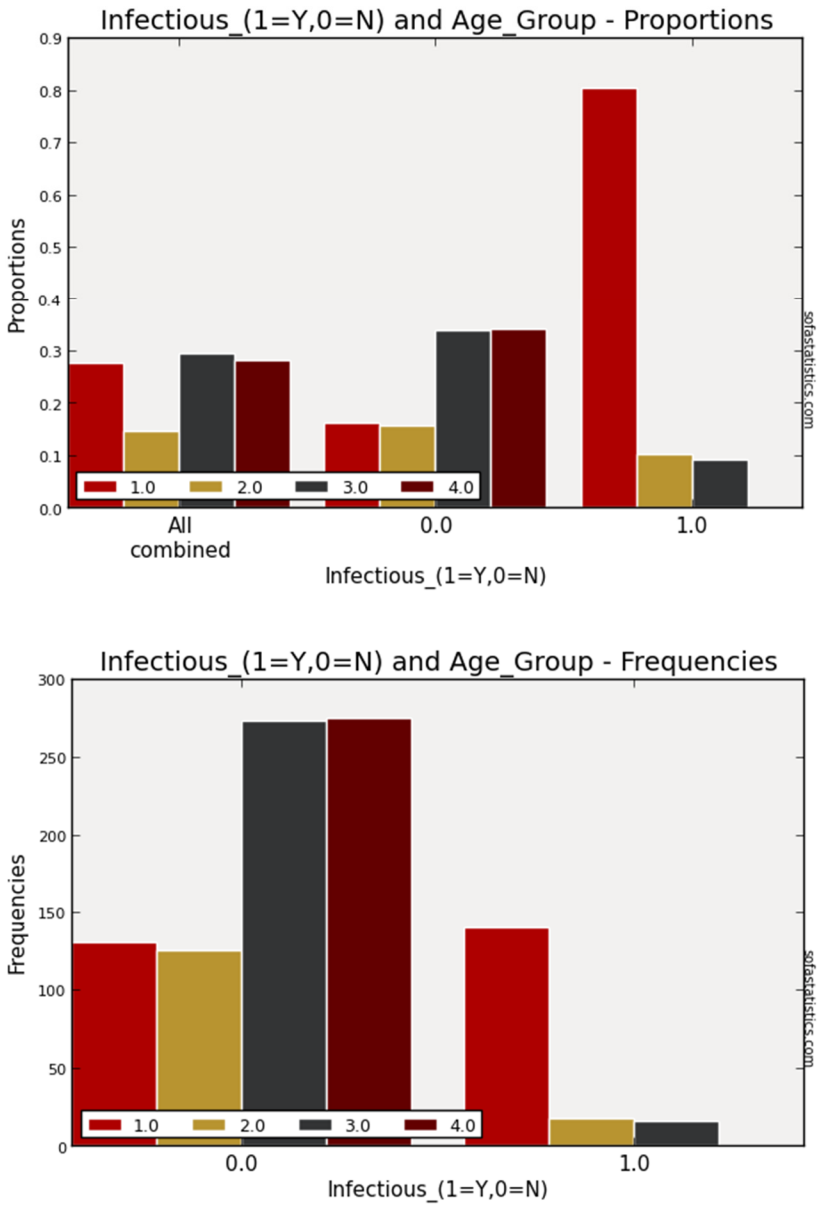


Fig 1.3.10. Results of Pearson's Chi Square Test of Association Between "Infectious diseases" and "Age_Group" ($p < 0.001$).

Malignant tumors were the fourth cause of death in the whole studied population of dogs, with different frequencies throughout the years (Figure 1.3.11). The main organ system involved by neoplasia as cause of death were the hemolinphopoietic and the vascular systems, with multicentric lymphoma (25,5%) and splenic hemangiosarcoma (18,6%) as the most frequent diagnosed tumors (results are summarized in Figure 1.3.11)

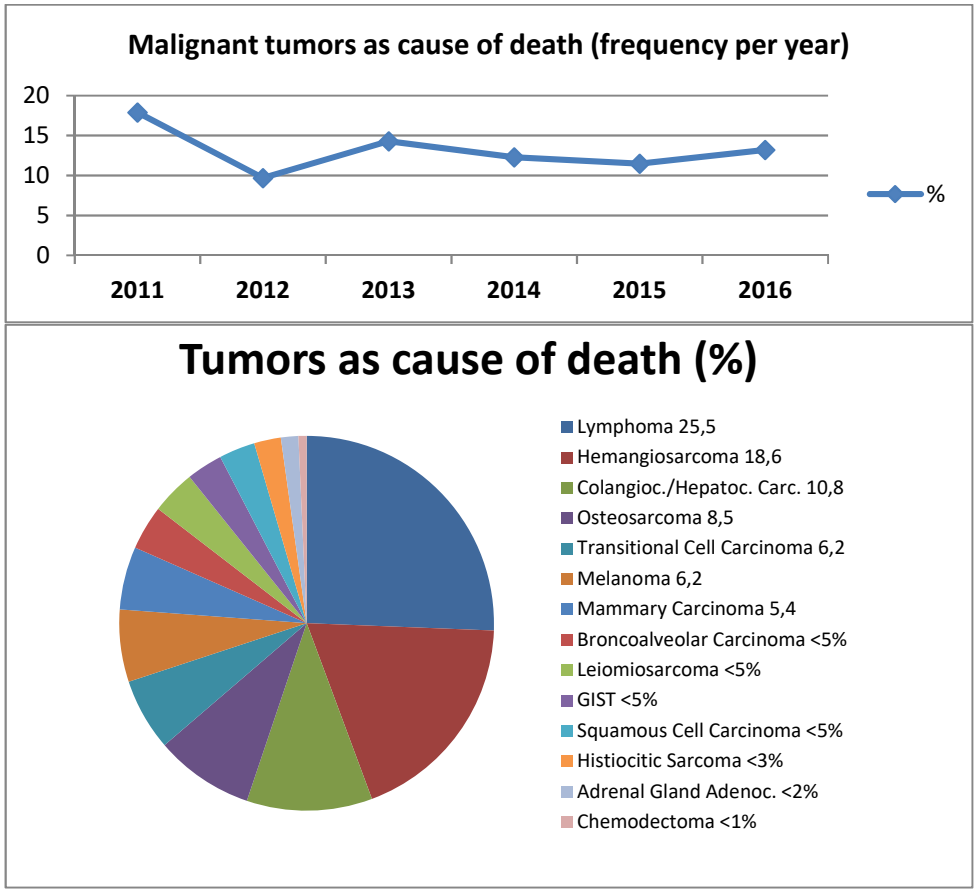


Fig 1.3.11. Frequencies (per year) of tumors as cause of death (on top). Overall frequencies of each tumor as cause of death in studied population

Out of the 129 neoplasm described, in 51 dogs were found tumors which could not be characterized due to the advanced state of putrefaction of the cadaver (n.18), and neoplasm which were not recognized as cause of death (i.e. n.9 leydigoma, n.15 mammary carcinoma with no macroscopic detectable metastasis, n.2 cutaneous Mast-cell tumor with no macroscopic detectable metastasis, n.5 Seminoma).

From January 2014, as stated in the Integrated Regional Plan of official controls on food, feed, animal health and welfare of Campania Region, the C.R.I.U.V. started a toxicological screening for Heavy Metals dosages on kidney and liver of dogs died from neoplastic or chronic/degenerative diseases. On a total population of 509 dogs (tot. n. 978 less 469 dogs died from 2011 to 2013) n.61 (11,9%) died for malignant neoplasia and n.45 (8,8%) for chronic or degenerative disease. On the basis of exclusion criteria described in materials and methods section toxicological screening was performed on 69 dogs (Table 1.3.3).

Tab 1.3.3 Case number, signalment, cause of death and provenience of studied dogs.

Case n.	**Breed**	**Age (years)**	**Sex**	**Diagnosis**	**Provenience**
1	Crossbred	11	F	Leiomyosarcoma	Secondo Vico Cavour – Parete (CE)
2	Crossbred	7	F	Renal Carcinoma	Via Montegrappa – Visciano (NA)

3	Crossbred	8	F	Lymphoma	Via Manzoni – Castello di Cisterna (NA)
4	Crossbred	9	M	Lymphoma	Via R. Sanzio – Orta di Atella (CE)
5	Crossbred	6	M	Lymphoma	Via dei Glicini – San Tammaro (CE)
6	Crossbred	11	F	Bronchioloalveolar C.	Via Santa Lucia – Aversa (CE)
7	Crossbred	8	F	Tansitional Cell Carc.	Via Marche – Ottaviano (NA)
8	Crossbred	13	F	Mammary Carcinoma	Via M. Spaccata – Pianura (NA)
9	Dogue De Bordeaux	10	F	Mammary Carcinoma	Via G.A. Campano - NA
10	German Shepherd	8	F	Mammary Carcinoma	Cont. S.Cumana - BN
11	German Shepherd	10	F	Mammary Carcinoma	C.so Umberto – Marano di Napoli (NA)
12	Rottweiler	6	F	Mammary Carcinoma	Via Palazzuolo – Scisciano (NA)

13	Crossbred	14	F	Mammary Carcinoma	ASL NA 1 District
14	Crossbred	7	F	Mammary Carcinoma	ASL NA 1 District
15	Crossbred	10	F	Mammary Carcinoma	Pianura (NA)
16	Crossbred	10	F	Renal Carcinoma	Pomigliano D'Arco (NA)
17	Beagle	9	F	Bronchioloalveolar C.	Via Jannelli – (NA)
18	Shih Tzu	10	F	Adrenocortical Carc.	Pomigliano D'Arco (NA)
19	Yorkshire	10	F	Adrenocortical Carc.	C.so A. Volta – Terzigno (NA)
20	Crossbred	14	F	Lymphoma	P.zza Santini – Capaccio (SA)
21	Boxer	13	F	Lymphoma	Via Villanova – (NA)
22	Crossbred	12	M	Colangiocarcinoma	Via V. Wolff – (NA)
23	Yorkshire	8	F	Colangiocarcinoma	San Giuseppe Vesuviano (NA)
24	Crossbred	11	M	Colangiocarcinoma	Ponticelli (NA)

25	Crossbred	10	F	Hemangiosarcoma	P.zza Leonardo (NA)
26	Crossbred	6	M	Hemangiosarcoma	Via Vaiani – Quarto (NA)
27	Crossbred	14	M	Hemangiosarcoma	V.co Trone alla Salute (NA)
28	Pitbull	12	M	Hemangiosarcoma	P.zza Cavour (NA)
29	Labrador	14	F	Hemangiosarcoma	Salita Arenella (NA)
30	Crossbred	16	F	Hemangiosarcoma	Via Torrione S. Martino (NA)
31	Crossbred	10	F	Hemangiosarcoma	Via G.Salvatore (NA)
32	Crossbred	7	F	Hemangiosarcoma	Via Sediari (NA)
33	German Shepherd	9	M	Hemangiosarcoma	Via Vecchia S.Rocco (NA)
34	Crossbred	12	F	Hemangiosarcoma	Via Roma (NA)
35	Crossbred	10	F	Hepatocarcinoma	Via Fieramosca – Cancello Arnone (CE)
36	Bulldog Inglese	9	M	Hepatocarcinoma	Via Nicolardi (NA)
37	Crossbred	4	F	Leiomyosarcoma	Via Vicinale Margherita (NA)

38	Crossbred	18	M	Lymphoma	NA
39	Crossbred	12	F	Lymphoma	Via delle Rep. Marinare (NA)
40	Crossbred	12	M	Lymphoma	V.le della Resistenza (NA)
41	Crossbred	11	M	Lymphoma	Volla
42	Crossbred	10	M	Lymphoma	Via del Campo – S.Giuseppe V. (NA)
43	Crossbred	8	M	Osteosarcoma	Via Ghisleri (NA)
44	Rottweiler	7	M	Osteosarcoma	Bosco di Capodimonte (NA)
45	Cavalier King	10	M	Osteosarcoma	Via A.Rocco (NA)
46	Crossbred	8	M	Transitional Cell Carcinoma	Via Chiaiano (NA)
47	Crossbred	6	F	Transitional Cell Carcinoma	V.le Europa – Aversa (CE)
48	Cocker Spaniel	4	F	Severe Hepatosis	Via P.Castaldi (NA)
49	Crossbred	16	F	Severe Hepatosis	Via Amato di Montecassino (NA)

50	Crossbred	7	M	Severe Hepatosis	Chiaiano (Metro) – NA
51	Setter Ing	9	F	Severe Hepatosis	Via di Crollanza – Gricignano (CE)
52	Crossbred	13	M	Severe Hepatosis	Via S.M. delle Catene (NA)
53	Crossbred	15	F	Chronic Kidney Disease Thyroid hyperplasia	Via Giuliano da Miano (NA)
54	Crossbred	14	M	Severe Hepatosis	Via Monte Russo – Pozzuoli (NA)
55	Siberian Huskey	10	F	Chronic Kidney Disease Thyroid hyperplasia	Via Santa Maria a Cubito (NA)
56	Crossbred	10	M	Chronic Kidney Disease Adrenal Gland hyperplasia	Via Toscanella – (NA)
57	Crossbred	13	M	Chronic Kidney Disease Prostatic hyperplasia	Via Eremo dei Camaldoli (NA)

58	Crossbred	11	F	Chronic Kidney Disease Adrenal Gland hyperplasia	Via E. Torricelli (NA)
59	Crossbred	10	M	Chronic Kidney Disease Prostatic Hyperplasia	P.zza Casanova – (NA)
60	Yorkshire	14	F	Severe Hepatosis	Via F.Saverio – Sant'Anastasia (NA)
61	German Shepherd	6	M	Chronic Kidney Disease Prostatic Hyperplasia	Ss Nola Villa Literno – Carinaro (CE)
62	Crossbred	12	M	Chronic Kidney Disease Thyroid hyperplasia	Casal di Principe (CE)
63	Crossbred	10	F	Chronic Kidney Disease Adrenal Gland hyperplasia	Via L.Caterino – S.Cipriano d'Aversa (CE)
64	Crossbred	14	F	Chronic Kidney Disease	Via Arenaccia 173 (NA)

				Adrenal Gland hyperplasia	
65	Crossbred	10	M	Chronic Kidney Disease Adrenal Gland hyperplasia	Via M.Diana – S.Cipriano d'Aversa (CE)
66	Poodle	10	F	Chronic Kidney Disease Thyroid hyperplasia	Via V.Emanuele – Parete (CE)
67	Crossbred	8	F	Chronic Kidney Disease Thyroid hyperplasia	Via Argine (NA)
68	Crossbred	13	F	Chronic Kidney Disease Adrenal Gland hyperplasia	Via del Seggio – Aversa (CE)
69	Crossbred	11	M	Chronic Kidney Disease Adrenal Gland hyperplasia	Via Marche – Ottaviano (NA)

Results of dosage for each metal (mg/Kg) are presented in the following table (Table 1.3.4) and were compared with minimum, medium and maximal level previously described (Serpe et al. 2012; Pablack N. et al. 2015).

Tab 1.3.4. Value (mg/Kg): ND (Not Detected); 0.000 (lower than the minimum level of range); ***0.000*** *(within the range and lower than median value);* ***0.000*** *(within the range and higher than median alue);* ***0.000*** *(within the range but very close to the maximum level);* ***0.000*** *(higher then the maximum level of range).*

		Liver				**Kidney**			
N.	**Lesion**	**Pb**	**Cd**	**Hg**	**As**	**Pb**	**Cd**	**Hg**	**As**
1	Leiomyosarcoma	0.069	**0.076**	**0.144**	ND	ND	**0.060**	ND	ND
2	Renal Carcinoma	**0.120**	**0.249**	**0.088**	ND	**0.051**	**0.092**	ND	ND
3	Lymphoma	ND	**0.539**	**0.072**	ND	ND	ND	ND	ND
4	Lymphoma	0.129	0.173	**0.056**	ND	0.060	0.159	ND	ND
5	Lymphoma	**0.116**	**0.080**	**0.037**	ND	**0.023**	**0.395**	**0.067**	ND
6	Bronchioloalveolar C.	0.071	**0.079**	ND	ND	0.028	**0.325**	**0.139**	ND
7	Tansitional Cell Carc.	0.063	ND	ND	ND	ND	ND	ND	ND

8	Mammary Carcinoma	0.093	0.103	**0.096**	ND	ND	**0.437**	**0.182**	ND
9	Mammary Carcinoma	**0.140**	**0.051**	ND	ND	**0.070**	**0.343**	**0.065**	ND
10	Mammary Carcinoma	0.091	0.055	ND	ND	0.090	**0.919**	ND	ND
11	Mammary Carcinoma	ND	ND	ND	ND	ND	ND	ND	ND
12	Mammary Carcinoma	ND	**0.122**	ND	ND	ND	**0.491**	ND	ND
13	Mammary Carcinoma	0.172	**0.156**	ND	ND	ND	**0.299**	ND	ND
14	Mammary Carcinoma	0.137	0.046	ND	ND	0.053	0.227	ND	ND
15	Mammary Carcinoma	**1.758**	0.038	ND	ND	ND	ND	**0.060**	ND
16	Renal Carcinoma	0.178	**0.103**	0.048	ND	0.100	0.028	**0.056**	ND
17	Bronchioloalveolar C.	0.076	**0.125**	0.040	ND	ND	**0.279**	ND	ND
18	Adrenocortical Carc.	0.062	**0.100**	**0.088**	ND	0.215	0.137	**0.080**	ND
19	Adrenocortical Carc.	0.153	0.090	**0.124**	ND	0.105	0.172	**0.192**	ND

