UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

DOCTORATE IN VETERINARY SCIENCE XXXIII CYCLE

THESIS

"GENETICS AND ENVIRONMENT: IMPACT ON MAMMALIAN SPERMATOZOA"

Candidate
Dott. Giacomo Galdiero

Tutor
Prof. Francesca Ciotola

DOCTORATE IN VETERINARY SCIENCES
Secretery *Dott.ssa Maria Teresa Cagiano*
Coordinator - *Prof. Giuseppe Cringoli*

INDEX

Preface

Zootechnics has the mission to improve animal production, according to the human needs, that will influence market demand and animal and human health. Animal reproduction can represent a surrogate model to address environmental pollution and lifestyle impacts and the effects of natural countermeasures in the prevention of reproductive disorders.
This thesis reports the research studies carried out during the doctorate, born from the collaboration between the Unit of Andrology and Reproductive Medicine and Male and Female Sexuality (FERTISEXCARES) and Laboratory of Veterinary Genetics and Biotechnology applied to Animal Productions (GENENVET) of the University Federico II of Naples. The general aim of the current research study was to determine the overall impact of the environment on semen quality and male fertility potential, by particularly addressing the effects of both environmental pollution and lifestyle components, and the contribution of genetic and epigenetic changes to such reproductive effects. Specific aims included the assessment of the potential epigenetic changes induced by heavy metals exposure, particularly cadmium (Cd) and to correlate epigenetic changes to semen quality and potential for male fertility (in humans and dogs). As reguard these specific aims, the human-dog pair represents an interesting study model, as the close relationship between owner and animal implies that the two elements of the couple share common environmental effects and are, therefore, plausibly exposed to a set of common pollutants/environmental factors. Lastly, a further specific aim was to assess the effects of healthy lifestyle, particularly the supplementation with nutraceuticals, on semen quality, in order to identify natural substances as alternative treatments for seminal impairment in animals. For this cause a horse model was used, by determining the effect of food supplementation with a plant rich in antioxidant substances: maca (Lepidium meyenii) on seminal parameters of Italian thoroughbred stallions. In particular, morphometric analyzes and sperm DNA fragmentation (SDF) tests were performed on the ejaculates in addition to the qualitative and quantitative evaluations routinely carried out.

Abstract

Aim of this doctorate has been to explore the links between environment, genetics and characteristics of spermatozoa in mammals and in particular three species have been studied: humans, dogs and horses. This study was in fact conceived following the collaboration of two important research areas: human reproduction and the genetic improvement of livestock species. We started from the premise of wanting to fill gap of information concerning semen quality of men living in the high environmental pressure area of the “Land of Fires” (LF), and to investigate the potential association withenvironmental pollutants, such as cadmium (Cd). Specific aims included the assessment of the potential epigenetic changes induced by heavy metals exposure, particularly Cd, and to correlate these changes to semen quality and spermatozoa charachteristics (in humans and dogs).
The study included two different cohorts of subjects comprising 730 (C1) and 512 (C2) participants belonging to the LF. All subjects in C1 were offered a diagnostic clinical examination in their domestic dogs. The dogs recruited (N=30) for the study had to be clinically healthy and with at least one litter in the last 12 months. In C2 subjects a strict correlation was demonstrated between seminal parameters, particularly sperm total count, and Cd burden in semen samples, testifying a potential harmful effect on reproductive health in humans. Moreover, reduced SDGM was associated to reduced semen quality, demonstrating an overall similar relationship between epigenetic changes and seminal parameters in men and their dogs. This is the first report demonstrating a correlation between SGDM percentage and conventional seminal parameters in dogs; this epigenetic finding could be of considerable interest also in the zootechnical field, due to the possible reproductive and economic repercussions.
Lastly, a further specific aim was to assess the effects of healthy lifestyle, particularly the supplementation with nutraceuticals, on semen quality, in order to identify natural substances as alternative treatments for seminal impairment in stallions. Four stallions were food supplemented with maca (Lepidium meyenii) during the breeding season. Maca food supplementation in stallions during breeding season reduced the percentage of spermatozoa with fragmented DNA, significantly increased sperm concentration and lengthened spermatozoa head, suggesting that food supplementation of maca could be useful in horse breeding.

CHAPTER 1

Introduction

1.1 Temporal and geographic trends in semen quality in human

Infertility is defined as the inability to conceive after 1 year of unprotected intercourses (*Zheng et al., 2000*). It affects millions of people worldwide, in particular, it is exstimated that about 15% of couples are afflicted, with male factors accounting for 50% of causes of fertility problems, alone or in combination with female factors. The 16% is due to genetic factors (1%), or to unexplained, or idiopathic, infertility, which is diagnosed in absence of a specific etiological determinant (*O'Flynn 2014*). Male infertility diagnosis is most commonly based on semen analysis, according to the 2010 World Health Organization laboratory manual guidelines (*WHO 2010*). The worldwide decline in human fertility has been recorded since the early 1950s, and reported by the international literature (*Bank 2019*), as well as a steady decay of birth rates in all European countries was reported by several demographic surveys (*Lutz et al., 2003*). Changes in lifestyle such as increased women occupational rate and maternal age at pregnancy, prevention of undesired parenthood, or increased socio-economic burden of parenthood did not justify and support the progressive worsening of couple fertility (Figure 1). Evidence suggested that worldwide sperm total counts have dropped by 50% since the 1930s, as reported by a 1992 metanalysis of 61 studies on semen quality published over a 50-year period (1938–1991), comprising almost 15.000 men from 23 different countries (*Carlsen et al., 1992*); in particular, has been reported an average sperm counts of 100 million, 75 million, and 50 million/ml in 1950, 1970, and 1990, respectively, in western countries (*Dindyal 2003*). The declining semen quality was afterward confirmed by an extended metanalysis of 101 studies (1934-1996) (*Swan et al., 2000*). The gross changes in sperm counts are not necessarily linked to corresponding changes in fertility trends, since successful pregnancy might also occur in case of low sperm counts; nevertheless, impairment of semen quality might result in longer waiting time to pregnancy, and, on a long-term basis, might eventually result in the observed decline in fertility trends (*Slama et al., 2004*). It is interesting how recent studies highlighted marked geographical variations in semen quality within both United States and Europe, suggesting the hypothesis of a possible impact of local persistent environmental pollution patterns on male fertility (*Nordkap et al., 2012*) (Figure 2). Therefore, the contribution

of concurrent, global, and local factors should be taken into account to explain differences among studies.

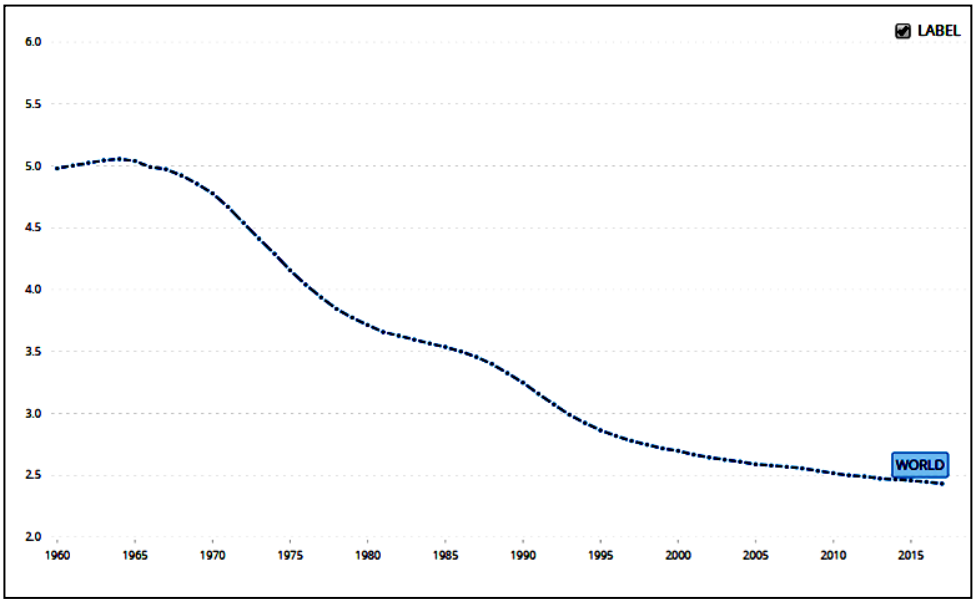

Figure 1: *Fertility rate, total (births per woman, weighted average). Trends in birth rate from 1960 to 2015, showing a progressive decline in worldwide total fertility rate. Total fertility rate represents the number of children that would be born to a woman if she were to live to the end of her childbearing years and bear children in accordance with age-specific fertility rates of the specified year. https://data.worldbank.org/indicator/SP.DYN.TFRT.IN.*

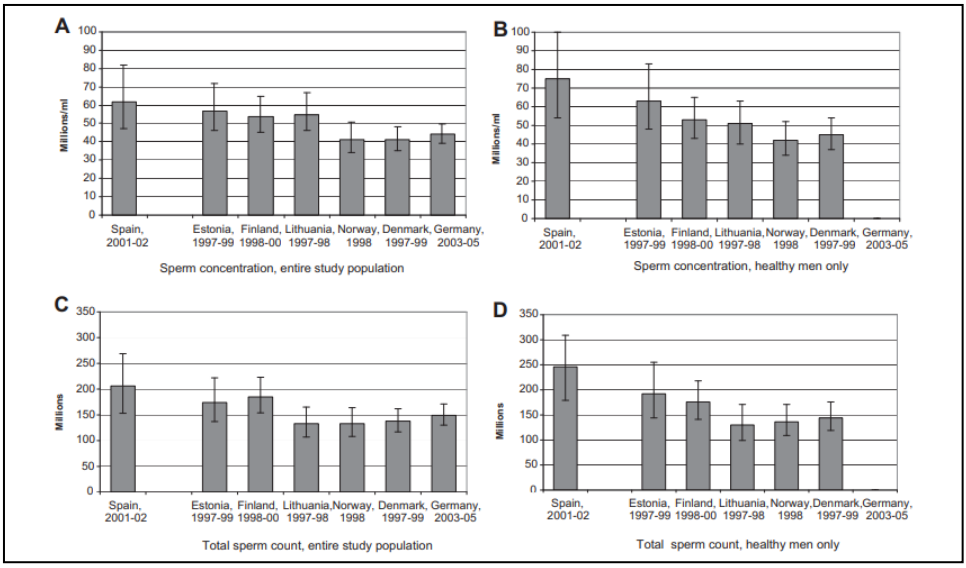

Figure 2: *Sperm concentrations and sperm total count of young men from seven European countries. The bars show the adjusted median sperm concentration (nx10^6/ml) and sperm total count (nx10^6/ejaculate) in the entire study populations (A and C) and in subgroups of men without any recent use of medicine or known andrological disease (B and D) (Nordkap et al., 2012).*

1.2 Male fertility as a target of environmental exposures: heavy metals

Human semen contains trace elements such as calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), manganese (Mn), copper (Cu), zinc (Zn), and selenium (Se), which are essential for normal spermatogenesis, sperm maturation, sperm motility and capacitation (*Mirnamniha et al., 2019*). Deficiencies of these trace elements might therefore be a relevant factor affecting semen quality, as demonstrated by studies in infertile patients, reporting reduced Ca, Mg, Zn, Cu and Se in seminal plasma (*Mirnamniha et al., 2019*). On the other hand, a number of different environmental chemicals, including trace elements belonging to the class of heavy metals,

have been claimed to exert sharp detrimental effects on male reproductive function.

A direct consequence of global industrialization has been the exponential increase of environmental pollutants, particularly in countries with poorer surveillance and regulations (*CDC 2019; 2019.2*). Epidemiological evidence has shown associations of human exposure to heavy metals with adverse reproductive outcomes due to widespread cumulative burden and low human body clearance. Based on this evidence, there is a growing concern for the effects of heavy metals on semen quality and male fertility, even at low environmental, non-occupational, level of exposure (*Wirth 2010*). Nevertheless, although the effects of high level exposure to several non-essential heavy metals, such as cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), and mercury (Hg), on male reproductive function have been clearly demonstrated by experimental studies in animal models and/or in human occupational exposure studies (*ATSDR 1999; 2005; 2012; 2012; 2019; 2019*), there is a lack of strong confirmatory clinical studies concerning low environmental level of exposure, as well as evidence quite sparse, conflicting or inconclusive, due to several shortcomings including heterogeneity in study design (mostly retrospective), small sample size, and lack of adjustment for potential confounders. Moreover, dose-response studies linking exposure to reproductive outcomes are limited, and contemporary exposure to multiple heavy metals potentially exerting synergistic or antagonistic effects is frequently disregarded (*Wirth 2010; Sun et al., 2017*).