20	Lymphoma	ND	0.095	ND	ND	ND	0.175	ND	ND
21	Lymphoma	ND	0.079	ND	ND	ND	0.813	0.082	ND
22	Colangiocarcinoma	0.041	0.010	ND	ND	ND	0.153	ND	ND
23	Colangiocarcinoma	0.065	0.064	ND	ND	ND	0.186	ND	ND
24	Colangiocarcinoma	0.102	0.062	ND	ND	0.025	0.186	ND	ND
25	Hemangiosarcoma	0.103	0.147	0.404	ND	0.067	0.108	0.324	ND
26	Hemangiosarcoma	0.342	0.032	0.115	ND	0.193	0.089	0.136	ND
27	Hemangiosarcoma	0.191	0.192	0.030	ND	0.100	0.226	0.076	ND
28	Hemangiosarcoma	0.242	0.047	ND	ND	0.092	0.198	ND	ND
29	Hemangiosarcoma	0.094	0.031	0.146	ND	0.025	0.155	0.137	ND
30	Hemangiosarcoma	0.060	0.152	0.088	ND	ND	0.016	0.046	ND
31	Hemangiosarcoma	0.072	0.092	ND	ND	0.044	0.201	0.048	ND
32	Hemangiosarcoma	0.324	0.116	ND	ND	0.125	0.311	ND	ND
33	Hemangiosarcoma	0.171	0.053	ND	ND	0.108	0.294	0.035	ND
34	Hemangiosarcoma	0.252	0.113	0.190	ND	0.063	0.555	0.212	ND
35	Hepatocarcinoma	0.262	0.026	ND	ND	0.106	0.289	ND	ND

36	Hepatocarcinoma	0.052	0.056	0.050	ND	0.060	0.405	0.125	ND
37	Leiomyosarcoma	0.079	0.067	ND	ND	0.071	0.409	0.071	ND
38	Lymphoma	ND	ND	ND	ND	0.073	0.427	0.049	ND
39	Lymphoma	0.064	0.102	0.127	ND	0.084	0.250	ND	ND
40	Lymphoma	0.203	0.048	ND	ND	ND	0.326	0.052	ND
41	Lymphoma	ND	0.099	ND	ND	ND	0.238	ND	ND
42	Lymphoma	ND	0.017	ND	ND	ND	0.304	ND	ND
43	Osteosarcoma	0.089	0.055	ND	ND	ND	0.058	ND	ND
44	Osteosarcoma	0.095	0.109	0.088	ND	0.072	0.491	0.046	ND
45	Osteosarcoma	0.335	0.009	0.011	ND	0.209	0.008	0.008	ND
46	Transitional Cell Carcinoma	ND	0.059	0.031	ND	0.020	0.171	0.233	ND
47	Transitional Cell Carcinoma	ND	ND	0.042	ND	0.275	1.197	ND	ND
48	Severe Hepatosis	0.078	ND	0.264	ND	0.060	0.173	0.296	ND
49	Severe Hepatosis	0.092	0.130	0.116	ND	ND	0.265	0.080	ND
50	Severe Hepatosis	0.156	0.047	0.033	0.30	0.074	0.597	0.182	ND

51	Severe Hepatosis	ND	ND	**0.034**	ND	0.042	ND	ND	ND
52	Severe Hepatosis	**0.656**	**0.013**	ND	ND	**0.140**	**0.149**	ND	ND
53	Chronic Kidney Disease Thyroid hyperplasia	0.041	**0.073**	**0.087**	ND	ND	**0.749**	**0.154**	ND
54	Severe Hepatosis	0.070	**0.153**	**0.592**	**0.663**	ND	**0.722**	**0.288**	**0.417**
55	Chronic Kidney Disease Thyroid hyperplasia	**0.190**	**0.179**	**0.065**	ND	**0.077**	**0.696**	**0.063**	ND
56	Chronic Kidney Disease Adrenal Gland hyperplasia	**0.123**	**0.162**	**0.122**	ND	**0.084**	**0.340**	**0.116**	ND
57	Chronic Kidney Disease Prostatic hyperplasia	**0.164**	**0.144**	**0.046**	ND	**0.064**	**0.622**	**0.058**	ND
58	Chronic Kidney Disease Adrenal Gland hyperplasia	**0.298**	**0.112**	**0.044**	ND	**0.058**	**0.329**	**0.080**	ND

59	Chronic Kidney Disease Prostatic Hyperplasia	**0.216**	**0.082**	ND	ND	**0.128**	**0.527**	**0.105**	ND
60	Severe Hepatosis	0.030	**0.041**	ND	ND	ND	**0.470**	**0.061**	ND
61	Chronic Kidney Disease Prostatic Hyperplasia	**0.476**	**0.086**	0.353	ND	**0.093**	**0.086**	**0.758**	ND
62	Chronic Kidney Disease Thyroid hyperplasia	**0.120**	**0.064**	ND	ND	**0.088**	**0.418**	ND	ND
63	Chronic Kidney Disease Adrenal Gland hyperplasia	ND	**0.037**	ND	ND	0.046	**0.215**	ND	ND
64	Chronic Kidney Disease Adrenal Gland hyperplasia	0.033	**0.072**	ND	ND	ND	**0.385**	**0.154**	ND
65	Chronic Kidney Disease	**0.282**	**0.132**	**0.062**	ND	ND	ND	**0.060**	ND

	Adrenal Gland hyperplasia								
66	Chronic Kidney Disease Thyroid hyperplasia	0.068	**0.024**	**0.133**	ND	**0.053**	**0.042**	**0.084**	ND
67	Chronic Kidney Disease Thyroid hyperplasia	**0.294**	**0.073**	**0.045**	ND	**0.065**	**0.218**	**0.057**	ND
68	Chronic Kidney Disease Adrenal Gland hyperplasia	**0.158**	**0.221**	**0.320**	ND	ND	**0.576**	**1.711**	ND
69	Chronic Kidney Disease Adrenal Gland hyperplasia	**0.299**	ND	**0.052**	ND	**0.106**	**0.404**	**0.035**	ND

Medium value, maximum and minimum levels and standard deviation for each metal, Arsenic excluded, was evaluated. Results are showed in table 1.3.5.

Tab 1.3.5. Media, Standard deviation, Minimum and maximal levels of each metal found in liver and kidney

	Lead		**Mercury**		**Cadmium**	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
Media	0,184386	0,086023	0,117974	0,163405	0,096161	0,326746
St. Dev.	0,242169	0,053008	0,120485	0,274704	0,07738	0,233564
Min	ND	ND	ND	ND	ND	ND
Max	1,758	0,275	0,592	1,711	0,539	1,197

The variability of all parameters was high, indicating large individual differences. Traces of Pb were found in the liver of 51 dogs (73,9%). In 5 dogs the value was within the range, but higher than the median value. The highest level was found in dog n. 15, a 10 years old, crossbred femal dog died for mammary carcinoma with macroscopic detactable metastasis in lungs and liver. Lead in kidney was found in 39 dogs (61.9%) and in all of these cases, levels where lower than the median or the minimum values of range. Lead was found in the kidney of 44 dogs (63,7%) and all the levels were lower than medium value of the range. The highest level (0.275 mg/Kg) was found in a 6 years old crossbred femal dog died for a Tranitional cell carcinoma.

Cadmium was found in the liver of 62 dogs (89,8%) and in 25 of these, levels were higher than the median value. The highest level, exciding the maximal value of range, was found in a 8 years old crossbred female dog (case n.3) which died for multicentric lymphoma. In the kidney of 63 dogs (91,3%) were present traces of cadmium, of which 28 had levels higher than the

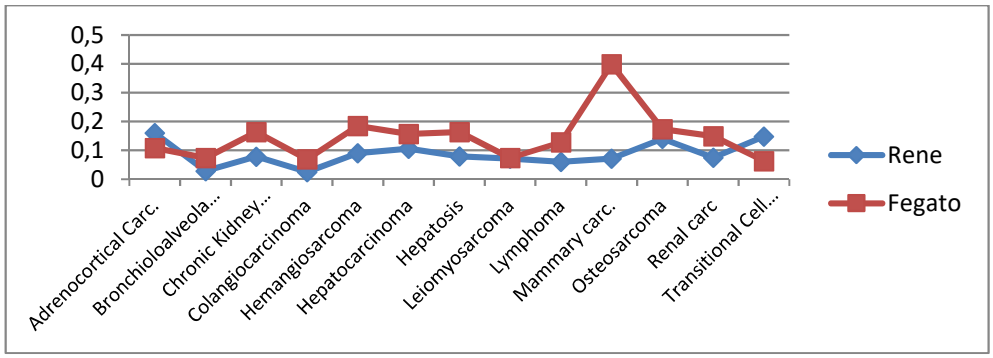
median value and 5 with levels within the range but with a value real close to the maximal one. In one case, a 6 year old crossbred female dog (case n.47) died for malignant tumor of urinary tract, the level of cadmium exceded the range. Cadmium was found in the kidney of 63 of the examined dogs (91,3%). In 29 cases (42%) the level was higher than the median value, in 4 cases it was close to the maximum level and in 1 case (same case of the highest value for Lead in the kidney) the range was exceded.

Mercury was found in the liver of 37 dogs (53,6%); in 20 cases (54%) the level was within the range and higher then the median value and in 1 case it was very close to the maximal one. The highest level of mercury, exciding the maximal range value, was found in case n. 54 (a 14 years old, crossbred male) died for severe hepatosis. Traces of mercury in kidney were found in 42 cases (60,8%); in 27 of these, the level of mercury was within the range and higher than median value. In two cases (a 6 year old male German Shepherd and a 13 years old female crossbred, both died for chronic kidney disease) the value was higher then the maximum level of range.

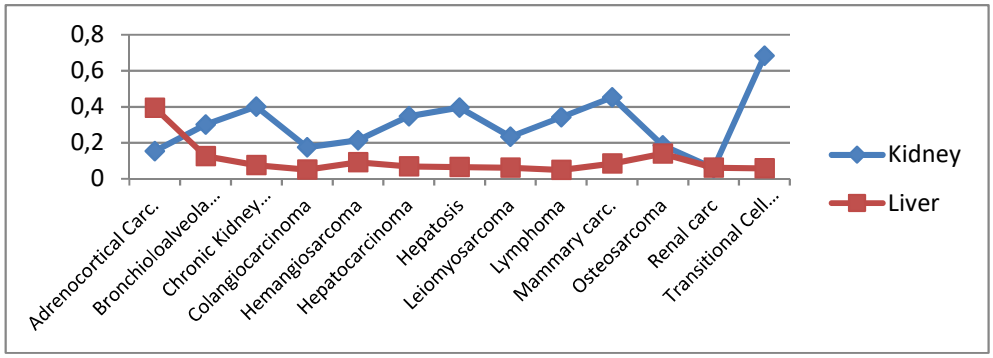
Arsenic was present only in the liver of two dogs; in one case (a 5 years old crossbred male – case n. 50), the level was within the range but lower than the median value. In the second case (n. 54) the value was higher than the maximum level of range; the latter presented in the liver high levels of Cd and Hg, and traces of Pb. The same dog was the only one to present As in the kidney, with a value within the range, higher than the median one and very close to the maximal level.

Here we report the associations between the anatomopathological lesions and the medium levels of each metal found in liver and kidney (Fig 1.3.12).

Lead



Cadmium



Mercury

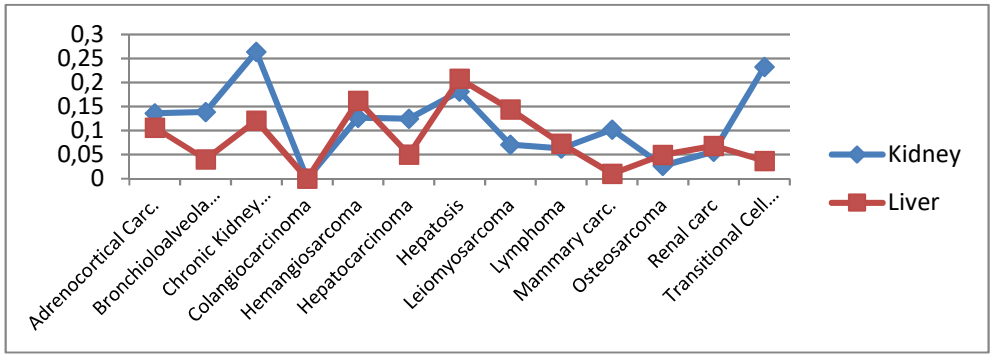


Fig 1.3.12. Comparison of medium levels of Lead, Cadmium and Mercury distributed per kind of neoplesia and chronic/degenerative diseases.

For each metal, in liver, there was no significant correlation between age and levels (Figure 1.3.13-15).

Source	Sum of Squares	gdl	Mean Sum of Squares	F	p
Liver	0.171	9	0.019	1.162	0.3693

Source	Sum of Squares	gdl	Mean Sum of Squares	F	p
Kidney	0.054	10	0.005	1.685	0.1071

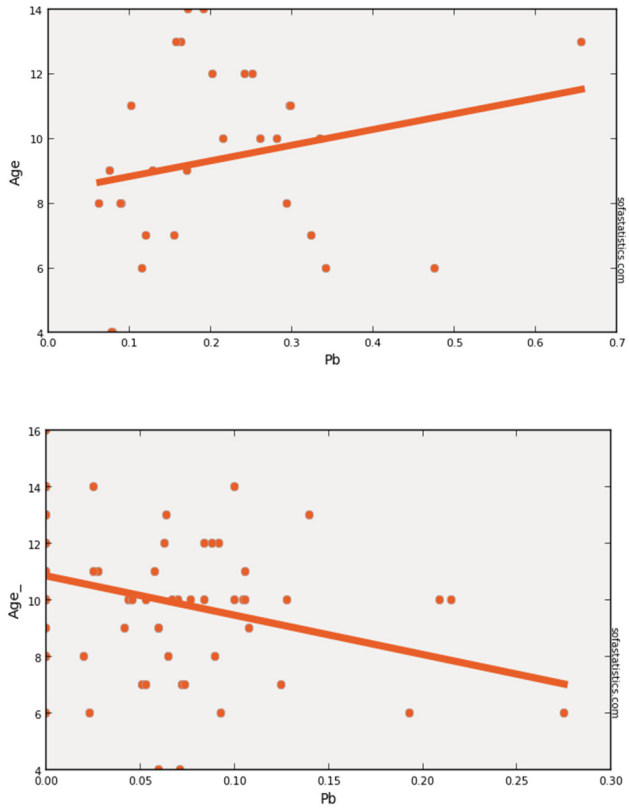


Fig 1.3.13 Results of ANOVA test of average Pb levels in liver and kidney for age and linear Personn's test

Source	Sum of Squares	gdl	Mean Sum of Squares	F	p
Liver	0.038	10	0.004	0.565	0.8351

Source	Sum of Squares	gdl	Mean Sum of Squares	F	p
Kidney	265.034.411	10	26.503.441	1.295	0.2556

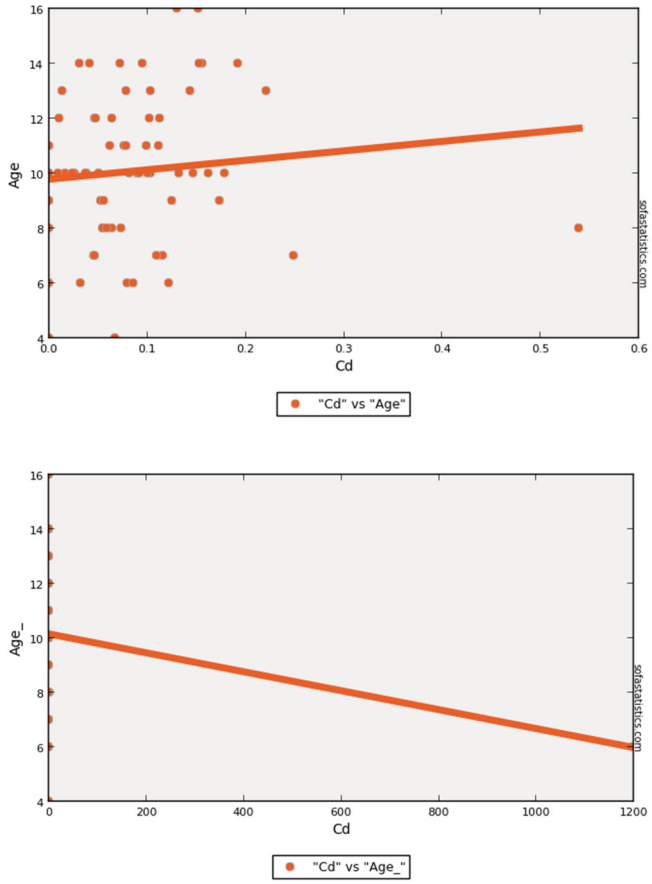


Fig 1.3.14 ANOVA test of average Cd levels in liver and kidney for age and linear Personn's test

Source	Sum of Squares	gdl	Mean Sum of Squares	F	p
Liver	0.064	10	0.006	0.511	0.8749

Source	Sum of Squares	gdl	Mean Sum of Squares	F	p
Kidney	541.777.597	10	54.177.760	1.296	0.2555

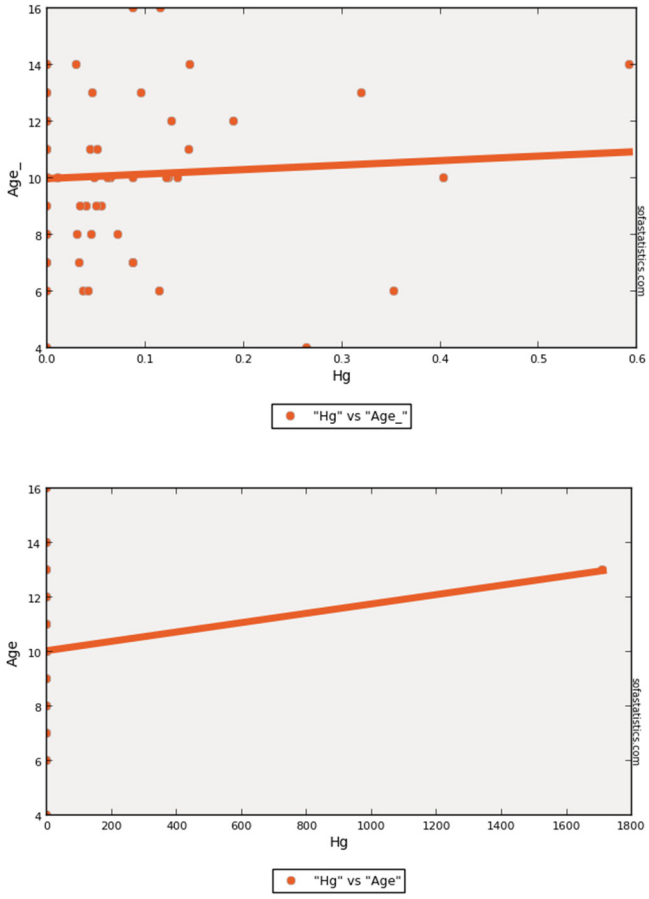


Fig 1.3.15 ANOVA test of average Hg levels in liver and kidney for age and linear Personn's test

All of the 69 cases tested for heavy metals were georepherred to visualize and compare the distance between the legal and illegal dumping sites and the area of provenience of dogs. As it's shawn in figure 20, the distribution of the population within the Campania Region it's not homogenus, considering the majority of dogs were from Naples and Caserta, which provinces falls within the so called "Terra dei Fuochi". In figure 1.3.16 it's possible to see the distribution of dogs died for malignancies or chronic diseases which where tested for heavy metals, scored for age (**4 to 6 years**; **7 to 9 y**; **10 to 12y**; **13 to 15y**; **16 to 18y**).

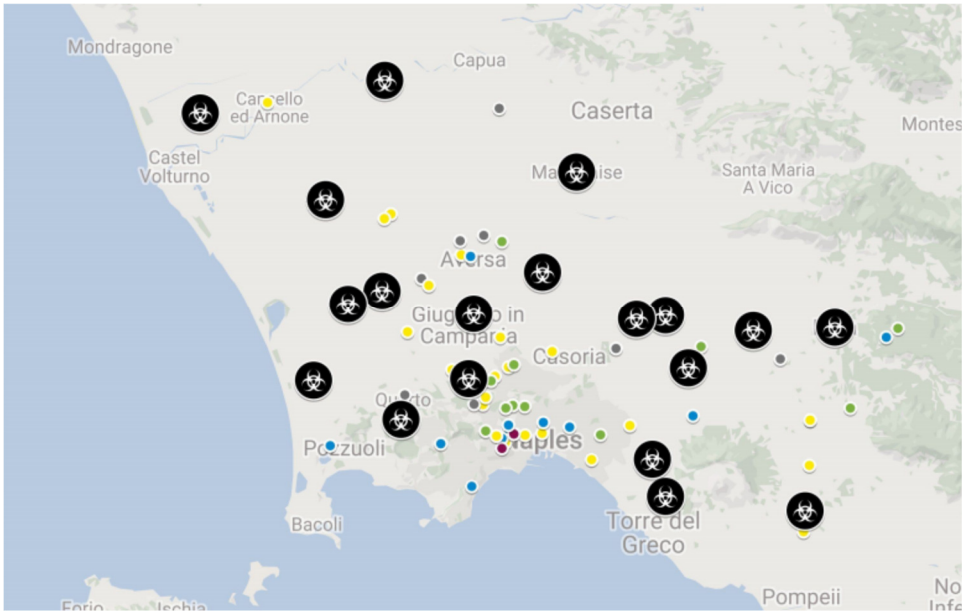


*Fig 1.3.16. Distribution of dogs died for malignancies or chronic diseases and tested for heavy metals, scored for age (**4 to 6 years**; **7 to 9 y**; **10 to 12y**; **13 to 15y**; **16 to 18y**).*

Even if the distribution is not homogenus it's possible to observe, in some area of Campania Region, a relative higher frequency of cases of younger dog died for malignancies lived nearby dumping sites compared to Naples city center (Figure 1.3.17).

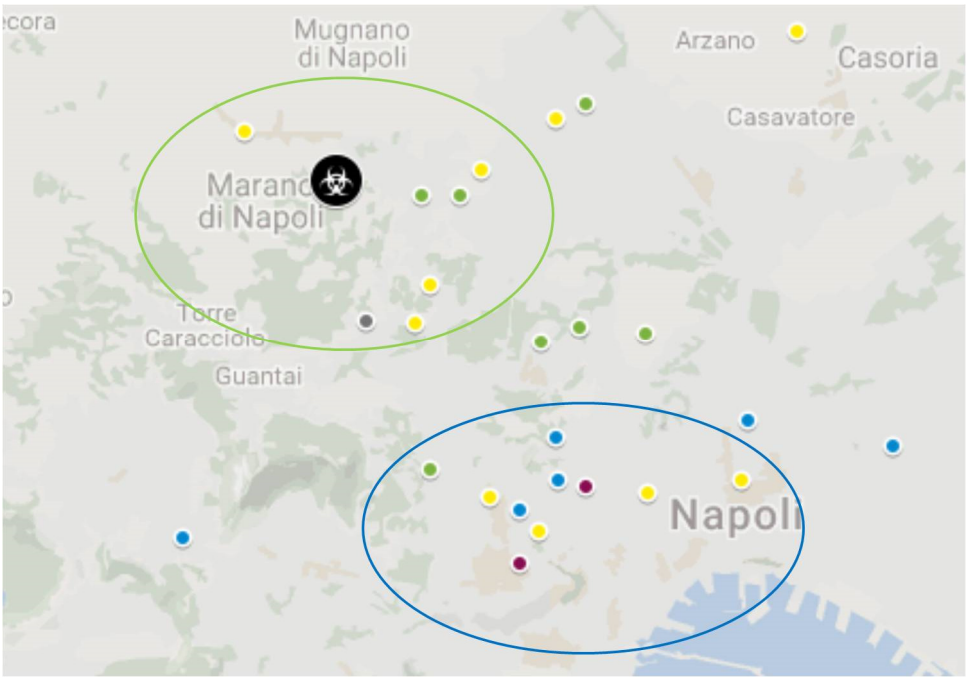


Fig 1.3.17 Geospatial representation of dogs died for malignancies, distributed per age, in two areas of Campania Region

Geospatial representation of provenience of dogs died for malignancies and levels of Lead found in liver and kidney (by colour, according to the score reported in table VI) was performed (Figure 1.3.18,19).

Fig 1.3.18 Provenience of dogs died for malignancies scored (by colour) according to the levels of lead found in liver

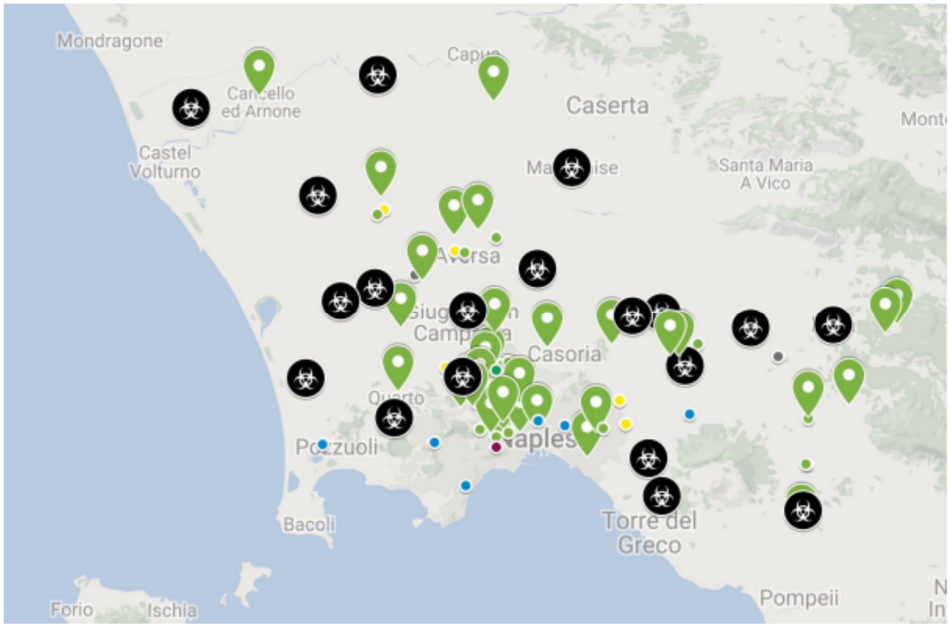


Fig 1.3.19. Provenience of dogs died for malignancies scored (by colour) according to the levels of lead found in kidney

The highest level of lead in the liver was found in dog n.15 which lived in Pianura at less than 2 Km from the dumping site of Pisani (Figure 1.3.20). The Pisani landfill in Pianura (in the province of Naples) was in use for over 50 years and closed in 1996 due to its saturation, suspected violation of norms, and the dangers it posed to the environment and human health..

Fig 1.3.20 Distance from Pisani dumping site and provenience of dog presenting the highest level of lead in liver

Geospatial representation of provenience of dogs died for malignancies and levels of Cadmium found in liver and kidney was performed (Figure 1.3.21,22).

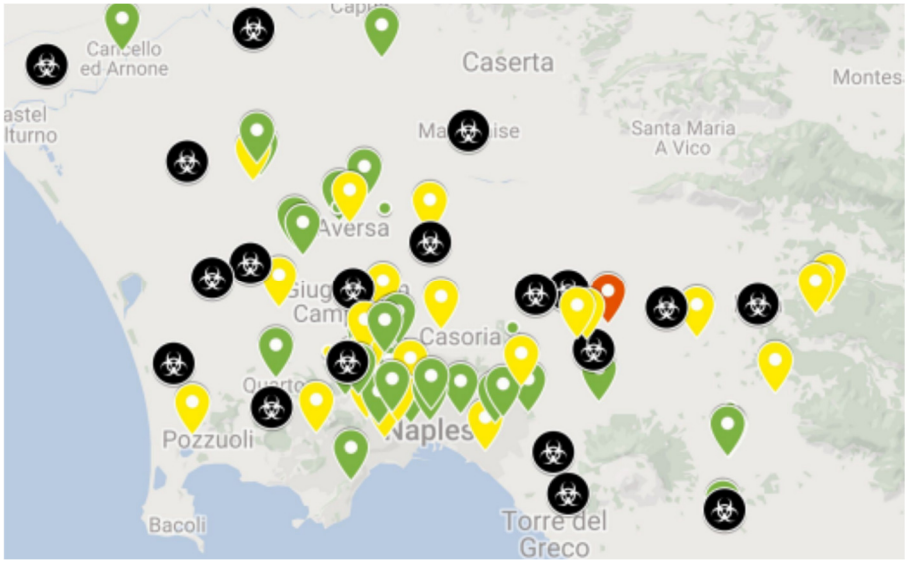


Fig 1.3.21 Provenience of dogs died for malignancies scored (by colour) according to the levels of Cd found in liver

Fig 1.3.22 Provenience of dogs died for malignancies scored (by colour) according to the level of Cd found in kidney

As for lead, even the highest level of Cadmium was found in a dog lived at less than 2 Km from a dumping site in Pomigliano D'Arco, and real close to the so colled "Triangle of Death" (Figure 1.3.23), an area comprising the municipalities of Acerra, Nola and Marigliano in which it has been observed an increase of the number of deaths caused by cancer and other diseases that exceeds the Italian national average; for years, the rise in cancer-related mortality has been thought to be mainly associated with pollution from illegal waste disposal by criminal organization.

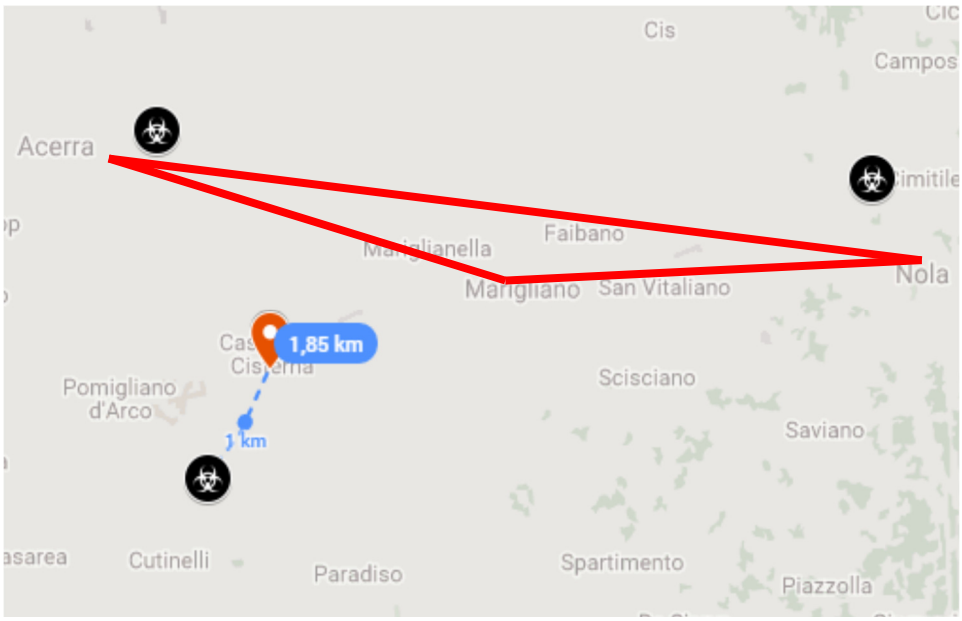


Fig 1.3.23. Distance from Chiaiano dumping site and "triangle of death", and provenience of dog presenting the highest level of Cd in liver

Geospatial representation of provenience of dogs died for malignancies and levels of Mercury found in liver and kidney (by colour, according to the score reported in table VI) was performed (Fig 1.3.24,25).

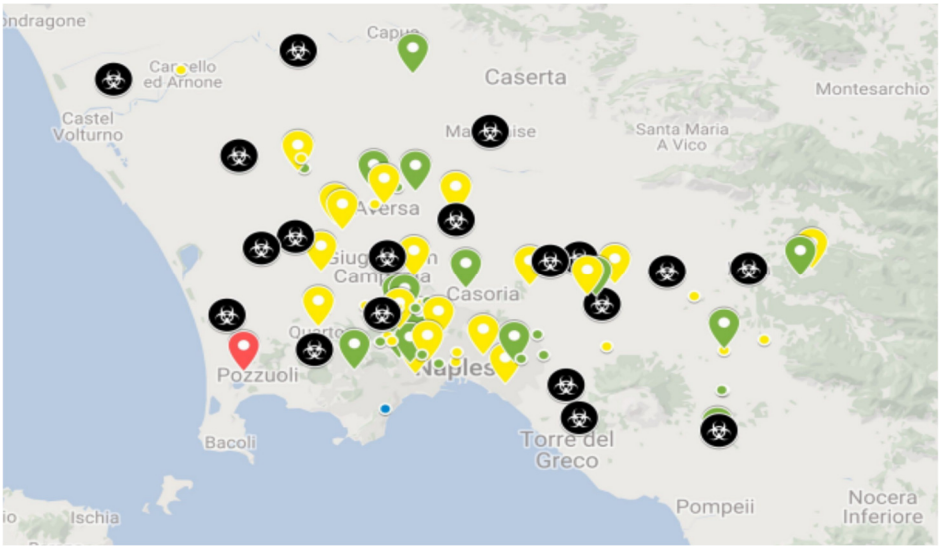


Fig 1.3.24 Provenience of dogs died for malignancies scored (by colour) according to the level of Hg found in liver

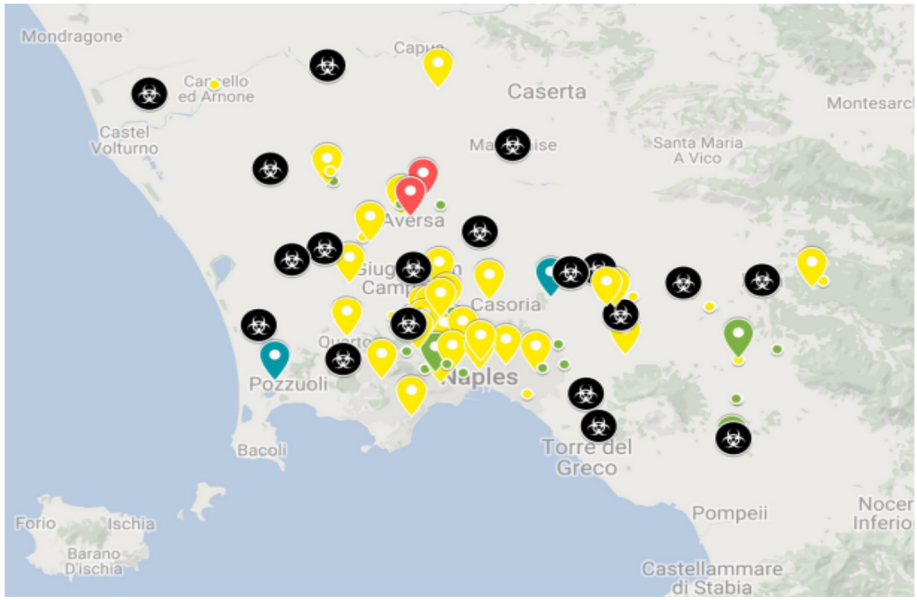


Fig 1.3.25. Provenience of dogs died for malignancies scored (by colour) according to the level of Hg found in kidney

The two highest levels of mercury detected in kidney were found in two dogs lived at less then 2 Km of distance from each other, in the heart of "Terra dei Fuochi (Land of Fire)" an area between Giugliano, Qualiano and Villaricca, notorious for illegal waste burning (Figure 1.3.26).

Fig 1.3.26. Geospatial representation of provenience of two dogs with the highest level of Hg detected in kidney

Geospatial representation of provenience of dogs died for malignancies and levels of Arsenic found in liver and kidney (by colour, according to the score reported in table VI) was performed (Fig 1.3.27).