The presence of Cd and its compounds in the environment is a consequence of both natural and anthropic processes. Natural sources of Cd include volcanic activity, weathering consumption of rocks, sea aerosols, forest fires and mobilization from soils and landfills, while anthropic sources include batteries, pigments, plastic stabilizers, pesticides and fertilizers, and photovoltaic devices, as well as spreading from rubber processing, galvanization process, fossil combustion and waste incineration (*de Angelis et al., 2017*). Among the male reproductive organs, testis is particularly susceptible to Cd poisoning. Indeed, it has been repeatedly shown, in experimental studies in animal models and human spermatozoa, that Cd is able to exert reproductive toxicity, mediated by multiple mechanisms, including structural damage to testis vasculature and

blood-testis barrier, inflammation, cytotoxicity on Sertoli and Leydig cells, oxidative stress mainly by means of mimicry and interference with essential trace elements, apoptosis, interference with selected signaling pathways and epigenetic changes of genes involved in the regulation of reproductive function, and disturbance of the hypothalamus-pituitary-gonadal axis (*de Angelis et al., 2017*) (Figure 3). Different epidemiological studies addressing the effect of environmental Cd exposure on semen quality reported conflicting results, although most of them have demonstrated a negative correlation between Cd burden and seminal parameters (*de Angelis et al., 2017*). Inconsistencies among studies might be addressed by discrepancies in cohort's selection, statistical drawbacks, and the inappropriate choice of the biological matrix for Cd burden quantification. The choice of the biological matrix or Cd burden quantification represents an interesting issue because the biological matrices commonly used for metal burden determination may not precisely reflect the real local exposure of the male reproductive tract, whereas semen certainly represent a more suitable substrate (*Mendiola et al., 2011; Oldereid et al., 1993; Minguez et al., 2012*); and, as largely demonstrated in different studies, heavy metals have a heterogeneous distribution within human body fluids and/or compartments, and particularly Cd, Pb, and Zn, display preferential accumulation in male reproductive organs (*Mendiola et al., 2011; Oldereid et al., 1993; Minguez et al., 2012*). A metanalysis of 20 case-control studies on heavy metal concentrations in semen from a total of 2.146 patients (1.538 for Cd, 832 for Pb, 1.029 for Zn, and 1.166 for Cu) with different fertility status, highlighted that significantly higher semen Pb and Cd, and lower semen Zn concentrations, are detected in patients with reduced fertility (*Sun et al., 2017)*. A huge contribution has been provided by animal studies in the identification of the specific Cd targets, and the characterization of the pathogenetic mechanisms underlying Cd reproductive toxicity. Nevertheless, conceivable differences in the susceptibility to adverse reproductive effects between humans and mammalian animals must be addressed; moreover, the precise correspondence between realistic human exposure levels and the experimental doses employed in animal studies remains to be fully established, by making it demanding to establish

a clear-cut safety reference value for semen quality and reproductive outcomes.

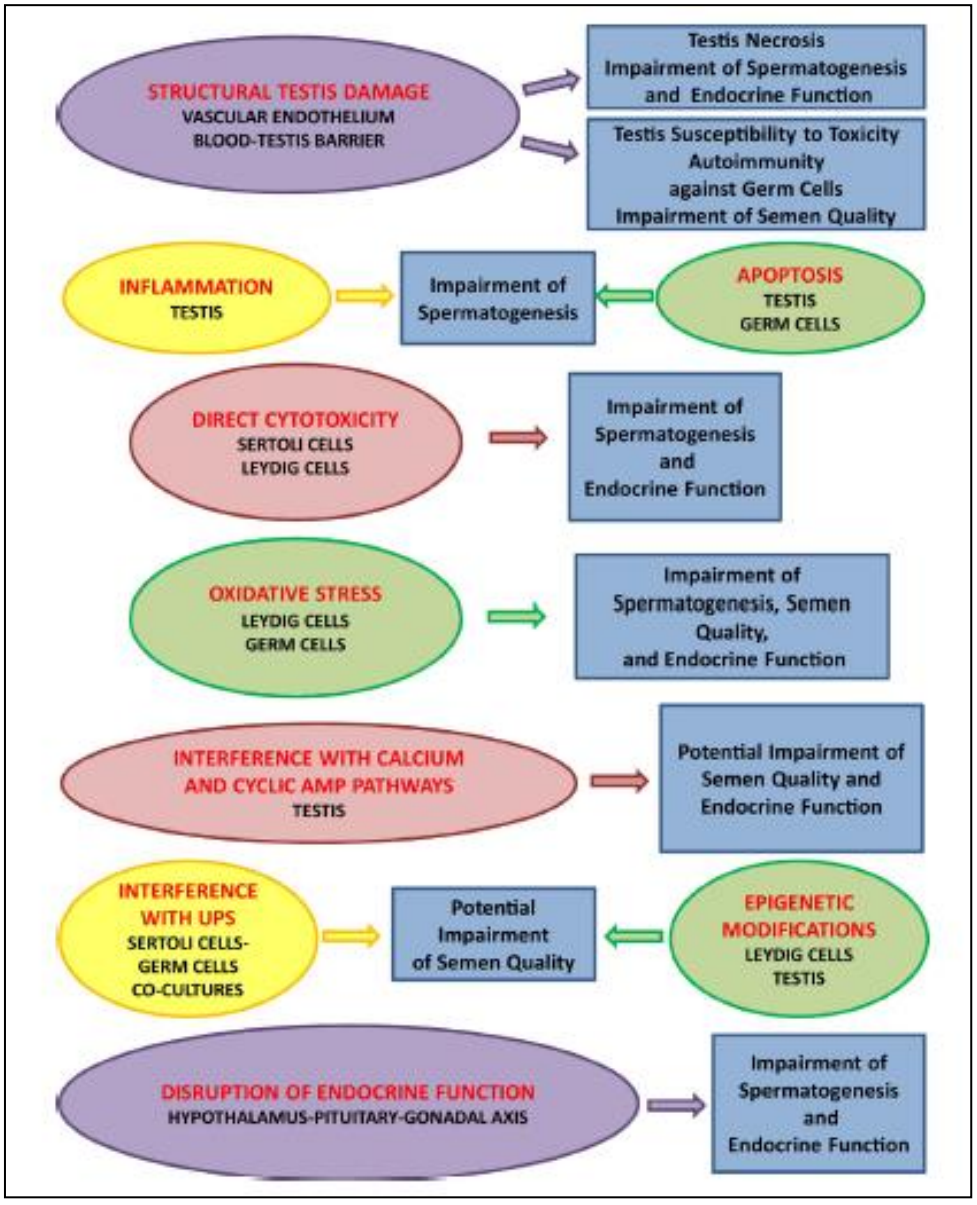

Figure 3: *Overview of the proposed pathogenetic mechanisms of cadmium reproductive toxicity. Colored circles report the main pathogenetic mechanisms of cadmium reproductive toxicity, along with the corresponding target organ or cell. The word "testis" refers to pathogenetic mechanisms demonstrated by experiments in vivo, or on whole testis homogenates. Light blue squares represent the proposed or hypothesized final effect on male reproductive function (de Angelis et al., 2017).*

1.3 The "Land of Fires" phenomenon

The Campania region has been the subject of great media interest in recent years, as well as intense scientific debate relating to the risk of harmful effects on human health related to exposure to environmental pollutants, due to the illegal disposal of urban, toxic and industrial waste, which includes several types of substances such as heavy metals, polychlorinated biphenyls, hydrocarbons etc. (*Triassi et al., 2015*); illegal burying of waste in inappropriates sites such as rural or agricultural areas, along with the diffuse practice of illegal waste burning, determined a significant increase in local environmental pressure (*Triassi et al., 2015*). Waste exposure exerts both short- and long-term health effects, which may include stress, anxiety, headache, dizziness, nausea, asthma, respiratory infections or irritation and congenital anomalies (short-term), as well as chronic respiratory and cardiovascular diseases, cancer, and even brain, nerves, liver, lympho-hematopoietic or kidneys diseases (*Triassi et al., 2015*). Growing media attention and pervasive concern among residents in regards of potential waste exposure-induced health problems led Italian authorities to establish emergency measures and to release a dedicated decree (L.n.6, February 2014), the "Land of Fires" (LF) Decree, issued with the main purposes of mapping contaminated sites and provide health screening of resident population, by also ensuring territorial remediation, and illegal waste disposal control within Campania Region. Ninety municipalities in the Province of Naples and Caserta are comprised within the LF area, which is characterized by high environmental pressure from both waste illegal disposal and high anthropic activity; despite a variety of studies attempted to address general health status of residents within the LF, just few investigations have been performed so far, concerning the male

reproductive function. Two studies demonstrated that traffic pollution exposure, particularly to nitrogen oxide and lead, was negatively correlated to sperm total count, total sperm motility, and sperm viability, and was associated to impaired sperm physiology, by exerting negative effects even at environmental concentration of nitrogen oxide below Italian legislation limits (*De Rosa et al., 2003; Boggia et al., 2009*). One study evaluating the relationship between the geochemical distribution of heavy metals in soils of the metropolitan area of Naples and human semen quality described a strong correlation between high Pb and antimony (Sb) concentrations in soil and poor seminal parameters, and a weaker correlation with high Hg and Zn, whereas other heavy metals in soil were not correlated with semen quality (*Giaccio et al., 2012*). In addition, a more recent pilot human biomonitoring study on trace elements (Zn, Cu, Cr, Fe) in blood and semen from a small cohort of men living in the LF, demonstrated significantly higher Zn, Cu, Cr and reduced iron (Fe) semen concentrations, increased percentage of immotile spermatozoa, higher sperm DNA fragmentation (SDF) index, and reduced redox biomarkers and antioxidant enzymes activity, in their semen, compared to individuals from a low environmental pressure area (*Bergamo et al., 2016*). No large investigations have been performed so far addressing semen quality of young men living within the LF, and the potential implication of heavy metals burden, quantitatively determined in a large number of semen samples.

CHAPTER 2

Semen parameters

2.1 Semen anlysis in human and animals (dogs and horse)

Semen analysis, or spermiogram, represents the starting point for a correct diagnostic and therapeutic classification of the infertile male. It is an operator-dependent examination, that is, the technician evaluates the number and characteristics of spermatozoa by means of microscopic observation. It is therefore essential that it must be carried out exclusively by experienced and specialized personnel, in order to reduce misleading results. Compared to human medicine, little is known in animal (as dogs and horse) medicine regarding specific findings on semen analysis and their correlation with fertility. Recommendation to optimize quality of semen analysis in veterinary practice include creating standardized protocols for evaluation of all seminal parameters and updating reference values where needed, eventually creating quality controls for the clinic laboratory. It is also necessary to inform owners that it is impossible to predict with 100% accuracy the fertilizing ability of animals with poor or those with excellent semen quality. Veterinary may perform seminal analysis as part of a complete animal evaluation thus verifying more accurately reproductive performances. Unfortunately, there are very few association studies between seminal parametes measured with standardized seminal analysis and issues like testicular function, fertilizing capability of spermatozoa, and likelihood that offspring will develop normally (*Amann 1981; England 1989*). Martinez et al. (*2004*) suggested that seminal analysis in the dogs can only be reliably predictive of fertility if the sperm quality is either very good or very bad. Results of tests that may be performed in a seminal analysis are influenced by sample collection technique and timing, concentration of spermatozoa in the sample, amount of time from sample collection to evaluation, temperature at which the sample was held, equipment used, and many other factors (*Rui et al., 1986; WHO 2010*). In humans, it is recommended that semen is collected after 2 to up to 7 days of sexual rest, and that two separate samples, collected 7–21 days apart, are evaluated before that any recommendations would be prescribed. There are no published guidelines for timing of semen collection in animals (relative to abstinence from sexual activity) as in humans; furthermore, the collection of animal semen takes place through manual ejaculation (*Kutzler 2005*).Currently, conventional seminal parameters, unlike those in human,

do not provide a reliable marker of fertility status in animals, mainly due to the lack of validated cut-off values and of a scientific evidence of a correspondence between seminal parameters and reproductive outcomes, namely, fertilizing capability, litter number and size, and offspring health status (*Martinez, 2004; Graham 1996*). The seminal parameters considered for analysis in both humans and animals are: pH, semen volume (ml), sperm concentration ($Nx10^6$/ml) sperm total count ($Nx10^6$/ejaculate), sperm motility (%) and normal sperm morphology (%). The first difference between human and animal is in the volume of the ejaculate, which changes in quantity according to the animal. A second difference concerns the fractions of the ejaculates; for example, in dog there are three fractions while in horse there are three fractions rich in spermatozoa and a fourth fraction consisting only of seminal fluid and gel, which is not used for analysis. In dog species the first (pre-sperm) fraction is small in volume and contains few to no spermatozoa; the second (sperm-rich) fraction comes from the epididymes and testes; the third (prostatic) fraction consists solely of prostatic fluid and also contains few to no spermatozoa. As regards the volume, it is not an indicator of semen quality in animals, while, in humans there is a cut-off for the volume parameter (1.5-6.8 ml), below/above which it is associated with specific andrological pathologies. Moreover, in humans there is only one fraction of ejaculated semen. Sperm concentration is inversely related to the volume collected (*Power 1963*), and it is a fuction of sperm total count in the ejaculate, which is obtained by multiplying sperm concentration for semen volume; sperm total count depends on testicular size (*Olar et al., 1983*), in humans, a normal value for this parameter is ≥39 million (WHO 2010), in dogs is ≥300 million (*Root Kustritz 2007*) and in horse ≥5 billion (*Viguer 1987*). Sperm concentration is traditionally assessed by using counting chamber such as Makler. The counting chamber Makler technique has been reported to be equally or more accurate than Computer-based automated semen analysis (CASA) systems and is considered the gold standard. For human and animal semen, motility is better maintained if samples are kept at room temperature than at body temperature (*BARTLETT 1962*); therefore, quick temperature fluctuations should be avoided. In humans the evaluation of sperm motility must be performed within 1 hour from collection. These indications are still lacking in the evaluation of animal sperm motility. The classification of