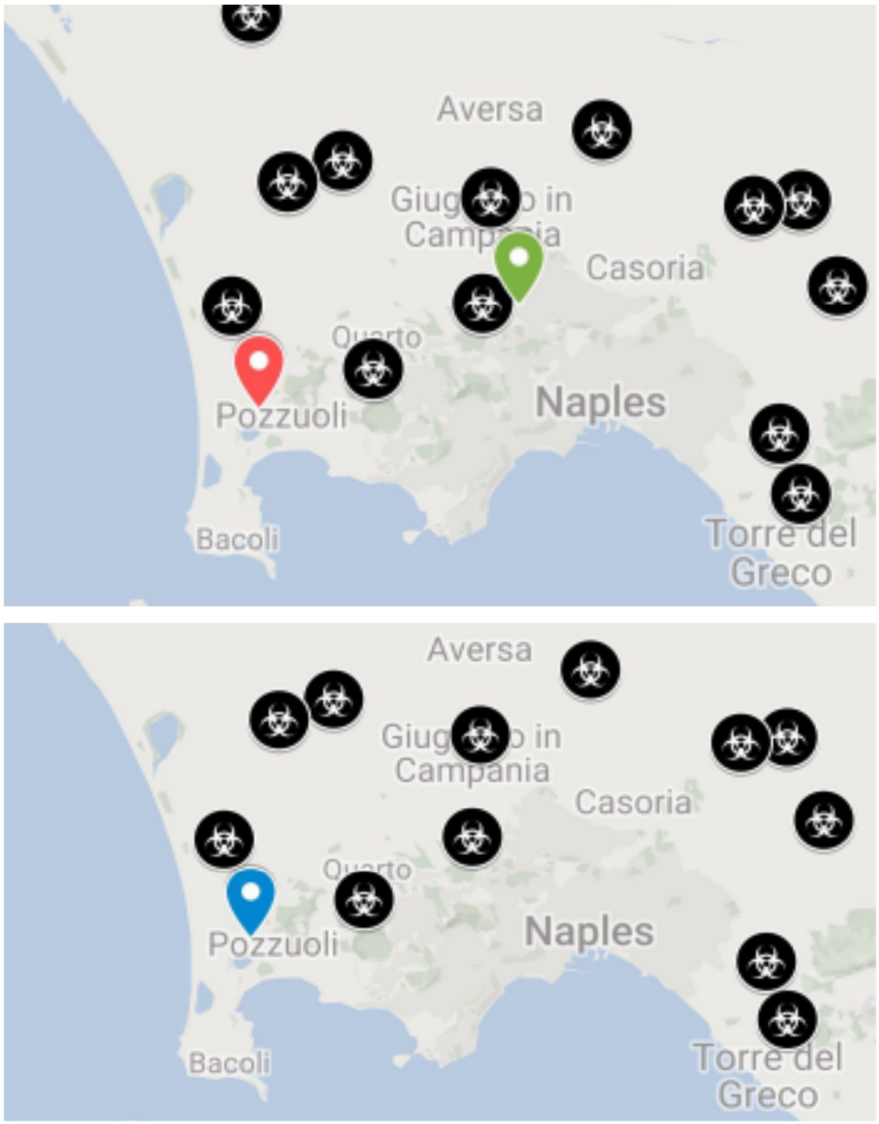


Fig 1.3.27. Provenience of dogs died for malignancies scored (by colour) according to the level of Arsenic found in liver (up) and kidney (down).

1.4 Discussion

The present study investigated the utility of necropsy on stray and owned dogs, to find out the causes of death and obtain information useful for epidemiological researches and possible correlation between environmental pollution and development of neoplastic or chronic/degenerative diseases. Unlike previous studies (Fleming et al.2011; Eleni C. 2014; Serpe et al. 2012; Pablack et al. 2015) this is the first work that encompasses the frequency of causes of death, that evaluates the levels of heavy metals in kidney and liver, and analyses the frequency of tumors and chronic/degenerative diseases. Moreover, the study underlines possible correlations between the levels of exposure to heavy metals and the possibility of developing neoplasia or chronic/degenerative lesions. Furthermore, Geographical Information System (GIS) data was used to observe the relationship between heavy metals values in dogs and distance from highly polluted sites, underlyning the possible presence of risk of exposure areas for public health.

As previously stated by Olsen and Allen (Olsen & Allen 2000), the cause-specific proportional mortality may be biased by the category of dead dogs received for post-mortem examination, so it's not possible to extend our data to the whole dog population living in Capania Region. The percentage of "undetermined" as cause of death is a little bit lower then the data reported by Olsen and Allen 2000 and Fleming et al. 2011. In the present study the macro-category "inflammatory" of unknown origin was the most frequent cause of death; most of the times it was not possible to define the etiology

of the inflammatory process due to the poor condition of the carcass, which did not allow the detection of specific characters of lesion or compromised the accuracy of microbiological tests.

"Trauma" was the second macro-category as cause of death, with a peack of incidence in dogs of group 2 (young). Such a high frequency is consistent with previous epidemiologic studies (Bonnet BN et al. 2005). These data may be due to the prevalence of stray dogs in the studied population, which most of the times lived in a urbanized area, at high risk of mothor vheichle accident (Aquila I et al. 2014).

"Infectious diseases" were the most common cause of death in puppies and younger dogs, as already reported by Walter and Kirchhoff in 1995. Parvovirus was the most frequent disease, which is charachterized by high morbidity and high mortality in litters (Merck Veterinary Manual). Canine distemper is also a highly contagious, systemic, viral disease of dogs seen worldwide, and in young puppies the prognosis is usually poor. Its epidemiology is complicated by the large number of species susceptible to infection (Canidae, Mustelidae, some Viveridae, Ailuridae, Elephantidae (Asian elephant) and primates (Japanese monkey), so systematic control of the disease by official veterinaries and pratictioners may be usefull for epidemiologic surveillance.

Within the same macro-category, 3,9% of dogs died for Leishmaniasis, the most frequent endemic zoonotic disease in Campania Region. Today, in Campania region canine Leishmaniasis undoubtedly has an increased incidence and a wider geographic distribution than before: new cases are now reported in areas that were previously non-endemic (Baldi et al. 2004).

Even ecological and environmental changes, large population movements and urbanization have led to an increased incidence and to importation into suburbs with high densities of people and sand-flies.

Lastly, for infectious disease macro-category, it's important to highlitght that the epidemiologic surveillance program through necropsy, leaded by C.R.I.U.V., allowed to to isolate pathogens responsible of zoonotic diseases that are often considered rare. At necropsy of a 3 years old male Bassethound, lived in Naples city center, humanly euthanized for severe organic wasting, was diagnosed an infection by *Mycobacterium avium*. According to the World Health Organization (WHO), more than 9 million new cases of Tuberculosis (TBC) are diagnosed annually, placing it second only to HIV/AIDS in terms of the global burden of infectious disease (Schmidt CV. 2008). Previous studies suggest that deteriorating air quality from rapid industrial growth and urban traffic coincide with high prevalence and incidence rates of endemic TBC in densely populated regions. There's epidemiological evidence that exposure to air pollutants (e.g., silica, indoor pollution from fossil fuel combustion, cigarette smoke, $PM_{2.5}$) increase the incidence of TBC (Sarkar S. 2012). Thus, we can speculate that environmental pollution may play a key role in the re-emerging of TBC and other zoonosis even in dogs.

"Malignant tumors" were the fourth overall cause of death, with a rising frequency from middle-age to adult dogs, and a decline in the oldest, which is quite similar with previous epidemiological study performed in other Region of Italy (Eleni C. 2014). Even the frequency within the macro-category was the same, with multicentric lymphoma and hemangiosarcoma

as the first two malignancies most frequently diagnosed. Canine malignant lymphoma is a common cancer of dogs and a model for non-Hodgkin's lymphoma in humans (Reif JS. 2011). A previous study described the increased risk for dogs living in areas exposed to hazardous waste emission substances to develop lymphoma (Marconato L. 2009). Chemical mixtures that have been identified at hazard waste landfills include organic solvents, polychlorinated biphenyls (PCB's) and heavy metals which can reach human and dog population through contaminated water, air or soil and have been casually related to adverse human health effects, including childhood lymphoma (Withrow and Mac Ewan's Small Animal Oncology). In studied cases, the higher concentration of metals found in liver and kidney of dogs died for malignant lymphoma, were Cadmium and Lead. In experimental models and in epidemiological studies, Cd and Pd exposure is hypothesised to be a risk factors for non-Hodgkin's lymphoma and multiple myeloma (Kelly RS. 2013).

Tumors of liver (including hepatocellular carcinoma and colangiocarcinoma) were diagnosed in 10,8% of cases and , in all of them, traces of Cadmium were found in the organ. In humans a rising incidence of hepatocellular carcinoma has been noted in recent years along with diabetes for which reasons and mechanisms are unclear. A correlation of cadmium exposure and increased hepatocellular carcinoma mortality risk has been noted in an ecological study while both hepatocellular carcinoma and hepatic adenoma occurred in cadmium-exposed mice (Satarug S. 2012). There are scientific evidences that cadmium can induce significant changes to both gene expression and epigenetic marks leading to development of

hepatic neoplasia (Cartularo L. 2015). Our results suggest that even in dogs there might be an association between cadmium-exposure and development of hepatic neoplasia.

Tumors of the urinary system were relatively frequent (6,2%); in transitional cell carcinoma observed in the study both metals, cadmium and mercury, had their spike. The risk of human bladder cancer is strongly associated with occupational exposures to chemicals and cigarette smoking. Canine bladder cancer may serve as a sentinel for chemical exposures in general and,more specifically, for exposure to pesticides in the home (Reif JS. 2011). By the way, a case-control study by Kellen E. et al. (2007), demonstrated the linear association between increased exposure to cadmium and the increased risk to develop urinary bladder cancer. Our epidemiological study highlighted the possible correlation even in canine specie.

Heavy metals are cathegorized as contaminants acting like endocrine disruptors (ED). An endocrine disruptor may be defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body (i.e. PCB, dioxin and dioxin-like compounds, pesticides) (Suck-Young Choe 2003). For their capability to mimic estrogen activity some heavy metals, like Cadmium, are also called “metalloestrogens”; most of the published studies address the ability of cadmium to activate the genomic and nongenomic pathways of estrogenic receptor-α and show that, similar to estradiol, cadmium induces the proliferation of estrogen dependent breast cancer cells (Byrne C. et al. 2013). Breast cancer in dogs and endometrial cancer in women, are tumors that feel the effects of hormonal activity (estrogen and progesterone). (Withrow,

2007 - Insabato L, 2012). In our study the 5,4% of tumors as cause of death was related to metastatic spread to liver or lungs (or both) of mammary carcinoma; moreover, out of the 129 tumors of macro-category "Neoplastic", 15 mammay carcinoma were described as tumors not associated with the cause of death. Our results showed, as expected, an high prevalence of mammary tumors in the studied population, and, in the selected cases for toxicological analysis, Cadmium was present in 87,5% of livers and in 75% of kidneys. It's interesting to notice that 3 dogs were spayed female and presented at least one value above the medium level of cadmium in kidney or liver. These data suggests that the role of cadmium may have a role in inducing and progression of breast cancer in dogs, as it has been described in humans.

"Degenerative diseases", as expected, were the most frequent cause of death in aged dogs as reported in other epidemiologic studies (Fleming JM et al. 2011; Eleni C. 2014). Chronic kidney disease (CKD) is an important cause of morbidity and mortality in dogs. The prevalence of CKD increases with age, with 15% of dogs over 10 years old being affected (Smets PM et al. 2010). Among the common environmental toxins associated with CKD, in humans, heavy metals are the most widely known (Soderland P et al. 2010). The kidney is the first target organ of heavy metal toxicity, because of its ability to reabsorb and accumulate divalent metals. Even in domestic and laboratory animal specie it's demonstrated that chronic exposure to heavy metals may lead to CKD (Frederick W. Oehme. 2009). In our cases in 93,3% levels of Cadmium or Mercury in kidney were higher then the reference values, and traces of at least one metal were found in 100% of cases. The

extent of renal damage by heavy metals depends on the nature, the dose, route and duration of exposure. Both in human and animals, acute and chronic intoxication have been demonstrated to cause nephropathies, with various levels of severity ranging from tubular dysfunctions to severe renal failure leading to death (Barbier O et al. 2005). Cdmium nephrotoxicity may follow chronic inhalation or ingestion; data from human studies suggest a latency period of approximately 10 years before clinical onset of renal damage, depending on intensity of exposure (CSEM 2008). Even for Mercury, the World Health Organization stated that most people are exposed to low levels of mercury, often through chronic exposure (continuous or intermittent long term contact) (WHO 2016). In the studied cases the 46,6% of dogs were younger than 10 years, with the youngest dog of 6 years old, presenting the overall highest level of mercury in kidney and coming from Villa Literno-Carinaro (CE), in the heart of Terra dei Fuochi. As a consequence, our results highlite the utility and potential role of dogs as early worning system for the association between heavy metal exposure and chronic kidney diseases.

In 46,6% of dogs died for liver disfunction levels of at least one of the heavy metal studied was higher then the medium value. Interestingly, liver disfunction due to severe hepatosis, in two cases was associated with high level of Arsenic in the organ. Arsenic poisoning is one of the more important causes of heavy metal poisoning in humans and domestic animals. It exists in several forms and has a long history of various uses, including insecticides for animals, wood preservatives, herbicides, and even some medicinal uses. Two species (dogs and cattle) are intoxicated more frequently than other

animals; yet sporadic instances of poisoning have been observed in cats, horses, and pigs (Merck Veterinary Manual). Arsenic poisons cells by binding the sulfhydryl groups on critical enzymes and through depletion of lipoate by trivalent arsenite. Lipoate is involved in the synthesis of key intermediates in the Krebs cycle. Hence the depletion of lipoate results in inhibition of the Krebs cycle and oxidative phosphorylation leading to ATP depletion. Thus so, inhibiting cellular respiration and uncoupling oxidative phosphorylation results in cellular energy depletion, resulting in cell death in high-energy dependent tissues like liver (Sharma B et al. 2014). Arsenic is one of WHO's 10 chemicals of major public health concern; the first symptoms of long-term exposure to inorganic arsenic (e.g. through drinking-water and food) are usually observed in the skin, and include pigmentation changes, skin lesions and hard patches on the palms and soles of the feet (hyperkeratosis). These occur after a minimum exposure of approximately five years and may be a precursor to skin cancer. Long-term exposure to arsenic may also cause cancers of the bladder and lungs (WHO 2016). Considering the minimum exposure period indicated by WHO, we can conclude that even for Arsenic poisoning, dog can be considered a good sentinel for early warning in public health surveillance programs.

Levels of metals detected in kidney and liver in the present study don't give rise to clinical manifestations of toxicity in dogs but are indicative of chronic heavy metal exposure of animals (Serpe FP 2012).

Higher medium concentration of lead was found in the liver; our data are allined with previous studies carried out in urbanized Italy, Spain and Germany (Ghisleni et al., 2004; Serpe FP 2012; Pablack N et al. 2015). Lead

exposure is estimated to account for 0.6% of the global burden of disease, with the highest burden in developing regions. Recent reductions in the use of lead in petrol (gasoline), paint, plumbing and solder have resulted in substantial reductions in lead levels in the blood. However, significant sources of exposure to lead still remain, particularly in developing countries (WHO – Lead). Pb is an important environmental contaminant. Depending on environmental pollution, varying Pb concentrations can be found in foods derived from plants and animals, resulting in variations of Pb uptake. Since it has been assumed that non-dietary factors are of minor importance for overall Pb exposure in Europe, measurement of Pb accumulation in organs might provide not only important reference data for clinical diagnostics but also an indication of Pb exposure of dogs through food.

Cadmium concentrations were significantly highest in kidney in agreement with previous studies (López-Alonso et al., 2007). Our data confirm its particular tropism for that organ (Serpe FP 2012). Cadmium can travel long distances from the source of emission by atmospheric transport, so the source of exposion can be difficult to detect. Cadmium contained in soil and water can be taken up by certain crops and aquatic organisms and accumulate in the food-chain, so humans and animals have a borad spectrum of risk to be exposed to this metal. The absorbed Cd persists or is only partly eliminated, and stored in the organism in various organs such as the liver and kidneys. Cd concentrations therefore increase in the storage organs over time. Under stressful conditions, Cd can be partly remobilized, resulting in signs of intoxication. Long-lasting intake can cause kidney damage

accompanied by mineral metabolism disorders. In addition, hepatic dysfunction or changes in the bone structure can appear.

Regarding mercury, our results are in agreement with previously published literature (Dunlap et al., 2007; López-Alonso et al., 2007). In particular, our study indicates low mercury levels in dogs, with highest concentrations in kidneys. Mercury is one of the most studied toxic heavy metals and the lethal effects of both acute toxic exposure and chronic low-level exposures are well documented. It is often used in household products that dogs are exposed to such as batteries, light bulbs, fabric softeners, latex gloves, paint, plastics, ink, and solvents. Mercury vapors are released from such things as home renovations involving old paint, broken thermometers and thermostats. It concentrate at floor level where dogs are lying or walking. It is also present in many commercial dog foods. On the basis of these data, we can consider dog as a valuable specie to monitor the environmental mercury exposure risk both for humans and other animals.

Traces of Arsenic in liver or kidney were found only in two dogs of studied cases, even if in one of them the level of the metal presented very high value. Arsenic is one of the most widespread environmental pollutant, and millions of people suffer from exposure to As, since it is a common pollutant in drinking water (Sabath E et al. 2012). In humans The causal association between As and the formation of tumours in the skin, lungs, bladder, liver and kidneys has been exhaustively described. Some epidemiological studies have shown an association between exposure to high levels of As and increased risk of cardiovascular disease and diabetes mellitus. The low number of cases encountered in our study is in contrast to previous report

performed in Campania Region (Zaccaroni A. et al 2014). This aspect may be due to the choice of studied cases by different criteria of inclusion or exclusion.

The use of GIS in the present study has been a valuable tool to check whether exposure to heavy metals could be correlated with the provenience of the dogs from potentially polluted areas. Even if the distribution of dogs within the Region was not homogenous, we provided interesting insights that could be better investigated. Middle aged dogs lived nearby Chiaiano dumping site presented levels of metals quite similar to aged dogs coming from areas not interested by illegal dumping activities. The highest levels of Lead, Cadmium and Arsenic were found in dogs lived less then 3 Km from legal or illegal dumping sites, while the highest levels of Mercury were found in two dogs lived in the heart of the supposed very polluted area Terra dei Fuochi. These aspects, if confirmed on wider scale, may suggest that effectively, dogs living nearby wasting dumping sites accumulate metals faster then dogs living in lower polluted areas. Furthermore, regarding Mercury in the kidney, we interestingly found two dogs that lived really close each other, and coming from an area relatively far from wasting dumping sites. This data confirms the utility of GIS not only to monitor already known polluted areas, but even to localize sites were environmental pollution is not considered to be a problem. Consisting of data input, management, analysis and presentation components, GIS act as an integrative thecnology useful to facilitate the incorporation of spatial relationship in epidemiological investigatios (Pfeiffer DU et al. 2002).