sperm motility in human and animal is the following: progressive motility, in situ sperm motility, and immotile spermatozoa. The reference cut-off for progressive motility (%) in humans, dogs and horses is as follows: ≥32%, ≥70% and ≥80% (*WHO 2010; Root Kustritz 2007; Viguer 1987).* The CASA systems also has been described for assessment of motility in animals (*Agarwal et al., 2003; Günzel et al., 1993; Rigau et al., 2001*). In humans, the CASA is only recommended for research purposes, while in animals it is also used for clinical evaluation due to the lack univoque guidelines. Human and animal spermatozoa comprise the following structures: head, which contains the genetic material and the acrosome; intermediate section and tail. The reference cut-off for normal sperm morphology (%) in humans, dogs and horses is as follows: ≥4%, ≥60% and ≥80% (*WHO 2010; Root Kustritz 2007; Viguer 1987)*. Sperm is a specialized cell in which chromatin is the main constituent, and its integrity is essential for successful fertilization and normal embryo development. SDF is the index used to evaluate chromatin integrity and it is inversely related to fertility. Unlike conventional semen analyses for quality assessment, such as sperm concentration, sperm motility and sperm morphology, SDF allows the evaluation of sperm genetic integrity; moreover, its rate is not necessarily linked to other sperm parameters. In fact, it has been observed that infertile men with normal semen may show a poor SDF index (*Agarwal et al., 2016*). Although structures such as the acrosome and flagellum are of great importance for spermatozoa functionality, the shape of the head, too, strongly influences their motility and fertilization ability. In a variety of mammal species it has been shown that sperm head morphometry is correlated with fertility (*Hirai et al., 2001; Ostermeier et al., 2001; Vicente-Fiel et al., 2014; Waheed et al., 2015*). Malo et al. (*2006*) affirmed that spermatozoa with more elongated heads may reach a higher swimming speed because they are more hydrodynamic. According to Yániz et al. (*2015*) morphometric analyses could be a useful predictive tool for semen fertility and storage, once the technique to perform them is standardized. Length, width, area and perimeter are the morphometric measures mainly used to objectively characterize sperm head shape.

Interestingly morphometric measures of spermatozoa is typical both in dogs and horses but not in humans. The morphometric measures has more value in the animal field given the high percentage of morphologically

normal forms of spermatozoa, whereas the human spermatogenesis is highly imperfect, for which morphometric studies have been abandoned over time.

CHAPTER 3
Epigenetics and Male fertility

3.1 What is epigenetics?

"The difference between genetics and epigenetics can probably be compared to the difference between writing and reading a book. Once a book is written, the text (the genes or DNA: stored information) will be the same in all the copies distributed. However, each individual reader of a given book may interpret the story slightly differently, with varying emotions and projections as they continue to unfold the chapters. In a very similar manner, epigenetics would allow different interpretations of a fixed template (the genetic code) and result in different read-outs, dependent upon the variable conditions under which this template is interrogated."
Thomas Jenuwein
(Max Plank institute of Immunobiology and Epigenetics, Freiburg, Germany)

The term "epigenetics" was first proposed by Conrad Waddington to describe the study of the processes by which the genetic information of an organism, defined as genotype, interacts with the environment in order to produce its observed traits, defined as phenotype (*Waddington 1942*). More recently, the term has been used to describe heritable changes in genome function that occur without a change in DNA sequence (*Holliday 1994*). These two definitions are closer than they seem. In eukaryotic cells, genomic DNA is packaged by histones and non-histone proteins into a dynamic polymer defined as chromatin. Several enzymes can modify the architecture and the composition of chromatin, both locally and globally, and they can direct the inheritance of local chromatin structures through cell division (*Turner 1993; Jenuwein 2001*). Thus, an individual's cells all share the same linear sequence of DNA nucleotides, the genome, but different cell types are characterized by the presence of different chromatin conformations of this genome, the epigenomes, that specify the characteristic functions of each cell type and allow the maintenance of the memory of these functions through cell division. Indeed, a large set of genome regulatory processes involve epigenetic determination and inheritance.
Epigenetic modifications are covalent modifications such as methylation, acetylation, phosphorylation and ubiquitination, present on the DNA itself,

or on associated proteins (such as histones in somatic cells and protamines in male germ cells), which modify gene expression, without alter its sequence (*Carrell 2010*).

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that transfer a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5mC (Figure 4). Dnmt3a and Dnmt3b can establish a new methylation pattern to unmodified DNA and are thus known as de novo Dnmt (Figure 4a). On the other hand, Dnmt1 functions during DNA replication to copy the DNA methylation pattern from the parental DNA strand onto the newly synthesized daughter strand (Figure 4b). All three Dnmts are extensively involved in the development of an embryo. By the time cells reach terminal differentiation, Dnmt expression is much reduced. This would seem to suggest that the DNA methylation pattern in postmitotic cells is stable. However, postmitotic neurons in the mature mammalian brain still express substantial levels of Dnmts, raising the possibility that Dnmts and DNA methylation may play a novel role in the brain (*Goto et al., 1994; Feng et al., 2005*).

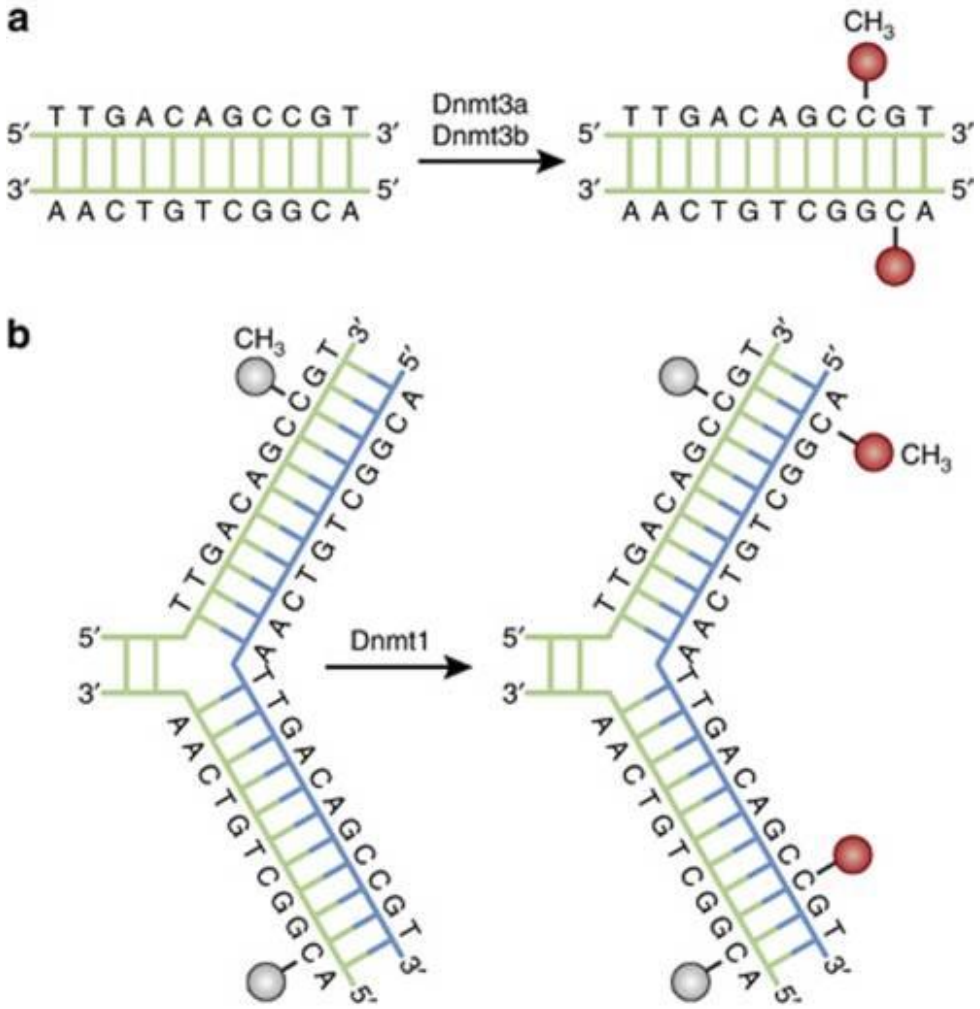

Figure 4: *DNA methylation pathways. A family of DNA methyltransferases (Dnmts) catalyzes the transfer of a methyl group from S-adenyl methionine (SAM) to the fifth carbon of cytosine residue to form 5-methylcytosine (5mC). (a) Dnmt3a and Dnmt3b are the de novo Dnmts and transfer methyl groups (red) onto naked DNA. (b) Dnmt1 is the maintenance Dnmt and maintains DNA methylation pattern during replication. When DNA*

undergoes semiconservative replication, the parental DNA stand retains the original DNA methylation pattern (gray). Dnmt1 associates at the replication foci and precisely replicates the original DNA methylation pattern by adding methyl groups (red) onto the newly formed daughter strand (blue) (*Moore et al., 2013).*

3.2 Methylation and male fertility

Early evidence for a link between epigenetic markers and male fertility was provided by studies in rodent models, which revealed that exposure to 5-azacytidine triggers a dose-dependent DNA hypomethylation in spermatozoa (*Egger et al., 2004*). Moreover, rats treated with 5-aza-2'-deoxycytidine showed impaired testicular histology, reduced sperm counts and infertility (*Doerksen 1996*). DNA methylation is an epigenetic modification consisting in changes, sometimes heritable, influencing gene expression that not causes changes in DNA sequence (*Holliday 1987*). Epigenetic changes not only have an impact on developmental processes and fetal growth but are also relevant in many different areas of biology and medicine, including cancer, aging, and environmental toxicology (*Herceg 2011; Liu 2011*). DNA methylationn is one of the most important epigenetic mechanisms called into question in numerous biological functions including spermatogenesis. As regard spermatogenesis, epigenetic changes are involved in the proper arrangement and maintenance of sperm genome and exert crucial effects on sperm quality and function and fertilization potential. A complex and precise epigenetic reprogramming takes place starting from germ cells during migration to the genital ridge and is essential for spermatogenesis completion (*Kelsey 2013; Seisenberger et al., 2013*). In humans, alteration of both sperm global DNA methylation (SGDM) and gene-specific (i.e. H19, MEST, BRDT, MTHFR) DNA methylation patterns have been related to poor quality semen, impaired seminal parameters, azoospermia and reduced fertility (*Boissonnas et al., 2010; Hammoud et al., 2010; Kobayashi et al., 2007; Marques et al., 2008; Minor et al., 2011; Poplinski et al., 2010*). Houshdaran et al. (*2007*) reported that poor semen quality samples displayed an abnormal global DNA sperm hyper-methylation, as assessed by the analysis of repetitive elements, and locus-specific sperm DNA at imprinted and non-imprinted

genes. Conversely, the majority of studies showed an abnormally reduced sperm global DNA methylation in poor semen quality samples. Most studies demonstrated that SGDM level is associated not only with sperm concentration but also with sperm motility: oligozoospermic and severely asthenozoospermic men have significantly lower levels of SGDM, compared to normozoospermic men or men with moderately impaired motility (*Marques et al., 2004; Montjean et al., 2015; Pacheco et al., 2011*). The first association between methylation levels and infertility was reported by Benchaib et al. (*2005*), who demonstrated that SGDM levels above an arbitrary threshold were seemingly linked to high pregnancy rates, suggesting that SGDM status independently affects embryogenesis. Urdinguio et al. (*2015*) showed significant differences in SGDM levels between fertile and unexplained infertile patients, with significantly lower methylation levels in spermatozoa from infertile individuals. In another study on 141 semen samples used for assisted reproductive technologies, El Hajj et al. (*2011*) found a significantly lower methylation level in semen samples resulting in abortions, compared to those leading to a delivery. Independently from the cause and the time of occurrence of the epigenetic alterations (in utero vs prepubertal vs adulthood), it has been demonstrated that sperm epigenetic landscape has transgenerational effects and is likely influential in the developing embryo. Indeed, mature sperm provide epigenetic marks that drive the activation/inactivation of specific genes by contributing to the pluripotency of the embryonic cells and by influencing its future adult health status, including fertility and reproductive disorders (*Kobayashi et al., 2007; Poplinski et al., 2010; Marques et al., 2004*). All these studies support the hypothesis that sperm DNA methylation pattern of both imprinted and non-imprinted genes is essential for normal sperm function, fertility, and embryo development. However, the etiology and whether DNA methylation errors are acquired during fetal or early post-natal development are still unresolved questions. An improved knowledge of sperm epigenetics is not only necessary to understand the physiology of reproduction, but also to provide clues on the potential causes of male infertility of unknown origin.