Aquila, I., Di Nunzio, C., Paciello, O., Britti, D., Pepe, F., De Luca, E., & Ricci, P. (2014). An unusual pedestrian road trauma: From forensic pathology to forensic veterinary medicine. Forensic science international, 234, e1-e4..

Baldi, L., Mizzoni, V., & Guarino, A. (2004). Canine leishmaniasis in Campania: new and old foci. Parassitologia, 46(1-2), 217-220.

Barbier, O., Jacquillet, G., Tauc, M., Cougnon, M., & Poujeol, P. (2005). Effect of heavy metals on, and handling by, the kidney. Nephron Physiology, 99(4), p105-p110.

Bonnett, B. N., Egenvall, A., Hedhammar, Å., & Olson, P. (2005). Mortality in over 350,000 insured Swedish dogs from 1995–2000: I. Breed-, gender-, age-and cause-specific rates. Acta Veterinaria Scandinavica, 46(3), 105.

Byrne, C., Divekar, S. D., Storchan, G. B., Parodi, D. A., & Martin, M. B. (2013). Metals and breast cancer. Journal of mammary gland biology and neoplasia, 18(1), 63-73.

Cartularo, L., Laulicht, F., Sun, H., Kluz, T., Freedman, J. H., & Costa, M. (2015). Gene expression and pathway analysis of human hepatocellular carcinoma cells treated with cadmium. Toxicology and applied pharmacology, 288(3), 399-408.

Choe, S. Y., Kim, S. J., Kim, H. G., Lee, J. H., Choi, Y., Lee, H., & Kim, Y. (2003). Evaluation of estrogenicity of major heavy metals. Science of the Total Environment, 312(1), 15-21.

CSEM 2008. Agency for Toxic Substances and Disease Registry Case Studies in Environmental Medicine (CSEM). Cadmium Toxicity.

De Felip, E., Bianchi, F., Bove, C., Cori, L., D'Argenzio, A., D'Orsi, G., ... & Parlato, A. (2014). Priority persistent contaminants in people dwelling in critical areas of Campania Region, Italy (SEBIOREC biomonitoring study). Science of the Total Environment, 487, 420-435.

Dunlap, R. E., & York, R. (2008). The globalization of environmental concern and the limits of the postmaterialist values explanation: Evidence from four multinational surveys. The Sociological Quarterly, 49(3), 529-563.

Eleni, C., Scholl, F., & Scaramozzino, P. (2013). Causes of death in dogs in the province of Rome (Italy). Veterinaria italiana, 50(2), 137-143.

Esposito, V., Maffei, A., Bruno, D., Varvaglione, B., Ficocelli, S., Capoccia, C., ... & Assennato, G. (2014). POP emissions from a large sinter plant in Taranto (Italy) over a five-year period following enforcement of new legislation. Science of the Total Environment, 491, 118-122.

Fazzo, L., Belli, S., Minichilli, F., Mitis, F., Santoro, M., Martina, L., ... & Bianchi, F. (2007). Cluster analysis of mortality and malformations in the Provinces of Naples and Caserta (Campania Region). Annali dell'Istituto superiore di sanita, 44(1), 99-111.

Fazzo, L., Santis, M. D., Mitis, F., Benedetti, M., Martuzzi, M., Comba, P., & Fusco, M. (2011). Ecological studies of cancer incidence in an area interested by dumping waste sites in Campania (Italy). Annali dell'Istituto superiore di sanita, 47(2), 181-191.

Fleming, J. M., Creevy, K. E., & Promislow, D. E. L. (2011). Mortality in North American Dogs from 1984 to 2004: An Investigation into Age-,

Size-, and Breed-Related Causes of Death. Journal of Veterinary Internal Medicine, 25(2), 187-198.

Ghisleni, G., Spagnolo, V., Roccabianca, P., Scanziani, E., Paltrinieri, S., Lupo, F., ... & Nageli, F. (2004). Blood lead levels, clinico-pathological findings and erythrocyte metabolism in dogs from different habitats. Veterinary and human toxicology, 46(2), 57-61.

Giovannini, A., Rivezzi, G., Carideo, P., Ceci, R., Diletti, G., Ippoliti, C., ... & Scortichini, G. (2014). Dioxins levels in breast milk of women living in Caserta and Naples: Assessment of environmental risk factors. Chemosphere, 94, 76-84.

Jubb, Kennedy & Palmer's Pathology of Domestic Animals-E-Book (2015). Elsevier Health Sciences.

Kellen, E., Zeegers, M. P., Den Hond, E., & Buntinx, F. (2007). Blood cadmium may be associated with bladder carcinogenesis: the Belgian case–control study on bladder cancer. Cancer detection and prevention, 31(1), 77-82.

Kelly, R. S., Lundh, T., Porta, M., Bergdahl, I. A., Palli, D., Johansson, A. S., ... & Chadeau-Hyam, M. (2013). Blood erythrocyte concentrations of cadmium and lead and the risk of B-cell non-Hodgkin's lymphoma and multiple myeloma: a nested case-control study. PloS one, 8(11), e81892.

López-Alonso, M., Miranda, M., García-Partida, P., Cantero, F., Hernández, J., & Benedito, J. L. (2007). Use of dogs as indicators of metal exposure in rural and urban habitats in NW Spain. Science of the Total Environment, 372(2), 668-675.

Marconato, L., Leo, C., Girelli, R., Salvi, S., Abramo, F., Bettini, G., ... & Zini, E. (2009). Association between waste management and cancer in companion animals. Journal of veterinary internal medicine, 23(3), 564-569.

Mitis, F., & Martuzzi, M. (2007). Population health and waste management: scientific data and policy options. Report of a WHO workshop. Rome, Italy, 29-30 March 2007. World Health Organization Regional Office for Europe. http://www. euro. who. int/healthimpact/MainActs/20070228_1.

Martuzzi, M., Mitis, F., Bianchi, F., Minichilli, F., Comba, P., & Fazzo, L. (2009). Cancer mortality and congenital anomalies in a region of Italy with intense environmental pressure due to waste. Occupational and environmental medicine.

Nature Publishing Group (2014). A toxic legacy. Nature 508, 431 doi:10.1038/508431a

Neugebauer, E. A. M. (2009). 2. Evidence-based medicine–a possible model for evidence-based toxicology? 2.1 Translation of evidence-based medicine into practice. Human & experimental toxicology, 28(2-3), 105-107.

Oehme, F. W., & Rumbeiha, W. K. (1999). Veterinary toxicology. General, Applied and Systems Toxicology.

Olsen, T. F., & Allen, A. L. (2000). Causes of sudden and unexpected death in dogs: a 10-year retrospective study. The Canadian Veterinary Journal, 41(11), 873.

Paßlack, N., Mainzer, B., Lahrssen-Wiederholt, M., Schafft, H., Palavinskas, R., Breithaupt, A., & Zentek, J. (2015). Concentrations of

strontium, barium, cadmium, copper, zinc, manganese, chromium, antimony, selenium, and lead in the liver and kidneys of dogs according to age, gender, and the occurrence of chronic kidney disease. Journal of veterinary science, 16(1), 57-66.

Pfeiffer, D. U., & Hugh-Jones, M. (2002). Infectious diseases of wildlife: Detection, diagnosis and management (Part One)-Diagnostics-Geographical information systems as a tool in epidemiological assessment and wildlife disease management. Revue Scientifique et Technique-Office International des Epizooties, 21(1), 91-102.

Reif, J. S. (2011). Animal sentinels for environmental and public health. Public Health Reports, 126(1 suppl), 50-57.

Rivezzi, G., Piscitelli, P., Scortichini, G., Giovannini, A., Diletti, G., Migliorati, G., ... & Black, D. M. (2013). A general model of dioxin contamination in breast milk: Results from a study on 94 women from the Caserta and Naples areas in Italy. International journal of environmental research and public health, 10(11), 5953-5970.

Sabath, E., & Robles-Osorio, M. L. (2012). Renal health and the environment: heavy metal nephrotoxicity. Nefrologia, 32(3), 279-286.

Sarkar, S., Song, Y., Sarkar, S., Kipen, H. M., Laumbach, R. J., Zhang, J., ... & Schwander, S. (2012). Suppression of the NF-κB pathway by diesel exhaust particles impairs human antimycobacterial immunity. The Journal of Immunology, 188(6), 2778-2793.

Satarug, S. (2012). Long-term exposure to cadmium in food and cigarette smoke, liver effects and hepatocellular carcinoma. Current drug metabolism, 13(3), 257-271.

Schmidt, C. W. (2008). Linking TB and the environment: an overlooked mitigation strategy. Environ Health Perspect, 116(11), A478-85.

Serpe, F. P., Russo, R., De Simone, A., Florio, S., Esposito, M., & Severino, L. (2012). Levels of heavy metals in liver and kidney of dogs from urban environment. Open veterinary journal, 2(1), 15-18.

Sharma, B. K. (2014). Environmental chemistry. Krishna Prakashan Media.

Smets, P. M. Y., Meyer, E., Maddens, B. E. J., Duchateau, L., & Daminet, S. (2010). Urinary markers in healthy young and aged dogs and dogs with chronic kidney disease. Journal of veterinary internal medicine, 24(1), 65-72.

Soderland, P., Lovekar, S., Weiner, D. E., Brooks, D. R., & Kaufman, J. S. (2010). Chronic kidney disease associated with environmental toxins and exposures. Advances in chronic kidney disease, 17(3), 254-264.

Walter, J. H., & Kirchhoff, A. (1995). Causes of illness in young dogs in necropsy files (1980-1993). Berliner und Munchener Tierarztliche Wochenschrift, 108(4), 121-126.

WHO. Head, K. W. (Ed.). (2003). Histological classification of tumors of the alimentary system of domestic animals (Vol. 10). Published by the Armed Forces Institute of Pathology in cooperation with the American Registry of Pathology and the World Health Organization Collaborating Center for Worldwide Reference on Comparative Oncology.

Withrow, S. J., Page, R., & Vail, D. M. (2013). Withrow and MacEwen's small animal clinical oncology. Elsevier Health Sciences.

Zaccaroni, A., Corteggio, A., Altamura, G., Silvi, M., Di Vaia, R., Formigaro, C., & Borzacchiello, G. (2014). Elements levels in dogs from

“triangle of death” and different areas of Campania region (Italy). Chemosphere, 108, 62-69.

Chapter 2

Dairy cattle as a sensitive warning system for nitrate environmental pollution: evaluation of risk for human health

2.1 Introduction

Nitrate and nitrite are toxicants that have become increasingly significant for environmental pollution. Their increase in environmental distribution, both in surface and ground water, has been attributed to the use of agricultural fertilizers and wastes produced by municipalities, industries, and feedlots (Costagliola A et al. 2014). Humans have always been exposed to nitrates and nitrites from the consumption of food (i.e. vegetables) in which these substances are naturally present. Smaller, even if not negligible, amounts come however from water and the foods in which these substances may be present as additives. Nitrates are commonly used in some food production processes because they preserve the color of meet and exert an antimicrobial effect, especially against *Clostridium botulinum*. In particular, they may be present in raw and processed meats, dairy products, fish and fish products and sometimes even in spirits and liqueurs (Cortesi ML et al. 2015). It is widely accepted that the presence of nitrates in meat, vegetables and in water, is a serious threat to human health. TheWorld Health Organization indicates that the toxic dose for humans is 0.4–200 mg/kg of body weight, and the lethal dose is 33–250 mg/kg of body weight (Speijers and van den Brandt 2006). Nitrates are not dangerous in themselves, as they are only irritant to gastrointestinal mucosa at high dosage, but become so when they are reduced to nitrites; the latter have high capacity to react with hemoglobin and oxidate Fe^{2+} to Fe^{3+} which causes formation of methemoglobin that is not capable to bind oxygen. For infants and children the risk of exposure to nitrates by dietary intake is higher beacuse the amount of food / b.w. is greater than that consumed by an adult. Moreover children are very sensitive

to this type of pathology, also known as “blue baby syndrome”, unlike adults who have more protection against the low pH of the stomach reduction of nitrate to nitrite by bacteria.

Many animal species are susceptible to nitrate and nitrite poisoning; however, cattle are much more likely to be affected than other animals (Ozmen et al. 2003). The most common cause of toxicosis from these compounds in farm animals is the consumption of feed or water containing high levels of nitrates (Costagliola A et al. 2014). Ingested nitrates may directly irritate the gastrointestinal mucosa and produce abdominal pain and diarrhea. Acute intoxication is manifested primarily by methemoglobin formation and resultant anoxia. Brown, cyanotic mucous membranes develop rapidly as methemoglobinemia exceeds 50%. Dyspnea, tachypnea, anxiety, and frequent urination are common. Affected animals may die suddenly without appearing ill, in terminal anoxic convulsions within 1 hr, or after a clinical course of 12–24 hr or longer (Merck Veterinary Manual). Although usually acute, the effects of nitrite or nitrate toxicity may be subacute or chronic and are reported to include retarded growth, lowered milk production, vitamin A deficiency, minor transitory goitrogenic effects, abortions and fetotoxicity, and increased susceptibility to infection. Chronic nitrate toxicosis remains a controversial issue and is not as yet well characterized, but most current evidence does not support assertions of lowered milk production in dairy cows due to excessive dietary nitrate exposure alone (Merck Veterinary. Manual).

The first international evaluation of the risks associated with the ingestion of nitrate and nitrite was conducted by the Joint FAO/WHO Expert

Committee on Food Additives (JECFA) in 1961. The Scientific Committee for Food (SCF) reviewed the toxicological effects of nitrate and nitrite and established an Acceptable Daily Intake (ADI) of 0–3.7 mg kg−1 b.w. for nitrate in 1990, retained the same ADI in 1995 and derived an ADI of 0–0.06 mg/kg for nitrite. The JECFA reconfirmed in 2002 an ADI of 0–3.7 mg kg−1 b.w. for nitrate and set an ADI of 0–0.07 mg kg−1 b.w. for nitrite.The European Union has always paid attention to food components and contaminants, nitrate and nitrite included, as shown by the directives and the regulations on the subject. In 2008 and 2010 EFSA was asked additional questions about the dangers of exposure to nitrates in order to revise, if necessary, the current legislative framework relating to food contaminants (Cortesi et al.2015). As it's shawn by FAO stats, milk is still a dairy cattle product consumed worldwide (Figure 2.1); even if the environmental pollution by nitrate compaounds is increasing in every part of the world, the possible presence of nitrates and nitrites in milk has never been investigated as possible hazard for human health.

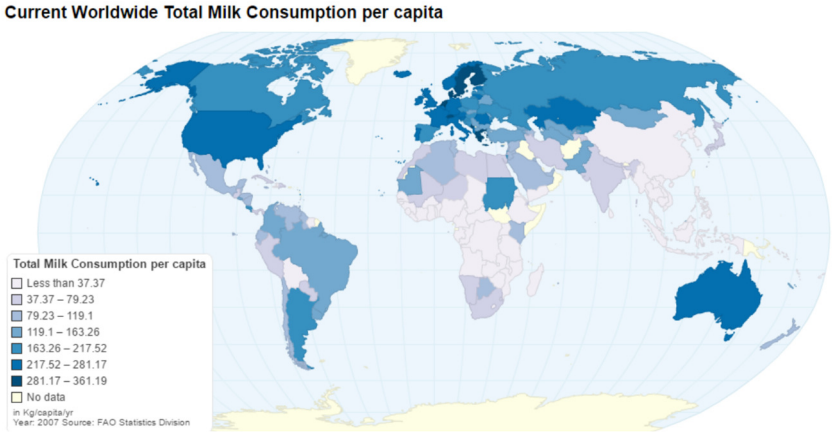


Fig 2.1 Current worldwide total milk consumption per capita (FAO)

The aim of this study is to evaluate if nitrate compounds accumulate in dairy cattle milk and may represent a possible risk for human health, and discuss the potential role of cattle in monitoring environmental pollution by nitrate in order to prevent diseases and toxicosis in humans.

2.2 Materials and Methods

Blood and milk samples were collected from n. 13 Italian Podolica breed dairy cattle, between 3 and 6 years of age, from a farmhouse of Campania Region. All of the cattle showed moderate to severe nitrate toxicosis clinical signs. The samples were transported to the laboratory and analyzed for nitrates and nitrites using the spectrophotometric AOAC official method (1995). Analytical grade reagents were used. Sodium nitrite, sodium nitrate, cadmium sulfate, sulfanilic acid, N-(1-naphthyl)-ethylenediamine dihydrochloride, glacial acetic acid solution 20%, a monia hydroxide 25%, sodium tetraborate decahydrate, zinc sulfate heptahydrate and zinc metal powder were purchased from Acros Organics (Geel, Belgium). Distilled water used throughout the procedure was supplied from the distillation apparatus of Purelab Option Elga (UK).

Nitrate stock solution (1000 mg L−1) Dissolve 137.1 mg of primary standard sodium nitrate, previously dried for 24 h at 105°C, in sufficient water and then dilute to 100 mL. For preparing standard nitrate solutions dilute nitrate stock solution with distilled water.

Nitrite stock solution (1000 mg L−1) Dissolve 150.0 mg of primary standard sodium nitrite, previously dried for 1 h at 110°C, in sufficient water and

dilute to 100 mL. For preparing standard nitrite solutions dilute nitrite stock solution with distilled water.

Color reagent

Color reagent was prepared according to the official method of AOAC International (993.03). Dissolve 600 mg of sulfanilic acid (C6H7NO3 S) in 50 mL hot water. Let it cool; add 20 mL glacial acetic acid and dilute to 100 mL with water. Dissolve 20 mg N- (1-naphthyl)-ethylenediamine dichloride (C12H16Cl2N2•CH3OH) in 20 mL glacial acetic acid and dilute to 100 mL with water. Mix equal volumes of sulfalinic acid solution and N-(1-naphthyl)-ethylenediamine dichloride reagent immediately before use. Discard any unused color reagent.

Sample preparation

Weigh a sample of 10 g into a 200 mL volumetric flask. Add 150 mL hot water and 10 mL of 5% (w/v) borax solution (Na2B4O7•10H2O) to precipitate protein. Then mix and heat for 15 min. Clarify the mixture by adding 4 mL of 30% (w/v) ZnSO4•7H2O solution and shaking for 15 s. After cooling to room temperature, dilute the sample to volume with distilled water, mix and filter through a Whatman (no. 40) filter paper. Prepare blank without sample material.