3.3 Methylation and dog male fertility

Reproduction and fertility are the most important traits in animals domestic, therefore a lot of studies are aimed to identify the causes of reproduction failure in these species. Among the most investigated fields there are the disorders of sex development (Albarella *et al., 2019; Albarella et al., 2020*) and reproductive performances (*Fuente-Lara et al. 2019*). As regards dog specie, the occurrence of male infertility is an emerging problem and the knowledge of underlying causes is the first step to identify new diagnostic and therapeutic strategies. To date, the main analyses used to evaluate semen quality in domestic animals might be classified as qualitative (semen volume, aspect, viscosity and pH) and quantitative (sperm concentration/count, motility, morphology, vitality, DNA fragmentation, morphometric) (*Root Kustritz 2007*). However, it has been observed that, in humans, infertility may occur even in the presence of parameters within the normal range, and it has been proven that SGDM is related to human fertility rate independently from other seminal parameters, such as sperm total count, progressive motility and morphology (*Urdinguio et al., 2015*). Furthermore, the use of animal models (such as the mouse) has made us lose sight of the relationships that exist between the environment and organisms and between the various parts of an organism, ignoring the individual as a whole. The animals used for the experiments are artificially selected, kept in cages, without those stimuli necessary to develop their potential self-defense. Thus, the laboratory animal does not correspond to the one that lives in its natural environment and all the experiments done on it are not even extrapolable to other animals of the same species, which live in a normal space-time context. Dog is considered as an advantageous model of study for human biology and disease (*Ostrander 1997*). Indeed, dogs generally cohabitate with their human owners, therefore representing a valuable model for the study of environmental factors on health, and receive medical care from them almost like a human; lastly, the use of pet animals reduce the requirement of experimental animals (*Tsai et al., 2007*), in fact, by using non invasive and harmeless methods for the analysis of owned dog we may have an indication of the effect that enviroment could have on people. An improved knowledge of sperm epigenetics is not only necessary to

understand the physiology of reproduction, but also to provide clues on the potential causes of male infertility of unknown origin. The total absence of studies on SGDM related to seminal parameters in dogs, and the relationship with fertility dogs, raises a series of questions but also numerous research opportunities on this topic.

CHAPTER 4
Nutraceuticals and male fertility

4.1 Nutraceuticals and male fertility

Nutraceuticals are food elements that that can provide medical or health benefits by potentially contributing to prevent the occurrence of several diseases by supporting physiological functions, and are therefore widely marketed as ingredients of dietary supplements (*EUR-Lex 2020*). A large number of recent studies have focused on the ability of nutraceuticals to improve the hormonal status and sperm parameters by different mechanisms (*Falsig et al., 2019*).

The improvement of semen quality and pregnancy rate by feed supplementation has been much disregarded in male livestock, in comparison to females. Nevertheless, the success of artificial insemination (AI) also depends on the livestock animals' semen production. On the other hand, removing bulls with insufficient sperm number or capability to fertilise from commercial semen production is costly and often results in the loss of interesting genotypes and animals with high breeding value. Kastelic and Thundathil (*2008*) stated that, in an unselected population, 20–40% of bulls are likely to have a reduced fertility due to impaired semen quality. Feed supplementation might offer benefits in the treatment of these male animals improving the abnormal semen. Indeed, nutritional status is of primary importance in determining semen quantity and quality (*Brown, 1994, Petherick, 2005, Robinson et al., 2006, Martin et al., 2010*). In adult animal conditions of under-nutrition, malnutrition and nutrient imbalances, may induce reduced androgen secretion and low semen quality (*Brown, 1994, Petherick, 2005*). In these last cases the approache to feed supplementation would consist of complementing animal diets with the lacking nutrient or energy (*Brown, 1994, Almeida et al., 2007*). Such improvements would help to avoid nutritionally-caused, but often reversible, sub- or infertility.

Currently, nutraceuticals -based dietary supplements are widely prescribed to improve physiological aspects related to male fertility, also in humans. Many dietary supplements are available on the market with various formulations, containing both nutrients and botanicals at different doses; despite many research studies demonstrating positive effects of some ingredients on semen parameters and fertility outcomes, many others have shown lack of efficacy of dietary supplements, mainly due to an extremely

heterogeneous formulation, containing up to a conspicuous number of different active ingredients reported to exert beneficial effects or with ineffective/unreported effects, and at variable concentrations often below the minimal effective daily dose (*Garolla et al., 2020*). In a recent position statement, the Italian Society of Andrology and Sexual Medicine (SIAMS) summarized the state of the art on each single ingredient currently used in the andrological field (*Calogero et al., 2017*). In particular, zinc, folic acid, N-acetylcysteine, coenzyme Q10, vitamins E and C, selenium, carnitines, and pentoxifylline, in various dosage and variable combinations have been demonstrated to efficiently improve seminal parameters in human (*Calogero et al., 2017*) (Table 1), as well as in several animal species (*Pereira et al., 2020; Pitel et al., 2020; Kendallet al., 2000; Aliarabi et al., 2019*). The most part of nutraceuticals currently used are antioxidant compounds supporting spermatogenesis by contributing to maintain a physiological balance between reactive oxygen species (ROS) and ROS scavenger activity. Indeed, oxidative stress (OS) has been widely recognized as one of the most common factors contributing to male infertility (*Hemingway, 2003*); OS is defined as the imbalance between the production of ROS and antioxidant activity (*Hemingway, 2003*; *Agarwal, 2010; Sikka, 2001*), and may occur within the male reproductive tract as a consequence of urogenital or systemic disorders, and exposure to environmental and lifestyle factors (*Hemingway, 2003*). Excessive ROS production determines spermatozoa lipid peroxidation, and consequent reduction of membrane fluidity, resulting in the inhibition of mechanisms required for fertilization, and DNA oxidative damage, including SDF, base modification, deletions, frame shifts, DNA crosslinks and chromosomal aberrations (*Hemingway, 2003*). Antioxidants have been increasingly introduced among the potential medical treatments for male infertility in the last decades (*Lanzafame et al., 2009*); they are generally recommended, without a specific preference concerning the bioactive compound, only after performing accurate diagnostic workup of infertility, in male with idiopathic infertility and documented alterations of seminal parameters and increased SDF, a marker of ROS-mediated DNA damage (*Calogero et al., 2017; Agarwal et al., 2019*). The high costs associated with assisted reproductive techniques for male infertility have led consumers to find less expensive alternatives for potential treatment. An attractive option has been pointed out,

concerning plants rich in antioxidant active components. Many studies have shown that using maca (*Lepidium meyenii*) and khat (*Catha edulis*) positively affect sperm production and semen quality in animals. Some evidence points to favourable effects of leucaena (Le*ucaena leucocephala and Leucaena pallida*), sesbania (*Sesbania sesban*), pomegranate (*Punica granatum*), tomato (*Solanum lycopersicum*) and amaranth (*Amaranthus hypochondriacus*) as well, but studies are either superficial or provide partially contradictory results (*Clément et al., 2012*). Taking together the available studies, increasing attention both in the public forum and in the scientific community, and increasing bodies of evidence are developing for some plants with beneficial effects on health (nutriceuticals); nevertheless, even though there are many claims of positive effects on semen quality, very few nutraceuticals have been subjected to rigorous scientific investigation and the promising effects can be deducted only from indirect indications (*Clément et al., 2012*).

Nutraceuticals	**Evidence**	**Possible indications**
Ascorbic acid (vitamin C)	Its administration positively correlates with sperm count and motility, and negatively with sperm DNA fragmentation index	OAT
Carnitine	Improves concentration, total sperm count, and progressive motility	OAT
Carotenoids (lycopene, β-carotene)	Improve sperm parameters	OAT
Coenzyme Q10	Improves sperm count, motility, and morphology [	OAT
Myoinositol	Improves sperm progressive and total motility and the mitochondrial function; increases the number of sperm recovered after swim-up technique	OAT

Nutraceuticals	Evidence	Possible indications
Glutathione	Improves sperm concentration, motility, and morphology	OAT
N-acetyl-cysteine	Improves sperm volume, viscosity] and motility. Prevents oxidative DNA damage	OAT
α-Tocopherol (vitamin E)	Increases sperm motility	OAT

Table 1: *Main nutraceuticals used for the treatment of male infertility* (*Calogero et al., 2017).*

4.2 Maca and male fertility

Reproduction and fertility are main concerns in horse breeding (*Albarella et al., 2018*) and the discovery of new plant-based food supplements that are safe, economically valid and able to improve these parameters is an aim both in human and livestock has. As antioxidants have a positive effect on semen storage and maintenance of the functionality of spermatozoa (*Del Prete et al., 2019*) close attention has been paid to plants that are rich in them, such as maca (*Lepidium meyenii*). This is a Peruvian plant, the hypocotyl of which has been used for centuries in the Andes for nutrition and fertility enhancement in humans and animals (*Gonzales, 2012; Tafuri et al., 2019a*). As regard semen characteristics it has been proven that the main effects of an oral supplementation with maca are: increased sperm total count and motility, improved SDF index, a better quality of semen after storage at 5°C up to 72 h (*Clément et al., 2012; Del Prete et al., 2018a*). All these beneficial effects are probably due to the antioxidant–oxidant balance induced by macamides and the lipid-extractable fraction of maca with an unknown mechanism of action (*Melnikovova et al., 2015; Tafuri et al., 2019b*). Although pregnancy rates are the preferable endpoint to evaluate the fertilizing capacity of a semen in human and animals, a lot of techniques have been used to assess the quality of fresh and cooled semen

(*Casey et al., 1997; Pauciullo et al., 2012; Yániz et al., 2015; Agarwal et al., 2016; Del Prete et al., 2018b*).

CHAPTER 5

Aim of the study

The general aim of the current research study was to determine the overall impact of the environment on semen quality and male fertility potential, by particularly addressing the effects of both environmental pollution and lifestyle components, and the contribution of genetic and epigenetic changes to such reproductive effects. Moreover, a further general aim of the current research was to put an effort in identifying potentially suitable animal species, as surrogate models to address environmental pollution and lifestyle impacts and the effects of natural countermeasures in the prevention of reproductive disorders.

Specific aims included the assessment of the potential epigenetic changes induced by heavy metals exposure, particularly cadmium, which represents an ideal toxicant when trying to determine the impact of both environmental and lifestyle burden of exposure, due to widespread presence in the environment and, in particular cigarette smoke. Moreover, a correlation between epigenetic changes and semen quality has been performed. The human-dog model represents an interesting study model, as the close relationship between owner and animal implies that the two elements of the couple share common environmental effects and are, therefore, plausibly exposed to a set of common pollutants/environmental factors.

Lastly, a further specific aim was to assess the effects of healthy lifestyle, particularly the supplementation with nutraceuticals, on semen quality, in order to identify natural substances as alternative treatments for seminal impairment in animals. For this reason a horse model was used, by determining the effect of food supplementation with a plant rich in antioxidant substances: maca (Lepidium meyenii) on seminal parameters of Italian thoroughbred stallions. In particular both conventional semen analyses, and non-conventional endpoints, such as SDF index and spermatozoa morphometric measures, has been used.

CHAPTER 6

Epigenetic and environment in male fertility

6.1 Participants and methods

6.1.1 Recruitment and enrolment of participants (humans and dogs)

The research study was carried out upon approval of the Ethical Committee of "Federico II" University of Naples, and included Caucasian healthy males of reproductive age (13-50 years old) resident in Campania Region. The study included two different cohorts of subjects, recruited within two awareness and prevention campaigns on infertility and testis cancer in high environmental impact (HI) areas, identified on the basis of the Campania Region Environmental Protection Agency (ARPAC) reports (*Arpac, 2016*) (Figure 5). Awareness campaigns were promoted by Unità di Andrologia e Medicina della Riproduzione e della Sessualità Maschile e Femminile (FERTISEXCARES) – Azienda Ospedaliera Universitaria "Federico II" of Naples by publication of the initiative on the official website of the FERTISEXCARES centre, by social media networks, and by locally distributed flyers. The clinical diagnostic investigations on dogs were carried out in collaboration with the Laboratory of Veterinary Genetics and Biotechnology applied to Animal Productions (GENENVET) of the University Federico II of Naples held at the University Veterinary Didactic Hospital (OVUD) of the Department of Veterinary Medicine and Animal Production (MVPA). Human subjects from both the awareness campaign attending to FERTISEXCARES centre had to meet the following inclusion criteria to be eligible for enrolment in the study cohorts: residence for at least 10 years in Campania Region; no known chronic diseases (diabetes, endocrine disease, other systemic diseases, fertility-related genetic disorders); no reported history of drug abuse. No "a priori" selection based on the presence/absence of infertility and/or andrological disorders was applied as a criterion for participants enrolment.

Among males approaching to the first awareness campaign, 730 participants were enrolled; study cohort (C1) consisted of 544 healthy males aged 13-33 years resident in a HI area comprising municipalities within the Province of Naples and Caserta belonging to the LF, officially recognized by the ARPAC as the Campania Region area with the highest concentration of illegal disposal sites of toxic waste, and also characterized by frequent illegal and uncontrolled waste burning (*Turner, 1993*), and 186

healthy males aged 17-33 years resident in a low environmental impact (LI) area comprising municipalities not belonging to the LF, hereinafter referred to as “Other Areas”. The relative geographical distribution within Campania Region of residential municipalities of enrolled participants, as well as the density of enrolments at each residential municipality (for both HI and LI groups), are depicted in Figure 6.

Within the second awareness campaign 512 participants were enrolled; study cohort (C2) consisted of healthy males aged 14-50 years resident in the HI municipalities of Acerra (N=197), Afragola (N=117), and Giugliano in Campania (N=162), belonging to the LF. Written informed consent was obtained from all participants attending to FERTISEXCARES centre enrolled into the study. Upon enrolment, a progressive code number was assigned to each participant by the recruiting andrologist; the examining biologist performed semen analysis blinded to participant identity, residential municipality, and clinical characteristics. All subjects in C1 were offered a diagnostic clinical examination in their domestic dogs. The dogs recruited for the study had to be clinically healthy and with at least one litter in the last 12 months (Table 2). To exclude external factors affecting DNA methylation, only dogs housed under standard conditions and not taking drugs were included in the study. The absence of adhesion by the dog owners, the selection made based on the state of fertility and with at least one litter, allowed to recruit only 30 dogs for the study.

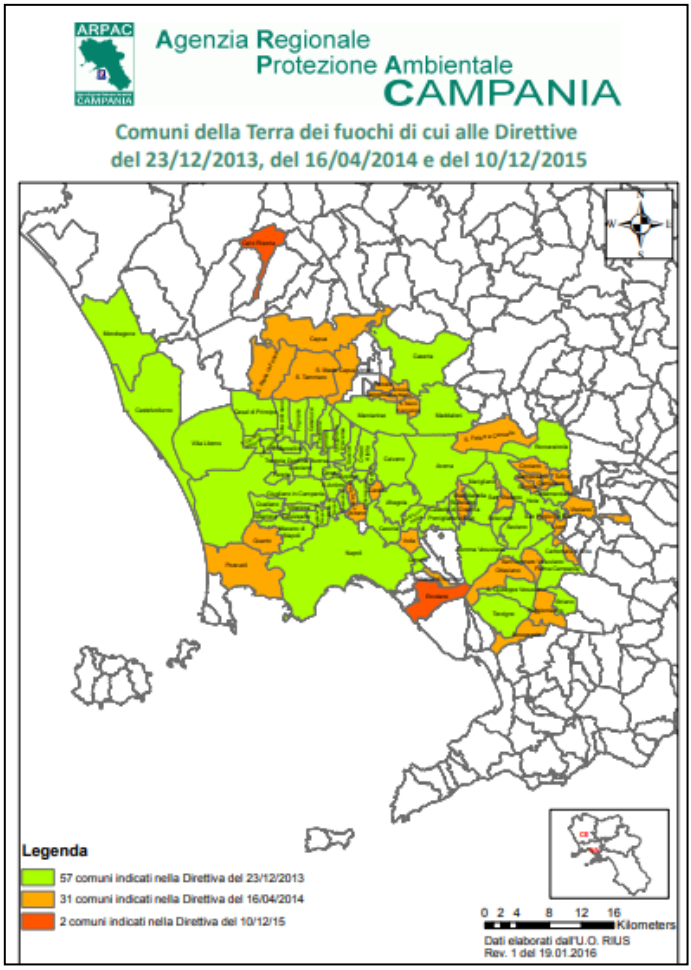

Figure 5: *Map depicting the 90 municipalities of the province of Naples and Caserta belonging to the "Land of Fires", an area officially recognized as a high environmental impact area on the basis of the Campania Region Environmental Protection Agency reports (2013-2014-2015), identifying the "Land of Fires" as the Campania Region area with the highest concentration of illegal disposal sites of toxic waste, and also characterized by frequent uncontrolled waste incineration.*

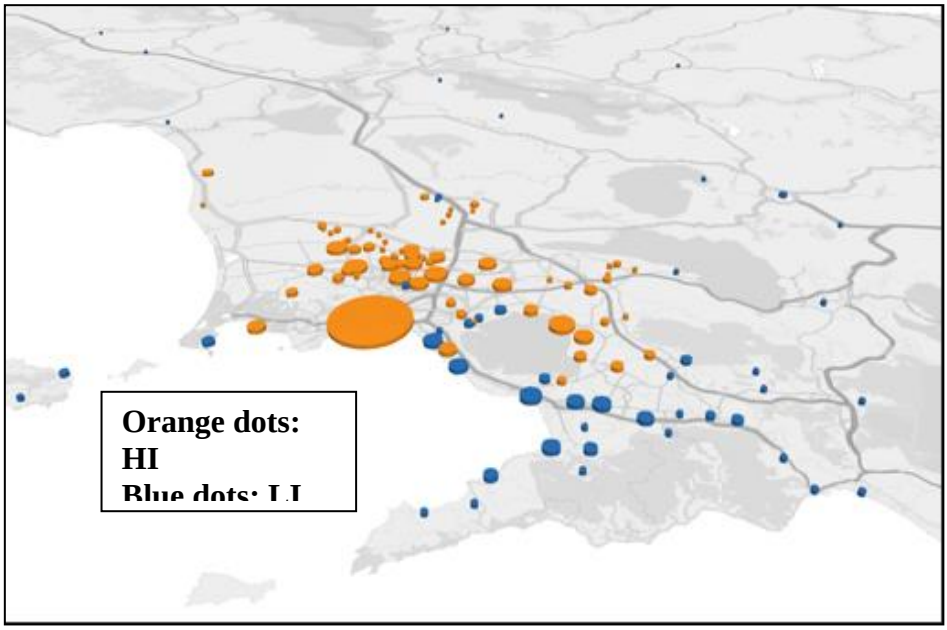

Figure 6: *Relative geographical distribution of research study participants residential municipalities within Campania Region, referred to the entire C1 cohort: 544 males resident in a high environmental impact (HI) area comprising municipalities within the Province of Naples and Caserta belonging to the so-called "Land of Fires" (LF), and of 186 males resident in a low environmental impact (LI) area comprising municipalities not belonging to the LF. Municipalities belonging to a HI area are displayed as orange dots, municipalities belonging to a LI area are displayed as blue dots. Dots radius is proportional to the density of enrolments at each residential municipality.*

Breed	N° of animals	Age ranges (Y)
Neapolitan Mastiff	8	2-4
German shepherd	5	1-6
English Bull dog	4	2-5
Dachshund	2	2-9
Beagle	1	8
Caucasian Shepherd dog	1	4
Maremmana Sheepdog	1	5
Pointer	1	3
Kangal	1	2
Labrador retriever	1	2
Half-breed	5	2-8

Table 2: *Breed and age of the analyzed dogs*

6.1.2 Clinical procedures and blood collection (human and dogs)

Participants were interviewed on medical history and underwent a complete physical examination including evaluation of anthropometric characteristics, weight, height, body mass index (BMI), urogenital examination, and scrotal ultrasonography (US); C1 also had prostate transrectal US (TRUS) performed. Peripheral venous blood samples were drawn by venipuncture using stainless steel needles and collected into tubes containing ethylene diamine tetra-acetic acid (EDTA), and tubes containing clot activator with separating gel, for whole blood and serum preparation, respectively, for routine blood tests and hormone profile assessment by chemiluminescence immunoassay (CLIA) [follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), total testosterone (TT), estradiol (E2), and sex hormone-binding globulin (SHBG)], in C1; trace elements quantitative determination but not hormone profile assessment was performed in serum, in C2.
For each dogs the following data were collected: anamnesis, clinical findings (general physical examination and objective examination of the reproductive apparatus) and instrumental examinations (ultrasound and radiography); blood samples for genetic and biochemical analysis; past surgical procedures, when present. The anamnesis data collected,

concerned physiological and pathological aspects of the single dog and family members such as sex, age, lifestyle (home, garden, sports), diet, present and / or past pathologies. With the general physical examination (EOG) constitution and skeletal development were evaluated; nutrition status (BCS) and muscle tone; state of the sensory; signs and attitudes; skin and subcutaneous connective tissue; explorable lymph nodes; apparent mucous membranes; body temperature; arterial pulse; breath; great organic functions. With the physical examination of the reproductive system (EOP), the reproductive organs were evaluated both by inspection and palpation and with the aid of diagnostic tools such as ultrasound.

6.1.3 Semen analysis in humans

Semen samples were collected on-site by masturbation directly into a sterile plastic container after 3–5 days of sexual abstinence. Semen was analyzed according to the 2010 World Health Organization laboratory manual guidelines (*WHO, 2010*). After collection, ejaculates were left to liquefy for 30'at 37°C. The following seminal parameters were considered for analysis: pH, semen volume (ml), sperm concentration (n $x10^6$/ml) sperm total count (n $x10^6$/ejaculate), total sperm motility (%), progressive sperm motility (%), normal sperm morphology (%) and abnormal sperm morphology (%). Sperm count and motility were evaluated with the Makler Counting Chamber; sperm morphology was evaluated at the optical microscope (100X) by Giemsa staining. WHO 2010 criteria for normozoospermia are as follow: sperm total count ≥ 39 $x10^6$/ejaculate or sperm concentration ≥15 $x10^6$/ml; progressive sperm motility ≥ 32% or total sperm motility ≥ 40%; normal sperm morphology ≥ 4% (*WHO, 2010*). In cases of azoospermia (absence of spermatozoa in the ejaculate), the analysis was repeated twice, and the diagnosis was made after evaluation of the entire post-centrifuge semen sample pellet. Factors potentially affecting semen quality (fever, medications, exposure to X rays etc.) were taken into account. Aliquots of semen were stored in metal-free tubes at -80°C for trace elements quantitative determination by ICP-MS, in C2.

6.1.4 Semen analysis in dogs

The semen was collected after 3 days of abstinence, obtained by a previous controlled manual manipulation performed as described by Kutzler (*2005*). Dog semen is ejaculated in three fractions. The first (pre-sperm) fraction is small in volume and contains few to no spermatozoa. The second (sperm-rich) fraction comes from the epididymes and testes. The third (prostatic) fraction consists solely of prostatic fluid and also contains few to no spermatozoa. The first and second fractions of the ejaculate were collected in the same conical tube, whereas the third fraction was collected in another tube using glass funnels. Semen quality was evaluated in the combined first and second fractions. Volume (ml) was determined using a graduated tube. Sperm count were evaluated with the Makler Counting Chamber; sperm motility was visually assessed under a phase contrast microscope (Nikon Eclipse 80i) at ×200 magnification; sperm morphology was evaluated at the optical microscope (x1000) by Giemsa staining. For the assessment of sperm motility, 10 µL of semen were pipetted onto a clean glass slide with a positive displacement pipette and the drop covered with a 22 mm × 22 mm coverslip; at least five fields were evaluated to classify at least 200 spermatozoa. Sperm concentration ($x10^6$/ml), followed by calculation of the sperm total count (sperm concentration × semen volume), and percentage of motile spermatozoa (%) were determined according to the WHO guidelines and procedures by classifying the spermatozoa in progressive motility, in situ sperm motility, and immotility based (*WHO, 2010*). We categorized dogs as normozospermic considering cut off of sperm count is greater than 300 x 10^6/ejaculate, the percentage of progressively motile spermatozoa is 70% or greater and the percentage of morphologically normal spermatozoa is 60% or greater based on dog's literature indexes (*Root Kustritz, 2007*). Dogs are defined Oligozoospermic when the sperm count is <300 x 10^6/ejaculate, Asthenozoospermic when percentage of progressively motile spermatozoa is <70% and Teratozoospermia when percentage of morphologically normal spermatozoa is <60% (*Root Kustritz, 2007*).