Reduction of nitrate to nitrite

For each sample, weigh ca 600 mg Zn powder into separate 50 mL volumetric flasks and spread powder over bottom of flask. Prepare additional flask for standards. Carefully add 4 mL of 10% (w/v) cadmium sulfate (3CdSO4•(H2O) solution to zinc powder in flask to obtain homogeneous mixture. Let newly formed spongy metallic cadmium stand for 10 min without moving. Add 2 mL 25% NH4OH and 10 mL sample solution to 1 flask. Prepare standard nitrate concentrations (containing 0, 50, 100, 150 and 200 µg NaNO3) by adding 10 mL of each standard solution to separate volumetric flasks prepared with spongy cadmium. Shake flasks for exactly 1 min to loosen spongy cadmium; then let stand for 10 min. Dilute to volume with H2O and filter. After use, pour contents of volumetric flasks into waste bottle. Dissolve possible residues in volumetric flasks with concentrated HCl to another waste bottle. Collect waste from color reaction in another bottle. Arrange for proper disposal of waste bottles.

Nitrite determination

Pipet 10 mL clear filtrates of samples and standard solutions (equivalent to 0, 10, 20, 30 and 40 µg NaNO2) to separate glassstoppered mixing cylinders. Add 10 mL color reagent to each, mix for 1 min by hand, and record absorbance at 530 nm using Spectrophotometer UV/VIS V-530 Jasco (Japan) and water blanks. Nitrate and nitrite concentrations were calculated using nitrate and nitrites calibration curves.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using NCSS software. The comparison of the means was carried out using the Tukey-Kramer multiple comparison test.

2.3 Results

Traces of nitrates and nitrites were found in all of the 13 cases selected both in blood and milk; in table n. 2.3.1 and figure n.2.3.1 are showed the levels of nitrate and nitrite in blood samples.

Tab 2.3.1 Levels of nitrate and nitrite in blood samples

	nitrate mg/Kg	nitrite mg/Kg
blood 1	38,6315914	20,31166598
blood 2	73,10058108	17,27249174
blood 3	63,76482667	14,38834676
blood 4	50,4000504	20,95347795
blood 5	54,18644472	16,34717968
blood 6	69,81899426	13,99954093
blood 7	20,35854999	5,073510409
blood 8	22,33011836	5,801083955
blood 9	27,28664388	5,551449796
blood 10	21,40766747	6,602011413
blood 11	20,89685346	6,099522865
blood 12	19,52793209	5,191690017
blood 13	21,35766747	5,602011413

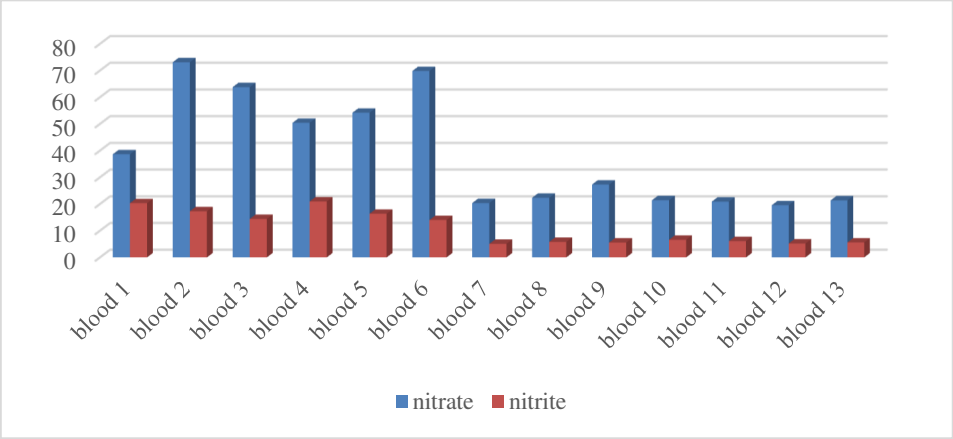


Fig 2.3.1 Graphical representation of nitrate and nitrite levels in blood samples

The medium level of nitrate in blood was 38,697 mg/Kg (Standard Deviation 20,767) with the highest level found in sample n.2 (73,1 mg/Kg). In the same matrix medium level of nitrites was lower (11,04 mg/Kg; Standard deviation 6,273) and the highest level was found in sample n.4 (20,953 mg/Kg). Nitrate and nitrite levels in blood were directly and strongly correlated (P_{XY}>*0,7)* (Figure 2.3.2)

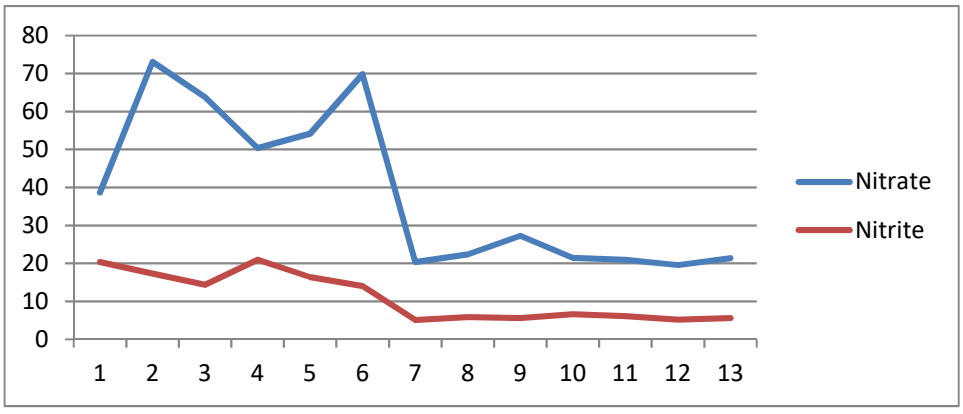


Fig 2.3.2 Correlation between nitrate and nitrite levels in blood

The medium level of nitrate in milk was 20,284 mg/Kg (Standard Deviation 3,22) with the highest level found in sample n.5 (30,152 mg/Kg). In the same matrix medium level of nitrites was lower (5,091 mg/Kg; Standard deviation 1,192) and the highest level was found in sample n.5 (8,001 mg/Kg) (Table n. 2.3.2 and figure n.2.3.3).

Tab 2.3.2 Levels of nitrate and nitrite in milk samples

	nitrate mg/Kg	nitrite mg/Kg
milk 1	20,71589463	5,24091953
milk 2	18,10895476	3,96386223
milk 3	18,75868854	4,13895775
milk 4	18,6721296	4,25160761
milk 5	30,15235582	8,00122093
milk 6	21,02912171	3,50017099
milk 7	19,14375218	5,11508951
milk 8	20,52247815	5,521049
milk 9	19,6276519	5,09013786
milk 10	20,16610502	4,25591176
milk 11	16,84285176	4,83867216
milk 12	21,16572299	6,03072409
milk 13	18,79367394	6,24107031

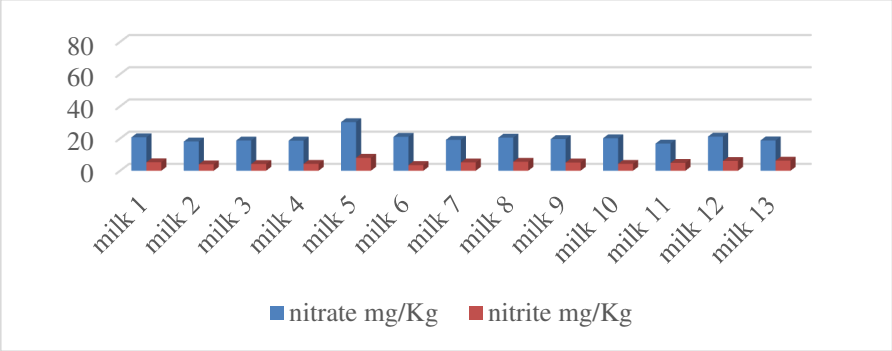


Fig 2.3.3 Graphical representation of nitrate and nitrite levels in blood samples

Nitrate and nitrite levels in milk were directly and strongly correlated ($P_{XY}>0,7$) (Figure 2.3.4)

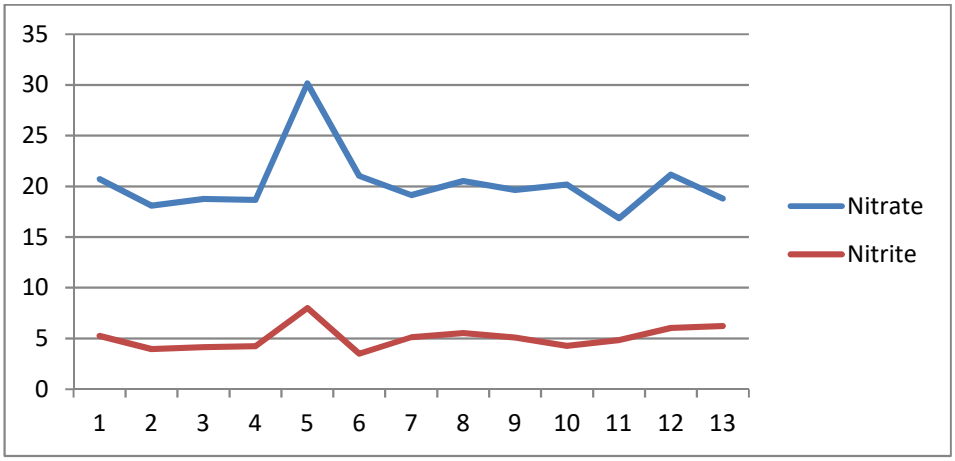


Fig 2.3.4 Correlation between nitrate and nitrite levels in blood

Correlation between blood and milk levels both for nitrates and nitrites was studied. Levels of nitrates in blood and milk were directly and weakly correlated (P_{xy}=0,17) (Figure 2.3.5)

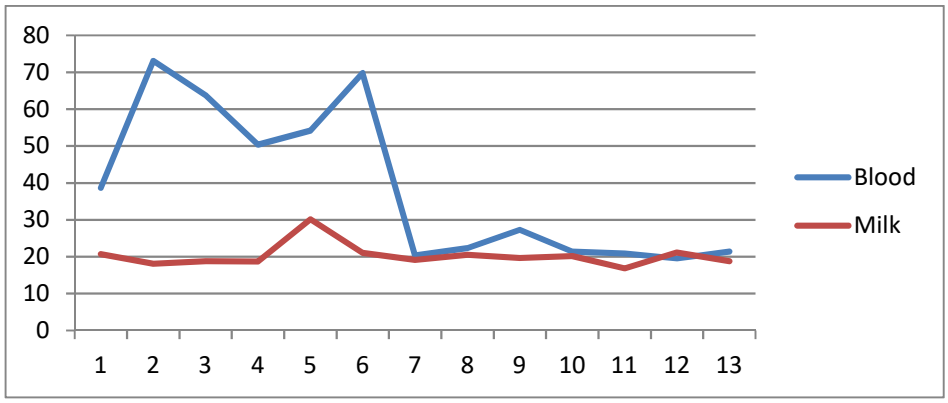


Fig 2.3.5 Correlation between nitrate levels in blood and milk from the same animals

Levels of nitrites in blood and milk were indirectly correlated (P_{xy}= -0,17) (Figure 2.3.6)

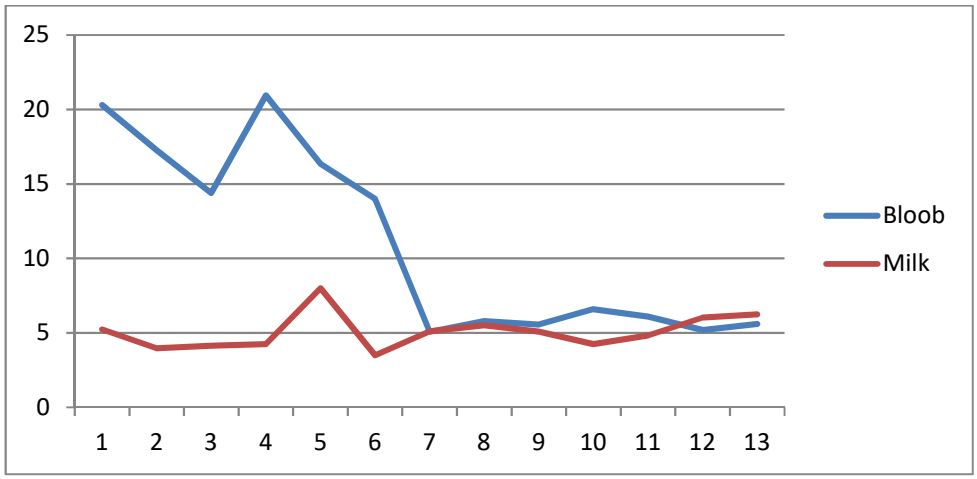


Fig 2.3.6 Correlation between nitrite levels in blood and milk from the same animals

2.4 Discussion

The presence of nitrates and nitrites in all of the examined samples confirms the high risk of exposure to these compounds for cattle. The most common cause of toxicosis in farm animals is the consumption of feed or water containing high levels of nitrates (Ozmen et al. 2003). Naturally, some vegetables easily concentrate nitrate (i.e. some cereal grasses like oat, some varieties of millet, rye, and corn). Furthermore, soils containing high levels of nitrate, environmental pollution, some weather conditions, and agricultural techniques (for example, the inappropriate use of certain fertilizers) tend to increase nitrate content in forage (Costagliola A. 2014).

In cattle, exposure to nitrates by dietary intake can be very dangerous as they can be reduced to nitrites in the rumen by ruminal microorganism (Kennett et al. 2005). According to Adams et al. (2012), stored forages containing more than 7,500 ppm of nitrates may cause acute toxicity; moreover, forage containing 5,000 to 10,000 ppm nitrate ion (NO3) can be considered potentially toxic if it is provided as the only feed (Adams et al. 2012; Whittier 2011). However, even forage concentrations of 1,000 ppm NO_3 dry weight basis have been lethal to hungry cows engorging themselves in a single feed within an hour, so the total dose of nitrate ingested is a deciding factor (Schneider 2012).Nitrate and nitrite compounds normally serve to the production of ammonia, which is used to synthesize microbial proteins (Ozmen et al. 2003).

If the nitrate intake exceeds the reduction capacity of the rumen, the process can lead to the accumulation of nitrites which enter the bloodstream and cause the oxidation of ferrous hemoglobin to ferric methemoglobin, which is unable to bind and transport oxygen to the tissues (McKenzie et al. 2009). Absence or low levels of oxygen in tissues leads to several injuries including pulmonary edema, degeneration and necrosis of epithelial cells lining the renal tubules and endothelial dysfunction (Costagliola A et al. 2014). Clinical signs may vary from moderate to severe depending on the quantity of nitrate/nitrite injested. Diagnosis of chronic exposure can be challenging due to the aspecific clinical signs which could not be detected by farmers or veterinarian pratictioners (Costagliola A et al. 2014). If sudden death occurs, expecially in acute toxicosis, a necropsy may be necessary for a definitive diagnosis of nitrate/nitrite poisoning; in addition to anatomopathological

findings, laboratory analysis of feed and water as well as assays of body fluids (i.e. serum, ocular fluids) must be considered to confirm the suspect of nitrate and nitrite poisoning (Villar et al. 2003) and detect the source of exposion. Differential diagnoses include poisonings by cyanide, urea, pesticides, toxic gases (eg, carbon monoxide, hydrogen sulfide), chlorates, aniline dyes, aminophenols, or drugs (eg, sulfonamides, phenacetin, and acetaminophen), as well as infectious or noninfectious diseases (eg, grain overload, hypocalcemia, hypomagnesemia, pulmonary adenomatosis, or emphysema) and any other cause of sudden unexplained deaths (Merck Veterinary Manual).

Results showed higher levels of nitrates and nitrites in blood reather then milk; as previously discussed nitrate compounds, once injested, if not completely methabolized, enter the bloodstream directly from rumen. Levels of nitrate found in the studied cases are much higher compared to previous study carried on healthy cow, in which nitrate concentration was 2,1 +/- 1.8 mg/L in serum (Boermans HJ. 1990). In the same study a severe case of nitrate poisoning in cattle was described and used to study the concentrations of nitrate and nitrite in samples obtained under natural conditions. Nitrate concentration of acutely poisoned cattle was 35% lower in ocular fluid at 158.1 +/- 51.4 mg/L, than in serum at 256.3 +/- 113.4 mg/L (Boermans HJ. 1990). Compared to the value reported by Boermans, cattle of our study presented values of nitrates in 6 cases indicative of severe poisoning and in 7 cases toxicosis was moderate. As described, cattle of present study effectively showed clinical signs of nitrate toxicosis, suggesting high exposure of cattle to these NO_3 compounds. As expected, results showed

that there's a strong and direct correlation between levels of nitrate and nitrite in blood and milk of studied cases. By the way, it must considered that high nitrate and nitrite values in postmortem specimens may be an incidental finding, indicative only of exposure and not toxicity. Bacterial contamination of postmortem specimens, especially ocular fluid, is likely to cause conversion of nitrate to nitrite at room temperature or higher; such specimens may have abnormally high nitrite concentrations with reduced to absent nitrate concentrations. Endogenous biosynthesis of nitrate and nitrite by macrophages stimulated by lipopolysaccharide or other bacterial products may also complicate interpretation of analytical findings; this should be considered as a possible maternal or fetal response to an infection (Merck Veterinary Manual).

Levels of nitrates in blood and milk were weakly directly correlated, while nitrites, in the same matrixes, were indirectly correlated. Furthermore, in case n. 12 levels of nitrates in milk were higher then in blood. This is, according to the author, the most interesting findings, suggesting that nitrates and nitrites accumulate in milk independentely from the level of these compounds circulating in the bloodstream. This aspect suggests that the dose of exposure of cattle to nitrate in the environment may not be enough to predict or to evaluate the levels of these compounds in milk. In humans, the ingestion and inhalation of nitrates and nitrites are the most frequent causes of

acute methemoglobinemia and our results confirmed that milk could be a source of exposure. The most sensitive people are infants and ill adults; theWorld Health Organization indicates that the toxic dose for humans is

0.4–200 mg/kg of body weight, and the lethal dose is 33–250 mg/kg of body weight (Speijers and van den Brandt 2006).