6.1.5 Trace elements analysis in humans

Quantitative determination of trace elements in serum and semen included lithium (Li), beryllium (Be), aluminum (Al), vanadium (V), chromium (Cr), manganese (Mn), Iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), rubidium (Rb), strontium (Sr), cadmium (Cd), tin (Sn), antimony (Sb), tellurium (Te), barium (Ba), lead (Pb), and uranium (U); mercury (Hg) was also determined in serum. Briefly, 500 µl of serum and/or semen sample were transferred into glass tubes and 1 ml of ≥ 69%, (v/v) nitric acid ($HNO_{3)}$ (TraceSELECT®) was added. Sealed tubes were subjected to sample acid oxidative digestions using an automated microwave digestion system (DISCOVER SP-D; CEM) and the following protocol: from room temperature (RT) to 160°C ramp time 3'; constant temperature 160°C hold time 2'; from 160°C to 80°C cooling time 2'; from 80°C to RT with no auxiliary cooling control. Once at RT, 2% (v/v) HNO_3 was added to the mix to final volume 10 ml. Trace element analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) (Aurora M90; Bruker), and elemental concentration was determined by comparison to certified standard solutions calibration curve; final concentrations were reported ad µg/l. The limits of detection (LoD) and limits of quantification (LoQ) were calculated as the blank signal plus three or ten times its standard deviation, respectively (*Curri, 1999*). LoD for trace elements of interest were as follow: Li 0,4; Be 2,6; Al 6,4; V 1,8; Cr 0,8; Mn 0,4; Fe 3,4; Co 0,4; Ni 4,2; Cu 4,4; Zn 1,8; As 0,2; Se 1,1; Rb 3,0; Sr 0,2; Cd 0,2; Sn 0,2; Sb 7,0; Te 3,0; Ba 6,6; Pb 0,1; U 0,2. Recovery was calculated to be within the range 70-120%.

6.1.6 Leukocytes depletion and spermatozoa purification and DNA extraction (humans and dogs)

Semen samples were processed with Dynabeads® CD45 magnetic beads (Invitrogen) in order to obtain purified spermatozoa samples, free from contaminating leukocytes. Thereafter, spermatozoa were separated from somatic cells using discontinuous two-layer (40:80: vol./vol.) density gradient (PureSperm) (Nidacon International AB, Molndal, Sweden). After centrifugation for 30 minutes at 300 g the spermatozoa pellet was

collected and washed twice with phosphate-buffered saline (PBS). Purified spermatozoa were subsequently used for DNA extraction.
Each spermatozoa sample was washed twice in 10 ml of Wash Buffer containing 150mM NaCl and 10 mM EDTA (ph 8.0) in DNAse and RNAse free water, and centrifuged at 750 x g for 10 minutes. Subsequently, samples were incubated for 2 hours in water bath at 56 °C in DNA Extraction Buffer (4,24 M guanidine thiocyanate, 100 mM NaCl, 1% Sarkosyl, 150 mM DTT, and 200 µg/ml proteinase K in DNAse and RNAse free water). DNA was precipitated in isopropanol, spooled with a U-shaped Pasteur pipette and transferred to a tube containing Sodium Citrate in 10% EtOH. DNA was then washed twice in 70% EtOH. DNA samples were resuspended in 500 µl of 10 mM Tris-HCl pH 8.0 and stored at 4° C until use (Carrell, 2013).

6.1.7 5-mC DNA ELISA (humans and dogs)

SGDM was evaluated by using an EZ DNA Methylation™ Kit, (Zymo research) according to manufacturer instructions. Briefly, the percentage of 5-Methylcytosine (% 5-mc), a surrogate marker of SGDM, was evaluated in each DNA sample by loading 100 ng of denatured, single-stranded DNA in a 96-well plate coated with an anti-5-mc monoclonal antibody and the HRP-conjugated secondary antibody. Detection of % 5-mc was performed after addition of the HRP developer and quantitation was performed by reading absorbance at 405-450 nm using an ELISA plate reader and the logarithmic equation of the line from the standard curve that was constructed with negative and positive controls and standards with known % 5-mc. Each DNA sample was assessed in duplicate.

6.1.8 Head area analysis and determination of SDF in dogs

At least 200 spermatozoa (50 per slide) from each ejaculate were observed in a bright field under a Nikon Eclipse 80i microscope (100x), captured with a digital camera (Nikon DS-Ri1) and analyzed with the software Nis Elements Imaging Software 4.00.02 (Nikon) for head area measurement.
SDF was evaluated by Halosperm® kit (Halotech® DNA SL, Madrid, Spain), according to manufacturer instructions. The slide was left to dry at room

temperature and therefore stained for direct microscopic observation under light microscopy. A minimum of 500 spermatozoa per sample were analyzed and scored.

6.1.9 Statistical analysis

Statistical analyses were performed with GraphPad Prism 7.0 (La Jolla, CA) and SPSS 22.0 [SPSS, Chicago, IL, USA] softwares. Descriptive analysis included calculating the mean ± standard deviation (SD) or median with 5°-95° percentile range. Distribution of continuous variables was assessed by D'Agostino & Pearson normality test. Independent groups were compared using parametric t-test/ANOVA or non-parametric Mann-Whitney/Kruskal-Wallis test for continuous variables. Ordered categorical variables (residential address, smoking habits) were analyzed in contingency tables, and Chi-squared or Fisher's exact tests were run to assess differences in the prevalence of pathological seminal parameters between groups. Participants were dichotomized on the basis of semen quality as per WHO 2010 reference cut-off values described above (*WHO, 2010*); normozoospermic participants who had values equal or greater than the reference cut-off value for all seminal parameters were considered as the comparison group. Pearson or Spearman correlation test was applied to determine the linear relationship between two continuous variables or between ranked values of ordinal variables, respectively. Serum and semen concentrations of trace elements were often below the LoD, therefore, these outcomes were treated as dichotomous variables, and participants were grouped as being below or above this LoD cutoff point, in order to detect differences in continuous variables and in the prevalence of pathological seminal parameters by Chi-squared or Fisher's exact tests. Two-tailed significance was set at $p < 0.05$ for all comparison.

6.2 Study design

The current research study was a single-centre, observational, cohort study, with a cross-sectional design. C1 cohort was recruited from 2013 to 2019, C2 cohort from 2018 to 2019. C1 participants were categorized into two groups, according to residential address being comprised within an HI area or LI area; a preliminary descriptive analysis of seminal parameters was performed and prevalence of pathological seminal parameters was determined in both groups, for comparison. To corroborate the results of preliminary analysis, a sub-analysis was carried out in both groups, by excluding participants with past or present andrological disorders known to affect semen quality, namely: unilateral and bilateral severe varicocele IV-V, according to Sarteschi grading, unilateral and bilateral testis hypotrophy (testis volume <12 ml) at scrotal US, hypotestosteronemia (TT< 3,5 ng/ml), cryptorchidism, testis cancer, orchitis, prostatitis, other urogenital infections, testicular trauma, history of testicular injury or surgery, Klinefelter Syndrome. All subjects in C1 were offered a diagnostic clinical examination of their domestic dogs, performed by a specialized veterinarian, in order to exclude dogs with andrological problems. In addition, a semen sample was collected from dogs for the analysis of conventional (volume, sperm concentration, sperm total count, motility and morphology) and non convention (SDF, head area and SGDM) seminal parameters. To exclude external factors affecting DNA methylation, only dogs housed under standard conditions and not taking drugs were included. In C2 cohort a preliminary descriptive analysis of seminal parameters was performed and prevalence of pathological seminal parameters was determined. No sub-analysis of results was carried out, due to small sample size. In C2 cohort, serum and semen burden of trace elements was determined, and correlated to semen quality and prevalence of pathological seminal parameters, by correcting results for potential confounders. Study design is depicted in Figure 7.

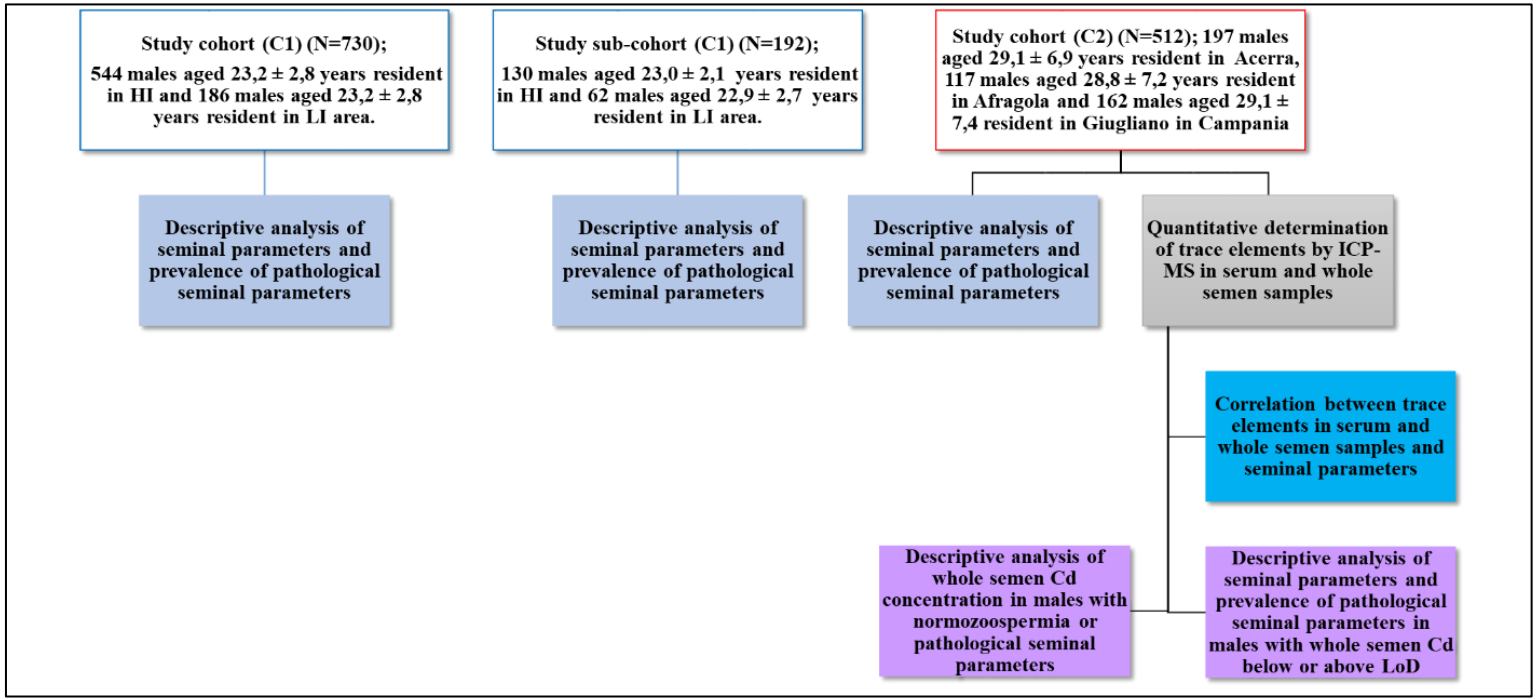
Study cohort (C1) (N=730);
544 males aged 23,2 ± 2,8 years resident in HI and 186 males aged 23,2 ± 2,8 years resident in LI area.
Study sub-cohort (C1) (N=192);
130 males aged 23,0 ± 2,1 years resident in HI and 62 males aged 22,9 ± 2,7 years resident in LI area.
Study cohort (C2) (N=512); 197 males aged 29,1 ± 6,9 years resident in Acerra, 117 males aged 28,8 ± 7,2 years resident in Afragola and 162 males aged 29,1 ± 7,4 resident in Giugliano in Campania
Descriptive analysis of seminal parameters and prevalence of pathological seminal parameters
Descriptive analysis of seminal parameters and prevalence of pathological seminal parameters
Descriptive analysis of seminal parameters and prevalence of pathological seminal parameters
Quantitative determination of trace elements by ICP-MS in serum and whole semen samples
Correlation between trace elements in serum and whole semen samples and seminal parameters
Descriptive analysis of whole semen Cd concentration in males with normozoospermia or pathological seminal parameters
Descriptive analysis of seminal parameters and prevalence of pathological seminal parameters in males with whole semen Cd below or above LoD

Figure 7: *Flow diagram of research study. Research study included Caucasian healthy males of reproductive age (13-50 years old) resident in Campania Region. Two different cohorts of subjects (C1, C2) were recruited within two awareness and prevention campaigns on infertility and testis cancer in high environmental impact (HI) areas, identified on the basis of the Campania Region Environmental Protection Agency (ARPAC) reports. Within the first awareness campaign 730 participants were enrolled; study cohort (C1) consisted of 544 males aged 13-33 years resident in a HI area comprising municipalities within the Province of Naples and Caserta belonging to the so-called "Land of Fires" (LF), and of 186 males aged 17-33 years resident in a low environmental impact (LI) area comprising municipalities not belonging to the LF. Within the second awareness campaign 512 participants were enrolled; study cohort (C2) consisted of males aged 14-50 years resident in the HI municipalities of Acerra (N=197), Afragola (N=117), and Giugliano in Campania (N=162), belonging to the LF. Both C1 and C2 cohorts were analyzed for semen quality and prevalence of pathological seminal parameters; a sub-analysis of results was carried out on C1 cohort, by excluding participants with past or present andrological disorders known to affect semen quality. In C2 cohort, serum and semen burden of trace elements was determined by inductively coupled plasma-mass spectrometry (ICP-MS), and elemental concentrations were correlated to seminal parameters. Further investigation was carried out by focusing on semen cadmium (Cd), which yielded the most consistent results at preliminary correlation analyses; semen quality and prevalence of pathological seminal parameters were assessed in C2 cohort, grouped as having semen Cd concentrations below or above Cd limit of detection (LoD). Moreover, semen Cd burden was assessed in C2 cohort, by stratifying participants based on semen quality.*

6.3 Results

6.3.1 Participants characteristics and semen quality in C1 cohort

In C1 cohort (N=730), participants from HI (N=544) and LI (N=186) groups did not differ for age (23,2 ± 2,8 vs 23,2 ± 2,8), BMI (24,6 ± 3,0 vs 24,6 ± 3,0), diet, physical activity, or self-reported occupational exposure to toxic chemicals. A higher prevalence of smokers was detected in HI, compared to LI group (39,6% vs 20,7%; p<0,0001). HI and LI groups showed similar values for pH (8,0 ± 0,4 vs 8,0 ± 0,4), semen volume (3,1 ± 1,4 vs 3,1 ± 1,6), sperm concentration (57,3 ± 47,9 vs 55,9 ± 42,3) sperm total count (167,0 ± 147,6 vs 164,3 ± 130,0), total sperm motility (56,9 ± 16,0 vs 57,9 ± 16,2), progressive sperm motility (48,0 ± 16,2 vs 48,4 ± 15,9), normal sperm morphology (9,4 ± 5,9 vs 9,5 ± 6,3) and abnormal sperm morphology (89,6 ± 10,5 vs 89,9 ± 9,2). C1 cohort characteristics and seminal parameters are shown in Table 3.

	Total C1 cohort (N=730)	HI group (N=544)	LI group (N=186)	p
Age (years)	23,2 ± 2,8	23,2 ± 2,8	23,3 ± 2,8	NS
BMI (Kg/m^2)	24,6 ± 3,0	24,6 ± 3,0	24,6 ± 3,0	NS
pH	8,0 ± 0,4	8,0 ± 0,4	8,0 ± 0,4	NS
Semen Volume (ml)	3,1 ± 1,4	3,1 ± 1,4	3,1 ± 1,6	NS
Sperm Concentration (nx10^6/ml)	57,0 ± 46,6	57,3 ± 47,9	55,9 ± 42,3	NS
Sperm Total Count (nx10^6/ejaculate)	166,3 ± 143,3	167,0 ± 147,6	164,3 ± 130,0	NS
Total Sperm Motility (%)	57,1 ± 16,0	56,9 ± 16,0	57,9 ± 16,2	NS
Progressive Sperm Motility (%)	48,1 ± 16,1	48,0 ± 16,2	48,4 ± 15,9	NS
Normal Sperm Morphology (%)	9,5 ± 6,0	9,4 ± 5,9	9,5 ± 6,3	NS
Abnormal Sperm Morphology (%)	89,7 ± 10,2	89,6 ± 10,5	89,9 ± 9,2	NS

Table 3: *C1 cohort characteristics and seminal parameters expressed as mean ± SD.*

Prevalence of normozoospermia and pathological seminal parameters did not differ in HI (73,4% and 26,7%), compared to LI (73,1% and 26,9%) group. More in detail, for pathological seminal parameters the following prevalence was detected in HI, compared to LI group: oligozoospermia (5,2% vs 5,4%); asthenozoospermia (7% vs 7%); teratozoospermia (2,4% vs 4,8%); oligo-asthenozoospermia (1,3% vs 2,2%); oligo-teratozoospermia (2,2% vs 2,2%); astheno-teratozoospermia (1,8% vs 0,5%); oligo-astheno-teratozoospermia (5,9% vs 4,3%); azoospermia (0,9% vs 0,5%) Prevalence of normozoospermia and pathological seminal parameters in C1 cohort is shown in Table 4.

	Total C1 cohort (N=730)	HI group (N=544)	LI group (N=186)	p
Normozoospermia (%)	73,3	73,4	73,1	NS
Pathological seminal parameters (%) any type	26,7	26,7	26,9	NS
Oligozoospermia (%)	5,2	5,2	5,4	NS
Asthenozoospermia (%)	7,0	7,0	7,0	NS
Teratozoospermia (%)	3,0	2,4	4,8	NS
Oligo_Asthenozoospermia (%)	1,5	1,3	2,2	NS
Oligo_Teratozoospermia (%)	2,2	2,2	2,2	NS
Astheno_Teratozoospermia (%)	1,5	1,8	0,5	NS
Oligo_Astheno_Teratozoospermia (%)	5,5	5,9	4,3	NS
Azoospermia (%)	0,8	0,9	0,5	NS

Table 4: *Prevalence of normozoospermia and pathological seminal parameters in C1 cohort.*

Sub-analysis of results was carried out in both HI and LI groups, by excluding participants with past or present andrological disorders known to affect semen quality, namely: unilateral and bilateral varicocele III-V, according to Sarteschi grading, unilateral and bilateral testis hypotrophy (testis volume <12 ml) at scrotal US, cryptorchidism, testis cancer, orchitis, prostatitis, other urogenital infections, testicular trauma, history of testicular injury or surgery, Klinefelter Syndrome. In C1 sub-cohort (N=192), participants from HI (N=130) and LI (N=62) groups did not differ for age (23,0 ± 2,1 vs 22,9 ± 2,7), BMI (24,3 ± 2,7 vs 24,2 ± 2,5), diet, physical activity, medical history, or self-reported occupational exposure to toxic chemicals. A higher prevalence of smokers was detected in HI, compared to LI group (45,4% vs 22,5%; p<0,01). HI and LI sub-groups showed similar values for pH (8,0 ± 0,4 vs 7,9 ± 0,3), semen volume (3,1 ± 1,3 vs 2,9 ± 1,5), sperm concentration (60,1 ± 41,9 vs 61,4 ± 45,6) sperm total count (180,7 ± 149,3 vs 177,4 ± 159,0), total sperm motility (59,6 ± 12,6 vs 57,0 ± 16,3), progressive sperm motility (50,5 ± 13,6 vs 48,9 ± 16,3), normal sperm morphology (10,8 ± 6,0 vs 9,5 ± 5,9) and abnormal sperm morphology (89,2 ± 6,0 vs 90,5 ± 5,9). C1 sub-cohort characteristics and seminal parameters are shown in Table 5. Prevalence of normozoospermia and pathological seminal parameters differed in HI (69,4% and 30,7%; p<0,05), compared to LI (83,1% and 16,9%; p<0,05) sub-group. Nevertheless, when analyzing results stratifying per each pathological seminal parameter, results were no longer significant. The following prevalence was detected in HI, compared to LI sub-group: oligozoospermia (8,1% vs 3,9%); asthenozoospermia (9,7% vs 4,6%); teratozoospermia (6,5% vs 1,5%); oligo-asthenozoospermia (1,6% vs 0,8%); oligo-teratozoospermia (0% vs 1,5%); astheno-teratozoospermia (0% vs 1,5%); oligo-astheno-teratozoospermia (4,8% vs 3,1%); azoospermia (0% vs 0%). Prevalence of normozoospermia and pathological seminal parameters in C1 sub-cohort is shown in Table 6.

	C1 sub-cohort (N=192)	HI sub-group (N=130)	LI sub-group (N=62)	p
Age (years)	22,9 ± 2,3	23,0 ± 2,1	22,9 ± 2,7	NS
BMI (Kg/m^2)	24,3 ± 2,6	24,3 ± 2,7	24,2 ± 2,5	NS
pH	8,0 ± 0,3	8,0 ± 0,4	7,9 ± 0,3	NS
Semen Volume (ml)	3,1 ± 1,4	3,1 ± 1,3	2,9 ± 1,5	NS
Sperm Concentration (nx10^6/ml)	60,5 ± 43,0	60,1 ± 41,9	61,4 ± 45,6	NS
Sperm Total Count (nx10^6/ejaculate)	179,7 ± 152,1	180,7 ± 149,3	177,4 ± 159,0	NS
Total Sperm Motility (%)	58,8 ± 13,9	59,6 ± 12,6	57,0 ± 16,3	NS
Progressive Sperm Motility (%)	50,0 ± 14,5	50,5 ± 13,6	48,9 ± 16,3	NS
Normal Sperm Morphology (%)	10,4 ± 6,0	10,8 ± 6,0	9,5 ± 5,9	NS
Abnormal Sperm Morphology (%)	89,6 ± 6,0	89,2 ± 6,0	90,5 ± 5,9	NS

Table 5: *C1 sub-cohort characteristics and seminal parameters expressed as mean ± SD.*

	C1 sub-cohort (N=192)	HI sub-group (N=130)	LI sub-group (N=62)	p
Normozoospermia (%)	78,7	69,4	83,1	*p<0,05
Pathological seminal parameters (%) any type	21,3	30,7	16,9	*p<0,05
Oligozoospermia (%)	5,2	8,1	3,9	NS
Asthenozoospermia (%)	6,3	9,7	4,6	NS
Teratozoospermia (%)	3,1	6,5	1,5	NS
Oligo_Asthenozoospermia (%)	1,0	1,6	0,8	NS
Oligo_Teratozoospermia (%)	1,0	0	1,5	NS
Astheno_Teratozoospermia (%)	1,0	0	1,5	NS
Oligo_Astheno_Teratozoospermia (%)	3,7	4,8	3,1	NS
Azoospermia (%)	0	0	0	NS

Table 6: *Prevalence of normozoospermia and pathological seminal parameters in C1 sub-cohort.*

6.3.2 Participants characteristics and semen quality in C2 cohort

In C2 cohort (N=512), mean residence period in a HI area was 25,6 years; no participants had lived for less than 10 years in the HI area of residence. Prevalence of never-, past-, and current-smokers was 43,2%, 9,7%, and 47,0%, respectively. Self-reported occupational exposure to toxic chemicals was 12,2%. Seminal parameters values are: pH (8,3 ± 0,3), semen volume (3,1 ± 1,7), sperm concentration (37,5 ± 30,2) sperm total count (111,2 ± 104,0), total sperm motility (56,8 ± 16,1), progressive sperm motility (50,2 ± 16,6), normal sperm morphology (8,0 ± 4,0) and abnormal

sperm morphology (92,0 ± 4,0). C2 cohort characteristics and seminal parameters are shown in Table 7. Prevalence of normozoospermia and pathological seminal parameters in the cohort 2 are 66,6% and 33,4. Prevalence the cohort 2 are: oligozoospermia (14,0%); asthenozoospermia (3,0%); teratozoospermia (0,6%); oligo-asthenozoospermia (3,4%); oligo-teratozoospermia (2,4%); astheno-teratozoospermia (1,2%); oligo-astheno-teratozoospermia (6,1%), and azoospermia (2,2%). Prevalence of normozoospermia and pathological seminal parameters in C2 cohort is shown in Table 8.

	Total C2 cohort (N=512)
Age (years)	29,1 ± 7,2
BMI (Kg/m^2)	25,8 ± 4,1
pH	8,3 ± 0,3
Semen Volume (ml)	3,1 ± 1,7
Sperm Concentration (nx10^6/ml)	37,5 ± 30,2
Sperm Total Count (nx10^6/ejaculate)	111,2 ± 104,0
Total Sperm Motility (%)	56,8 ± 16,1
Progressive Sperm Motility (%)	50,2 ± 16,6
Normal Sperm Morphology (%)	8,0 ± 4,0
Abnormal Sperm Morphology (%)	92,0 ± 4,0

Table 7: *C2 cohort characteristics and seminal parameters expressed as mean ± SD.*

	Total C2 cohort (N=512)
Normozoospermia (%)	66,6
Pathological seminal parameters (%) any type	33,4
Oligozoospermia (%)	14,0
Asthenozoospermia (%)	3,0
Teratozoospermia (%)	0,6
Oligo_Asthenozoospermia (%)	3,4
Oligo_Teratozoospermia (%)	2,4
Astheno_Teratozoospermia (%)	1,2
Oligo_Astheno_Teratozoospermia (%)	6,1
Azoospermia (%)	2,2

Table 8: *Prevalence of normozoospermia and pathological seminal parameters in C2 cohort.*

6.3.3 Trace elements burden in serum and semen and semen quality

In C2 cohort, quantitative determination of trace elements by ICP-MS was performed in 258 serum (Li, Be, Al, V Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Cd, Sn, Sb, Te, Ba, Pb, U, Hg) and 386 semen samples (same trace elements determined in serum, except for Hg). Trace elements burden in serum and semen was correlated to seminal parameters in the entire C2 cohort. In a preliminary analysis, the most consistent results in terms of statistical significance and available number of samples were found for Cd and Se; therefore, further analyses were performed on these selected trace elements. For both Cd and Se, serum burden did not correlate to semen burden, in C2 cohort (Table 9). Conversely, a negative correlation was found between Cd and Se burden in semen in C2 cohort ($r= -0,280$; $p<0,01$)

(Table 9). Mean Cd concentration was significantly higher in semen, compared to serum, in total C2 cohort (1,0 µg/l vs 0,8 µg/l; p<0,01) (Figure 8), as opposite, mean Se concentration was significantly higher in serum, compared to semen, in total C2 cohort (67,6 µg/l vs 54,7 µg/l; p<0,0001) (Figure 8). In regards to semen quality, semen Cd, but not serum Cd burden, was negatively correlated to sperm concentration (r= -0,211; p<0,05) and sperm total count (r= -0,177; p<0,05), in the entire C2 cohort (Table 10) (Figures 9, 10). Conversely, semen Se, but not serum Se burden, was found to be positively correlated to sperm concentration (r= 0,398; p<0,0001), sperm total count (r= 0,312; p<0,0001), progressive sperm motility (r= 0,120; p<0,05), and normal sperm morphology (r= 0,224; p<0,0001), and negatively correlated to pH (r= -0,209; p<0,0001) and abnormal sperm morphology (r= -0,224; p<0,0001), in the entire C2 cohort (Table 10). Based on these results, additional analyses were carried out to further determine the relationship between semen Cd burden and semen quality. Cd concentrations in semen were frequently below the LoD (0,20 µg/l) across C2 cohort; therefore, Cd concentration was set as a dichotomous variable, and participants were grouped as having semen Cd concentration below or above Cd LoD cutoff. Participants were grouped as follow: entire C2 cohort had 33,4% (129/386) detectable and 66,6% (257/386) undetectable semen samples.