In our cases levels of nitrates and nitrites were lower than the maximum value of the range established by WHO. Moreover, it must considered that within an entire heard, levels of nitrates and nitrites in milk from a certain number of poisoned cows is diluted in the so called bulk milk, making the levels detected lower. By the way, some production procedures may re-concentrate and increase nitrate levels. For example, nitrate levels increase in dried milk when direct-fired processes are employed; the temperature of combustion of natural gases currently available in many countries are sufficiently high for the combination of nitrogen and oxygen to produce quantities of oxides of nitrogen capable of producing nitrate and nitrite in contact with milk (Hill MJ. 1991). Dried (or powdered) milk is a product that belongs to the macrocategory of baby-food, which were analyzed for nitrate and nitrite levels in a recent study (Cortesi et al. 2015). Even in that study none of the examined samples exceeded the maximum admitted level of 200 mg kg−1 set for nitrate by the Regulation 1881/2006 for processed cereal-based foods and baby foods intended for infants and young children, as defined in European Commission Directive No 96/5/EC. Despite the wide distribution in the environment and their hazard for human and animal health, to date in the European Community there's no regulamentation on official controls of nitrates and nitrites in milk

Adams, C. A., Andrews, J. E., & Jickells, T. (2012). Nitrous oxide and methane fluxes vs. carbon, nitrogen and phosphorous burial in new intertidal and saltmarsh sediments. Science of the Total Environment, 434, 240-251.

Boermans, H. J. (1990). Diagnosis of nitrate toxicosis in cattle, using biological fluids and a rapid ion chromatographic method. American journal of veterinary research, 51(3), 491-495.

Cortesi, M. L., Vollano, L., Peruzy, M. F., Marrone, R., & Mercogliano, R. (2015). Determination of nitrate and nitrite levels in infant foods marketed in Southern Italy. CyTA-Journal of Food, 13(4), 629-634.

Costagliola, A., Roperto, F., Benedetto, D., Anastasio, A., Marrone, R., Perillo, A., ... & Paciello, O. (2014). Outbreak of fatal nitrate toxicosis associated with consumption of fennels (Foeniculum vulgare) in cattle farmed in Campania region (southern Italy). Environmental Science and Pollution Research, 21(9), 6252-6257.

Hill, M. J. (1996). Nitrates and nitrites in food and water (Vol. 7). CRC Press.

Kahn, C. M. (2005). Toxicology: Nitrate and nitrite poisoning. The Merck Veterinary Manual. New Jersey: Merck & Co.

Kennett, E. C., Ogawa, E., Agar, N. S., Godwin, I. R., Bubb, W. A., & Kuchel, P. W. (2005). Investigation of methaemoglobin reduction by extracellular NADH in mammalian erythrocytes. The international journal of biochemistry & cell biology, 37(7), 1438-1445.

Ozmen O, Mor F, Ayhan U (2003) Nitrate poisoning in cattle fed Chenopodium album hay. Vet Hum Toxicol 45:83–84

McKenzie R, Gordon A, Burren B, Gibson J, Gardner M (2009) Alpaca plant poisonings: nitrate-nitrite and possible cyanide. Aust Vet J 87:113–115

Schneider NR (2012) Overview of nitrate and nitrite poisoning in the Merck Veterinary Manual. http://www.merckmanuals.com/

Speijers, G., & Van den Brandt, P. A. (2003). Nitrite and potential endogenous formation of N-nitroso compounds; Safety evaluation of certain food additives, JECFA. WHO Food Additives Series, 50, 49-74.

Villar, D., Schwartz, K. J., Carson, T. L., Kinker, J. A., & Barker, J. (2003). Acute poisoning of cattle by fertilizer-contaminated water. Veterinary and human toxicology, 45(2), 88-90.

Whittier, J. C. (2011). Nitrate poisoning. Colorado State University Extension.

Chapter 3
Animal Biomonitoring and Epidemiological Surveillance in polluted areas of Basillicata Region

3.1 Introduction

Research and production of hydrocarbons in Italy through industrial techniques began in the second half of the nineteenth, and has been growing dramatically in the century after World War II, because of the significant quantities of natural gas discovered, especially in Basilicata and Calabria regions (Van Dijk 2012). The mining of oil in Italy takes place both on the ground (in 2009 there were extracted 4 million tons) and on the sea background (about 525,000 tons). In addition to Basilicata, the regions where there are onshore wells are: Emilia Romagna, Lazio, Lombardy, Molise, Piedmont and Sicily. On the sea there are 9 active platforms for a total of 76 wells from which crude oil is extracted. Two are located in front of the Marche coast, three in front of the Abruzzo region (Vasto - CH) and the other four in the Strait of Sicily in front of the coast between Ragusa and Gela.

Italy, compared to major producing countries, has not only modest but also large deposits of oil and natural gas, including those in the Val d'Agri in Basilicata, the largest in continental Europe, and in the area of Crotone in Calabria (Il Campo Luna - Hera Lacinia), ranking fourth among the European countries and oil producers as the world's 49th largest oil producer in quantity (0.1% of total world production). In Italy oil production in 2013 was 5.48 million tons; of these, 71% comes from Basilicata's fields, owned by Eni and Shell, of which over 2 million tons of oil and 1 million cubic meter of natural gas (16 % of the total extracted in Italy) have been extracted from the 3 active oil concessions: Gorgoglione, Serra Pizzuta and above all

from the Val d'Agri, from which derives more than 99% of the oil extracted in the region. The areas given in concession for the extraction of oil take about one thousand square kilometers, but the hypothecated area to oil activities could increase in the coming years (Figure 3.1).

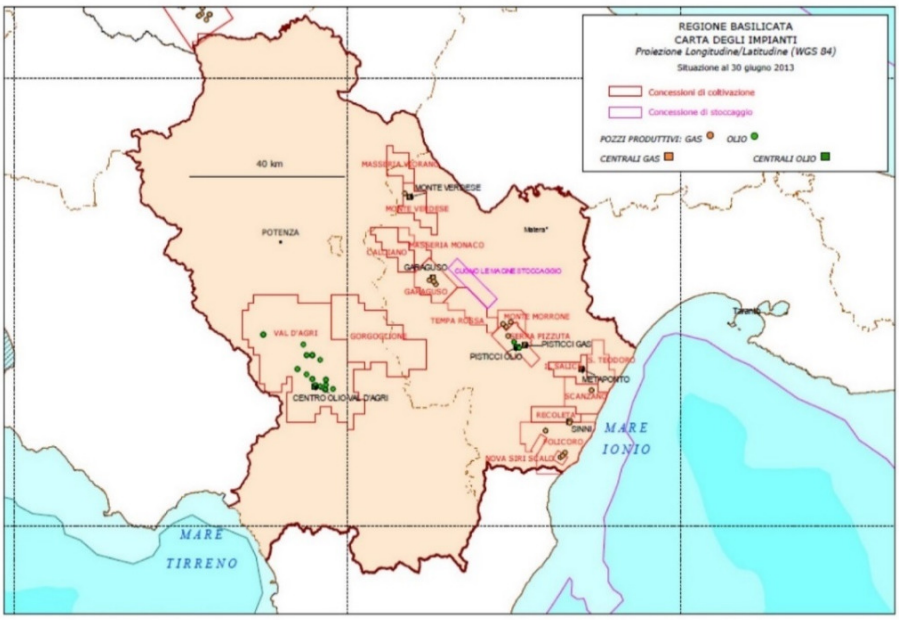


Fig 3.1 Map of active oil installations in Basilicata Region. Red lines show production licenses, the purple storage concessions. Orange circles productive gas wells, the green ones the oil producing wells. Orange squares active gas centrals, green ones, the oil plants. Source Ministero dello sviluppo economico DGS-UNMIG.

Activity and extraction of crude oil and natural gas storage in the region of Basilicata are listed as follows:

1. GARAGUSO (69, 62 km2) Holders: EDISON and GAG PLUS ITALIANA
- Average annual production: 24.527.356
2. MASSERIA VIORANO (41, 61 km2) Holder: PENGAS ITALIANA
- Average annual production: 81,553
3. MONTE MORRONE (29, 72 km2) Holder: GAS PLUS ITALIANA
- Average annual production: 9.472.349
4. RECOLETA (44, 62 km2) Holder: GAS PLUS ITALIANA
- Average annual production: 4.431.642
5. SERRA PIZZUTA (62, 55 km2) Holder: ENI
- Average annual production: 21.274.550
6. VAL D'AGRI (660, 15 km2) Holders: ENI and SHELL ITALIA E&P
- Average annual production: 1.058.929.111
7. SERRA PIZZUTA (62, 55 km2) Holder: ENI
- Average annual production: 18.232.799
8. VAL D'AGRI (660, 15 km2) Holders: ENI and SHELL ITALIA E&P
- Average annual production: 3.676.292.566
9. GORGOGLIONE (290,59 km2) Holders : TOTAL E&P ITALIA,SHELL ITALIA E&P and MITSUI E&P ITALIA B.
- Average annual production: 5.888.348

From these data emerges, that the most industrial activity is carried out in the heart of Val D'Agri, where there is the Val d'Agri Oil Centre located in

Viggiano, "Production area for industrial settlements" with a size of about 180,000 m^2, in which occur the oil treatment product from wells and conveyed here through the collection network (Figure 3.2). The fluid extracted from the field and coming to the implant, is a "multi-phase" mixture, containing different proportions of the three phases: oily, aqueous and gaseous. The extraction treatment consists in oil separation from the gas and the water layer which is naturally associated, in order to export stabilized gas and oil.

Fig 3.2. Active wells in Val D'Agri (source DGS-UNMIG Ministero dello sviluppo economico)

The municipalities interested by the presence of wells and /or the passage of the transport pipelines are: Viggiano, Calvello, Marsico Nuovo, Grumento

Nova, Marsicovetere, Montemurro, Paterno and Tramutola. With the establishment of the National Park Val d'Agri Lagonegrese in 2007, seven of these positions fall within the protected perimeter. Most of the industrial activities associated with oil extraction is then concentrated in the two provinces of Potenza and Matera, especially in the Val d'Agri area and Monteverdese in Potenza province, Gorgoglione, Garaguso, Pisticci, Recoleta and the Serra Pizzuta areas. Environmental pollution by chemicals released during these industrial activities in Basilicata Region is an issue of serious concern.

One of the major problem with gas drilling is the production of Polycyclic aromatic hydrocarbons (PAHs). PAHs are ubiquitous environmental pollutants generated primarily during the incomplete combustion of organic materials (e.g. coal, oil, petrol, and wood). Emissions from anthropogenic activities predominate; nevertheless, some PAHs in the environment originate from natural sources such as open burning, natural losses or seepage of petroleum or coal deposits, and volcanic activities. Major anthropogenic sources of PAHs include residential heating, coal gasification and liquefying plants, carbon black, coal-tar pitch and asphalt production, coke and aluminum production, catalytic cracking towers and related activities in petroleum refineries as well as and motor vehicle exhaust (Hussain L. 2012). PAHs are emitted to the atmosphere also from the incomplete combustion (either natural or anthropogenic) of organic matter. Thus, the main sources of exposure for animals and humans to PAHs is by inhalation of polluted air or injestion of contaminated food. Due to their molecular structure PAHs are highly lipid soluble and thus readily absorbed

from the gastrointestinal tract of mammals. They are rapidly distributed in a wide variety of tissues with a marked tendency for localization in body fat. Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation as the first step. Many PAHs have toxic, mutagenic and/or carcinogenic properties, and are potent immune-suppressants (Hussain L. 2012); published reports indicate that the immune-suppression is the most frequent toxic effect associated with exposure to PAHs. These agents, typified by benzo(a)pyrene (BaP), have been shown to alter antigen and mitogen receptor signaling pathways, leading to suppression of humoral and cell-mediated immunity, and at high exposure levels to activation of genes involved in apoptosis in lymphoid cells. The mechanism of toxicity is considered to be an interference with the function of cellular membranes as well as with enzyme systems which are associated with the membrane. Although unmetabolized PAHs can have toxic effects, a major concern is the ability to induce carcinogenesis, due to the capacity of the reactive metabolites, such as epoxides and dihydrodiols, of some PAHs to bind to cellular proteins and DNA. [34] Biochemical disruptions and cell damage occurrence lead to mutations, developmental malformations, tumors, and cancer. Evidence indicates that mixtures of PAHs are carcinogenic to humans. The evidence comes primarily from occupational studies of workers exposed to mixtures containing PAHs. Such long-term studies have shown an increased risk of predominantly skin and lung as well as bladder and gastrointestinal cancers. Nevertheless, it is not clear from these studies whether exposure to PAHs was the main cause as

workers were simultaneously exposed to other cancer-causing agents (e.g. aromatic amines) (Bach PB 2003).

In 2015 the biomonitoring surveillance project “S.E.BIO.VET.” (Study of Veterinary Environmental Biomonitoring) started in Basilicata region with the interdisciplinary collaboration of University of Naples Federico II - Department of Veterinary Medicine and Animal Production, Unit of Pathology, Unit of Parasitology and Unit of Infectious Diseases, the ASL of Basilicata and Zooprophylactic Institute of Basilicata, whose collaboration aims to achieve a sampling and an efficient and effective clinical approach to achieving the goals. Furthermore, very important for this activity was the role of the veterinarian in the Regional Breeders Association of Basilicata, for their knowledge of the territory and the radicalism within the region farms. The project aimed to use the sheep as sentinel animals for environmental monitoring because of their grazing characteristics, which make them excellent biological indicators of the effects of polluted water, air and soil. Throguh the evaluation of health condition of the animals and a complete post-mortem anatomopathological evaluation of organs at the slaughterhouse, it was try to identify informations to define the state of the environment of the Region. So, depending on the results obtained, it could properly be defined critical areas to enhance, or healthy sites to be preserved.

3.2 Materials and Methods

594 elderly sheep were selected using a distribution grid and georeferenced sampling from farms belonging to the different ASL districts of Basilicata Region. (Figure 3.2.1)

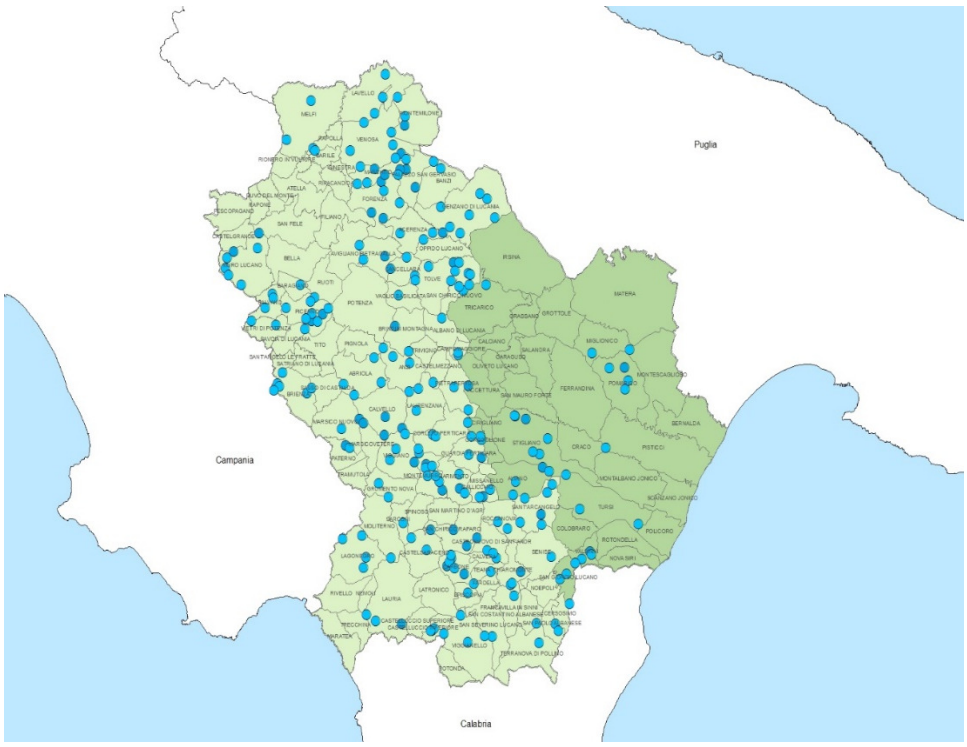


Fig 3.2.1 Distribution of Sheep Farms (blu circle) selected for the study

For the epidemiological study 7 districts belonging to the National Health Service System (ASL) were selected (Figure 3.2.2):

District 1: POTENZA

District 2: VILLA D'AGRI

District 3: SENISE

District 4: LAURIA

District 5: MELFI

District 6: VENOSA

District 7: MATERA

Fig 3.2.2 Distribution of districts and municipalities selected for epidemiological analysis

All the animals were properly identified through the verification of transport documents (DDT) and the ear-tag number. Once they have started the slaughter line, they received a progressive number reported, in addition to the signaling data (including ear-tag and holding of provenance) on a "diagnostic report" together with the findings of the ante-mortem inspection, the pathological examination results and information about the sampling for histopathological, toxicological and microbiological investigations. After stunning, at the time of the jugulation, at least 5cc of blood venous samples were collected by gravity, in suitable sterile containers with a screw cap (falcon from 15cc or similar).

Blood samples were used for further studies such as "entry profiles": Standard haematological tests and blood chemistries (complete blood count, glucose, AST, ALT, bilirubin, BUN, creatinine, total protein, Alb./ glob.) and also for the toxicological investigations for the search of hydrocarbons (PAHs: benzo [e] pyrene and benzo [a] pyrene), heavy metals (arsenic, cadmium, strontium) and endocrine disruptors (Dioxins and PCBs).

For the toxicological investigations, at the time of blood sampling, 5 ml of milk and at least 5 g of wool picked up from the back region of the animals, at the base, as close as possible to the skin, were collected. The samples were placed in special plastic containers with screw cap, suitable for the transport of biological material (type falcon from 50cc.). Each package reported the sequence number assigned to the animal and a record card which identifies precisely the animal from which samples were taken, and breeding of membership.

After evisceration, all the organs were inspected for anatomopathological examination. In particular the following organs were systematically analyzed:

- Lungs (including tracheobronchial and mediastinal lymphonodes)
- Heart (including the pericardium and the atrioventricular valves)
- Liver (including hepatic and pancreatic lymphonodes)
- Spleen
- Kidneys
- Stomachs
- Intestine (including the mesenteric lymphonodes)
- Male and Female reproductive apparatus
- Thyroid

The macroscopic examination results were reported on the diagnostic report where the pathologist indicates the observed lesions and their morphological diagnosis. After pathological examination sampling for microscopic (histopathology), toxicological and microbiological examination was performed.

Statistical analysis was performed using chi-square test (or Fisher test) with programm SPSS version 24.0.

3.2 Results

The macroscopical examination of organs results are summurized as follows (Lesion; number of cases observed/total number):

1) Lungs:

- ✓ Intestizial pneumonia n° 210 / 594
- ✓ Fibrinous pneumonia n° 96 / 594
- ✓ Catarral broncopneumonia n° 4 / 594
- ✓ Parasitic broncopneumonia n° 32 / 594
- ✓ Pleuropneumonia n° 24 / 594
- ✓ Granulomatous pneumonia n° 4 / 594
- ✓ Pulmunary abscesses n° 19/ 594
- ✓ Purulent broncopneumonia n° 102/ 594
- ✓ Pleuritis n° 27 / 594
- ✓ Corynebacterium pseudotuberculosis n° 23 / 594
- ✓ Mucopurulent tracheitis n° 1 / 594
- ✓ Pulmunary oedema n° 1 / 594
- ✓ Enfisema n° 2 / 594

2) Liver

- ✓ Interstitial hepatitis n° 71 / 594
- ✓ Chronic cholangitis and hepatitis n° 191 / 594
- ✓ Parasistic hepatitis n° 17 / 594
- ✓ TBC n° 2 / 594
- ✓ Diffuse chronic hepatitis n° 75 / 594
- ✓ Hepatosis n° 21 / 594
- ✓ Multifocal chronic hepatic abscesses n° 13 / 594
- ✓ Chronic active fibrinopurulent cholangitis n° 2 / 594
- ✓ Corynebacterium pseudotuberculosis n° 2 / 594
- ✓ Perihepatitis filamentosa ,Fibrous Tags n° 7/ 594
- ✓ Multifocal necrotic hepatitis n° 5 / 594

3) Spleen:

- ✓ Lymphoid Hyperplasia n° 103 / 594
- ✓ Multiple infarctions with hemorrhage n° 1 / 594
- ✓ Spleen congestion n° 5 / 594

4) Kydney

- ✓ Glomerulonephritis with hydronephrosis n° 24 / 594
- ✓ Renal calculi n° 3 / 594

- ✓ Serous atrophy of fat n° 27 / 594
- ✓ Acut to subacute tubulonephrosis n° 6 / 594
- ✓ Nephrosis n °33 / 594
- ✓ Polycystic kidney n ° 8/ 594
- ✓ Chronic cortical interstitial nephritis n° 8/ 594

5) Heart

- ✓ Serous atrophy of fat n° 97/ 594
- ✓ Haemoragies (sub/epi/endo) n° 39 / 594
- ✓ Miocardium fibrosis n ° 2/ 594
- ✓ Endocarditis and myocarditis n° 30/ 594
- ✓ Endocardiosis n ° 16 / 594
- ✓ Miocarditis TBC n° 1 / 594
- ✓ Hydropericardium n° 7 / 594
- ✓ Pericarditis n ° 7/ 594

6) Mammary glands

- ✓ Chronic mastitis n ° 53 / 594
- ✓ Acute mastitis n ° 22 / 594

Figure 3.2.3 shows some anatomopathological findings of macroscopical examination

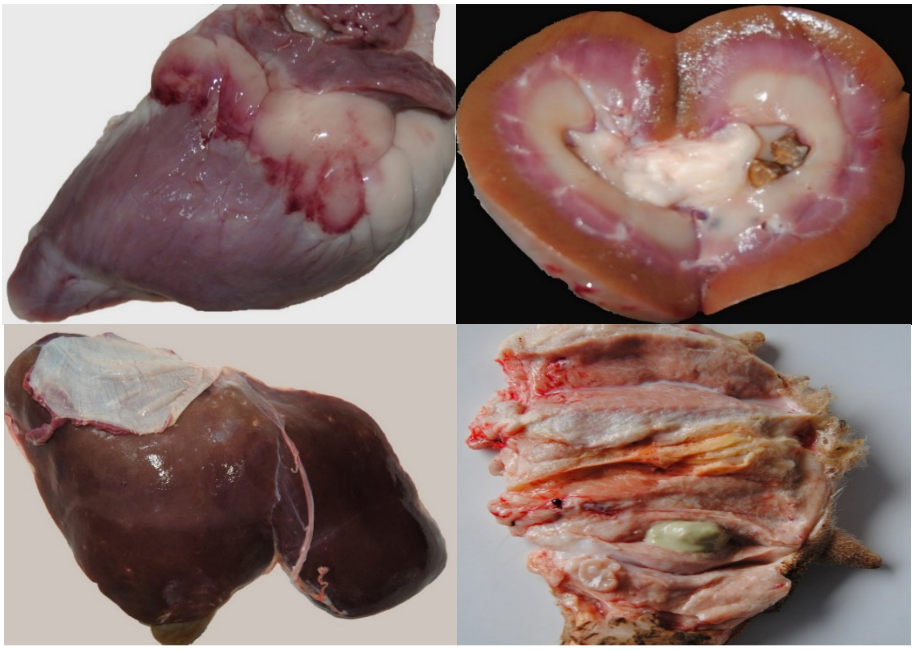


Fig 3.2.3 Heart. Serous fat atrophy associated with subepicardial hemorrhages. Kidney. Nephrosis associated with calculosis. Liver. Parasitic hepatitis. Mammary Gland. Abscess.

Statistical examination of data has allowed to observe an homogeneous distribution of anatomo-pathologic lesions in the various ASL districts. Only among lung inflammatory lesions a statistically significant difference has been highlighted; in particular for Interstizial Bronchopneumonia and Interstitial Pneumonia, Purulent Bronchopneumonia and Parasitic Bronchopneumonia, a different distribution was found in the various

districts. Statistically significant correlation (P <o.oo1) (Figure 3.2.4) have been found between :

- **District 5** of **Melfi** with **Interstitial Pneumonia** (9 of 30 positive animals).
- **District 6 of Venosa** with **Interstitial Pneumonia** (13 positive out of 45)
- **District 3 (Figure 6) of Senise** (16 of 36 positive animals) and **District 4** of **Lauria with Purulent Bronchopneumonia**, (12 of 18 positive animals).
- **District 2 (Figure 6) of Val D'Agri** with **Parasitic Bronchopneumonia** (22 positive out of 55).
- **District 7 of Matera** with **Interstitial Bronchopneumonia** (20 of 30 positive)

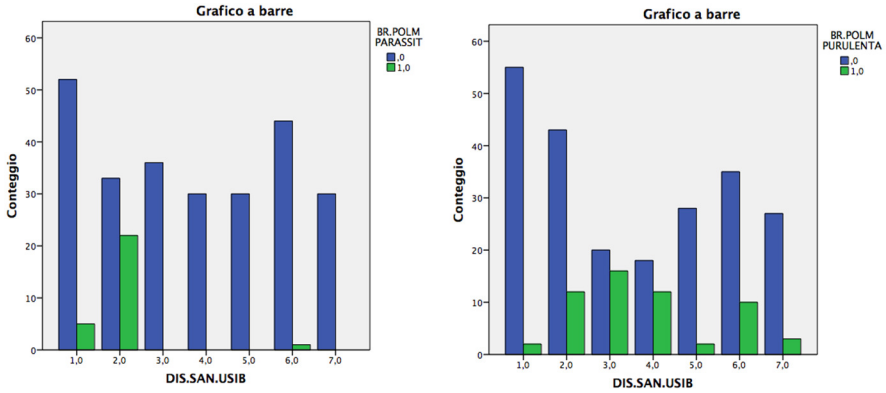


Fig 3.2.4 (Left) Prevalence of parasitic bronchopneumonia, underlined by the green column, in the District 2 of Val D'Agri. (Right) Prevalence of purulent bronchopneumonia, underlined by the green column, in the District 3 of Senise (PZ) , and District 4 of Lauria (PZ).

3.3 Discussion

The most striking finding of our investigations was the difficulty in obtaining definitive information on the link between hydrocarbon gas drilling and health effects. However, the results point to a number of ways policies can be changed to facilitate better data collection and to avoid obvious risks to animal and human health. The purpose of the present work was to study the use of animals to appraise the presence of environmental contamination, potentially dangerous for human health, with particular attention to the use of the sheep as "system sentinel animal" (SSA) in Basilicata Region, in Southern Italy.

This study represents a preliminary stage of a much larger project, which aims to highlight a correlation between anatomo-patological lesions, observed in these animals, and environmental pollution, illustrating one meaningfully statistic distribution of some lesions in a specific geographical area that is believed to be more polluted for the presence of oil wells.

The animals have been selected in homogeneous way by the various farms distributed in the only two existing provinces: Potenza and Matera. An increased prevalence of some inflammatory pulmonary pathologies observed in the results, which could recognize different causes (such as: bad management and the age of the animals, or a different incidence of etiological agents in relationship to the environment). In this study, we assumed a correlation among anatomo-patological pulmonary lesions and immunodeficiency probably caused by environmental pollution and the

geographical distribution of oil wells in Basilicata sheep farms. An equal distribution of anatomo-pathological lesions has emerged from the study except for some pulmonary illnesses (i.e. Parasites, Purulent and Interstitial Bronchopneumonia) which can be predisposed by a condition of immunodeficiency.

In fact different authors have underlined as the presence of environmental pollutants, particularly of the PHAs (Polycyclic aromatic hydrocarbons) which are generated primarily during the incomplete combustion of organic materials, particularly from oil and petroleum, is responsible for damages to the immune system (Hussein I. 2016).

The presence of a different distribution of anatomo-patological lesions in lungs but not in other organs (in which the lesions resulted equally distributed) could be explained with a different distribution of the environmental pollution on the territory in relationship to the location of the oil wells in some districts of the region. The atmosphere is the most important means of PAH dispersal, which are emitted to the atmosphere primarily from the incomplete combustion of organic matter. (Armstrong BJ 2004)

An higher distribution of Interstitial Bronchopneumonia in Val D'Agri, where most oil installations are located, compared to Matera's district (where industrial activities are scarce) was found. Moreover, a lower Purulent Bronchopneumonia frequency in the not polluted district of Lauria, in which there is the municipality of Lagonegro, compared to Senise area, another site next to these industrial activities, could be explained by a

correlation between immunodeficiency caused by a high level of environmental pollutants and the presence of these oil fields.

It is therefore interesting to note that in the province of Potenza there's a presence of lung diseases concentrated in Val d'Agri, where most oil fields are located, but not in other areas of the province, which contributes to strengthen the idea of a major concentration of illnesses in more polluted areas.

The most important requirement for an assessment of the impact of gas drilling on animal and human health is complete testing of air and water prior to drilling and at regular intervals after drilling has commenced. This includes chemicals used in the drilling muds, fracturing fluid and wastewater (the latter contains heavy metals and radioactive compounds normally found in a particular shale. Currently, the extent of testing (particularly for organic compounds) is frequently inadequate and limited by lack of information on what substances were used during the drilling process. Thus, sheep may be a good animal sentinel system to monitor the impact of industrial activities on the environment.

Abdel-Shafy, H. I., & Mansour, M. S. (2016). A review on polycyclic aromatic hydrocarbons: source, environmental impact, effect on human health and remediation. Egyptian Journal of Petroleum, 25(1), 107-123.

Ankley, G., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., ... & Dickerson, R. (1998). Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environmental Toxicology and Chemistry, 17(1), 68-87.

Bach, P. B., Kelley, M. J., Tate, R. C., & McCrory, D. C. (2003). Screening for lung cancer: a review of the current literature. CHEST Journal, 123(1_suppl), 72S-82S.

Bamberger, M., & Oswald, R. E. (2012). Impacts of gas drilling on human and animal health. New solutions: a journal of environmental and occupational health policy, 22(1), 51-77.

Barth, A., Brucker, N., Moro, A. M., Nascimento, S., Goethel, G., Souto, C., ... & Duarte, M. (2017). Association between inflammation processes, DNA damage, and exposure to environmental pollutants. Environmental Science and Pollution Research, 24(1), 353-362

Battisti, A., & Sala, M. (2007). Environmental pollution in the zootechnical productions: the experience of the b-exachlorecycloexane contamination in Sacco River Valley. In Proceedings IX National Congress SIDiLV (pp. 23-24).

Brambilla, G., Fochi, I., Falce, M., De Filippis, S. P., Ubaldi, A., & Di Domenico, A. (2008). PCDD and PCDF depletion in milk from dairy cows according to the herd metabolic scenario. Chemosphere, 73(1), S216-S219.

Burchiel, S. W., & Luster, M. I. (2001). Signaling by environmental polycyclic aromatic hydrocarbons in human lymphocytes. Clinical Immunology, 98(1), 2-10.

DeNardo, P., Bruni, B., Paoletti, L., Pasetto, R., & Sirianni, B. (2004). Pulmonary fibre burden in sheep living in the Biancavilla area (Sicily): preliminary results. Science of the total environment, 325(1), 51-58.

Esposito, M., Cavallo, S., Serpe, F. P., D'Ambrosio, R., Gallo, P., Colarusso, G., ... & Serpe, L. (2009). Levels and congener profiles of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and dioxin-like polychlorinated biphenyls in cow's milk collected in Campania, Italy. Chemosphere, 77(9), 1212-1216.

Esposito, M., Serpe, F. P., Neugebauer, F., Cavallo, S., Gallo, P., Colarusso, G., ... & Serpe, L. (2010). Contamination levels and congener distribution of PCDDs, PCDFs and dioxin-like PCBs in buffalo's milk from Caserta province (Italy). Chemosphere, 79(3), 341-348.

International Agency for Research on Cancer. (2010). Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures (Vol. 92). IARC Press, International Agency for Research on Cancer.

Kim, K. H., Jahan, S. A., Kabir, E., & Brown, R. J. (2013). A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. Environment international, 60, 71-80.

Scaramozzino, P., Rombolà, P., Battisti, S., Sala, M., Ferrari, G., & La Torre, G. (2012). Surveillance model for environmental contaminants

through their monitoring in animal production. Italian Journal of Public Health, 7(1).

Stahl Jr, R. G. (1997). Can mammalian and non-mammalian “sentinel species”; data be used to evaluate the human health implications of environmental contaminants?.

Van der Schalie, W. H., Gardner Jr, H. S., Bantle, J. A., De Rosa, C. T., Finch, R. A., Reif, J. S., ... & Stokes, W. S. (1999). Animals as sentinels of human health hazards of environmental chemicals. Environmental health perspectives, 107(4), 309.

Van Dijk, J. P., Affinito, V., Atena, R., Caputi, A., Cestari, A., D’Elia, S., ... & Picone, S. Cento Anni di Ricerca Petrolifera.

Wang, Z., Yang, C., Yang, Z., Sun, J., Hollebone, B., Brown, C., & Landriault, M. (2011). Forensic fingerprinting and source identification of the 2009 Sarnia (Ontario) oil spill. Journal of Environmental Monitoring, 13(11), 3004-3017.

World Health Organization. (1998). Polycyclic aromatic hydrocarbons, selected non-heterocyclic. Environmental Health Criteria, 202.

CONCLUSIONS

Conclusions

Necropsy, currently is still the best diagnostic investigation to obtain a definitive and accurate diagnosis of cause of death (Silas AO et al. 2009). In our reports it's shown the undiscussible utility of "environmental necropsy", an anatomopathological procedure based whose goal is to obtain not only the cause of death of the cadaver, but also to evaluate the presence of pathologies strictly linked with environment.

In study n.1 the dog was used for environmental biomonitoring of urban areas. Our data shows that in a discrete number of cases the cause of death was related to diseases which could be associated with exposure to environmental pollution. Canine specie has been confirmed a good animal sentinel system to monitor environmental pollution in the provinces of Naples and Caserta, but generally speaking within the whole Campania Region. Studies of the effects of environmental exposures on domestic and wild animals can corroborate or inform epidemiologic studies in humans in the concept of ONE HEALTH. Increasing the number of necropsies and make sampling of dogs more homogeneous within the entire Campania Region are crucial points to make these data more significant from an epidemiological point of view. Toxicological analysis are essential to associate exposition to environmental pollutants and development of neoplastic or chronic/degenerative disease; by the way further investigations are still needed to clearly define the molecular pathway involved in the pathogenesis of some tumors to clearly relate it to the exposure to environmental pollutants (i.e. heavy metals; Dioxins; PCBs) which is usually difficult due to cost of analysis and the multifactorial ethiology of cancer.

In report n.2 dairy cattle was studied as a possible early warning system for public health. The report focuses on the environmental risk of nitrate and nitrite accumulation, which is considered hazardous both for humans and animals since these compounds may accumulate in fruits, vegetables, drinking water (Katan 2009). Data on nitrate and nitrite residues in milk are very scarce and our study confirmed that this food could be a possible source of exposure for humans, even if it is thought that their accumulation in muscle tissues and animal products is very low in normal condition (Cockburn et al. 2013). Nevertheless, we are still concerned that the concentration of nitrate and nitrite in animal products could be a problem for human health. The use of sentinel animal data can be very helpful in monitoring the pollution in certain areas and in evaluating the long-term impact of some chemicals on human health. Environmental pathologists may compare the frequency and the pattern of some diseases in animals with those corresponding in humans and help detect new polluted areas and monitor the environmental pollution impact of anthropic activities.

Study n.3 was performed using sheep grazed around potentially polluted areas by oil drilling in Basilicata region. One of the worst concern during this activity is the release in the environment of PAHs. PAHs may have a great impact on human and animal heatlh so a constant monitoring of these compounds in soil, air and water should be required. By the way, most of the times, analytical research of pollutants in the environment is not cost effortable (especially if the area to monitorize is large) and could be biased by sampling. The anatomopathological findings, even if they must be considered as preliminary data, may help to identify, in such a big area as

Basilicata Region is, unknown polluted sites to be analysed, or to monitor places where the risk for human and animal health has already been assessed.