	Serum Cd	***Semen Cd***	***Serum Se***	***Semen Se***
Serum Cd	r= 1	r= 0,254	r= 0,244	r= -0,074
Semen Cd	r= 0,254	r= 1	r= -0,183	r= -0,280**
Serum Se	r= 0,244	r= -0,183	r= 1	r= -0,017
Semen Se	r= -0,074	r= -0,280**	r= -0,017	r= 1

Table 9: *Correlation matrix of serum and semen cadmium (Cd) and selenium (Se) within the same participants in total C2 cohort; a negative correlation was found between Cd and Se in semen (r= -0,280). **p<0,01*

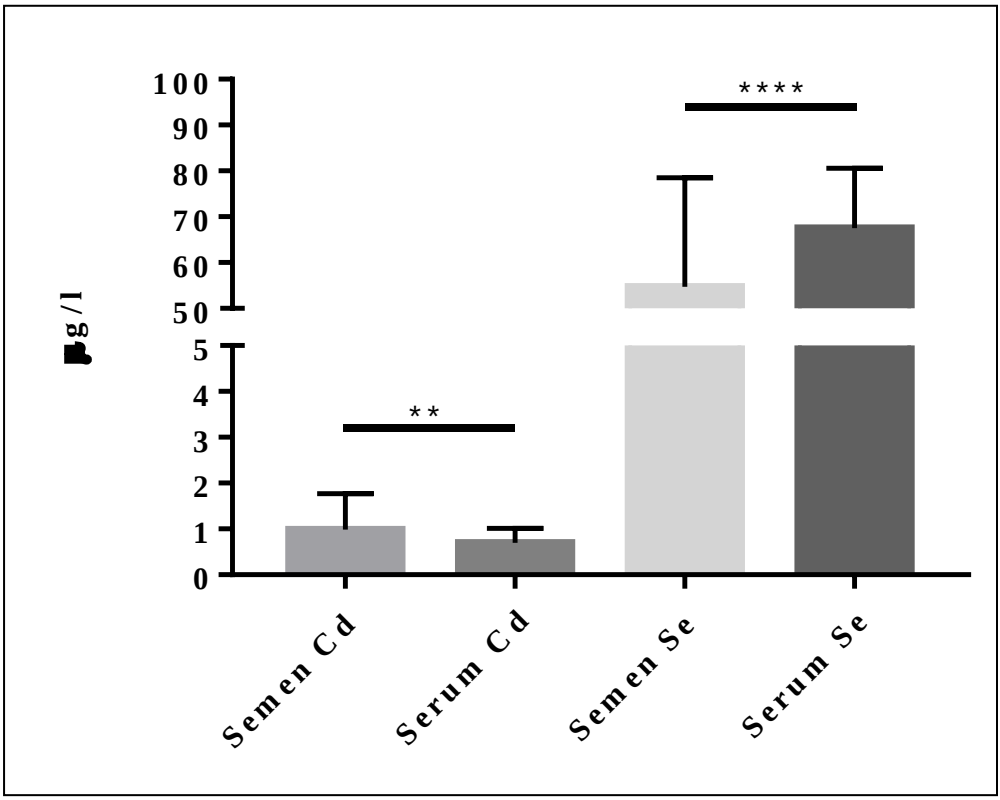

Figure 8: *Comparison between semen and serum cadmium (Cd) and selenium (Se) concentrations (µg/l), expressed as mean ± SD, within the same participants in total C2 cohort; mean Cd concentration was significantly higher in semen, compared to serum (1,0 µg/l vs 0,8 µg/l), whereas mean Se concentration was significantly higher in serum, compared to semen (67,6 µg/l vs 54,7 µg/l). **p<0,01, ****p<0,0001.*

	Serum Cd	*Semen Cd*	*Serum Se*	*Semen Se*
Sperm Concentration (nx10^6/ml)	r= -0,087	r= -0,211*	r= 0,017	r= 0,398****
Sperm Total Count (nx10^6/ejaculate)	r= -0,191	r= -0,177*	r= -0,041	r= 0,312****
Total Sperm Motility (%)	r= 0,085	r= 0,027	r= -0,004	r= 0,094
Progressive Sperm Motility (%)	r= 0,067	r= 0,029	r= -0,037	r= 0,120*
Normal Sperm Morphology (%)	r= -0,151	r= -0,159	r= -0,014	r= 0,224****
Abnormal Sperm Morphology (%)	r= 0,151	r= 0,159	r= -0,014	r= -0,224****

Table 10: *Correlation matrix of serum and semen cadmium (Cd) and selenium (Se) within the same participants in total C2 cohort; semen Cd, but not serum Cd, was negatively correlated to sperm concentration (r= -0,211) and sperm total count (r= -0,177), Conversely, semen Se, but not serum Se, was positively correlated to sperm concentration (r= 0,398), sperm total count (r= 0,312), progressive sperm motility (r= 0,120), and normal sperm morphology (r= 0,224), and negatively correlated to abnormal sperm morphology (r= -0,224). *p<0,05, ****p<0,0001.*

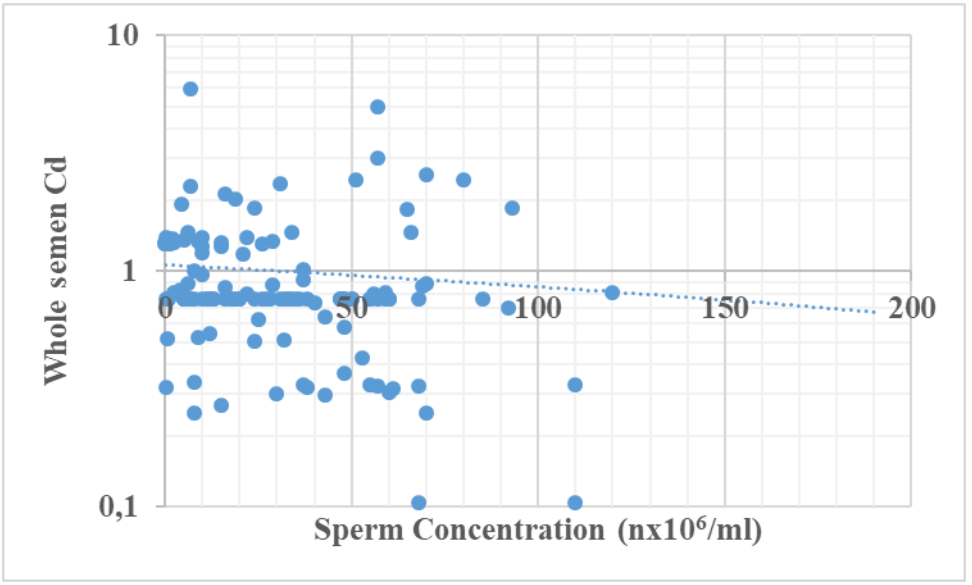

Figure 9: *Distribution of semen cadmium (Cd) concentration plotted on a Log10 scale against sperm concentration. Semen Cd was negatively correlated to sperm concentration (r= -0,211; p<0,05), in total C2 cohort.*

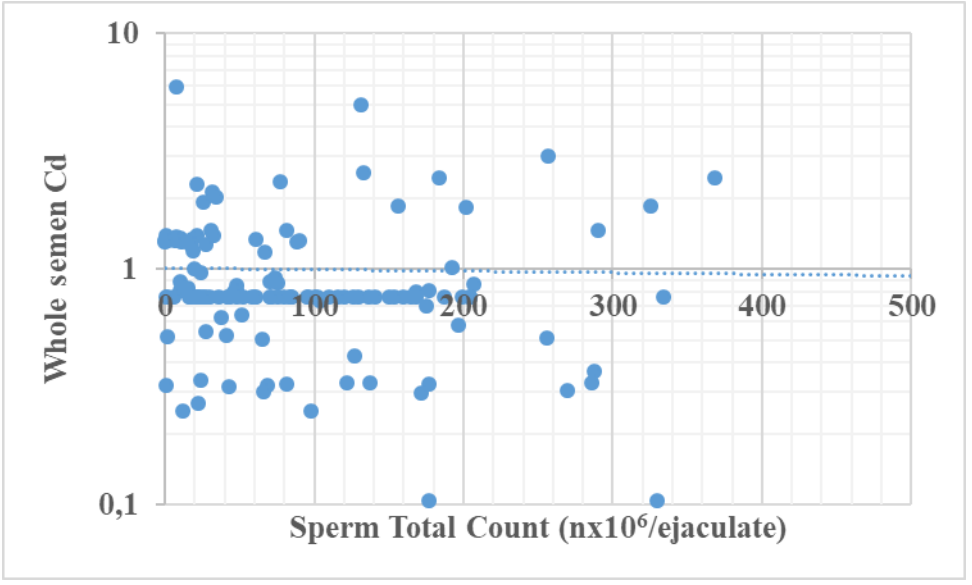

Figure 10: *Distribution of semen cadmium (Cd) concentration plotted on a Log10 scale against sperm total count. Semen Cd was negatively correlated to sperm total count (r= -0,177; p<0,05), in total C2 cohort.*

Due to the small sample size, mainly determined by the few samples with detectable semen Cd concentrations, no sub-analysis was performed in single municipality. In the entire C2 cohort, participants with detectable and undetectable semen Cd concentrations did not differ for age (30,5 ± 7,5 vs 29,5 ± 7,0), BMI (26,0 ± 3,8 vs 26,0 ± 4,1), diet, physical activity, self-reported occupational exposure to toxic chemicals (11,1% vs 15,6%). Conversely, a higher prevalence of smokers was found in participants with undetectable, compared to detectable, semen Cd concentrations (48,2% vs 36,7%; p<0,05). Participants with detectable semen Cd concentrations displayed worse semen quality, compared to those with undetectable concentrations. Specifically, participants with detectable and undetectable semen Cd concentrations had significantly different values for sperm total count (92,8 ± 85,1 vs 113,5 ± 101,5; p<0,05), normal sperm morphology (7,3 ± 3,7 vs 8,2 ± 3,9; p<0,05) and abnormal sperm morphology (92,7 ± 3,7 vs 91,8 ± 3,9; p<0,05); no differences were found for pH (8,4 ± 0,3 vs 8,4 ± 0,2), semen volume (3,1 ± 1,6 vs 3,3 ± 1,6), sperm concentration (32,2 ± 26,6 vs 35,4 ± 26,3) total sperm motility (56,4 ± 16,2 vs 57,9 ± 15,9), and progressive sperm motility (49,6 ± 16,9 vs 51,8 ± 16,4). Participants characteristics and seminal parameters are shown in Table 11.
Moreover, in the entire C2 cohort the prevalence of normozoospermia and pathological seminal parameters differed significantly between detectable and undetectable semen Cd concentrations, although no significant differences were detected when considering each pathological seminal parameter separately; specifically, normozoospermia was significantly less prevalent (59,7% vs 72,4%; p<0,05), whereas, as expected, pathological seminal parameters were significantly more prevalent (40,3% vs 27,6%; p<0,05) in detectable, compared to undetectable samples. Prevalence of normozoospermia and pathological seminal parameters is shown in Table 12.
In the attempt to further characterize the relationship between semen Cd burden and semen quality, an inverse approach was adopted; semen quality was set as a dichotomous variable, and participants of the entire C2

cohort were grouped as being normozoospermic (N=329) or having pathological seminal parameters (N=165), in order to detect differences in semen Cd burden and to determine a cut-off concentration, potentially predictive of poor semen quality. Semen Cd concentration was significantly higher in participants belonging to the pathological seminal parameters group, compared to those within the normozoospermic group (1,08 µg/l vs 0,93 µg/l; p<0,05) (Figure 11).

	Undetectable semen Cd (N=257)	Detectable semen Cd (N=129)	p
Age (years)	29,5 ± 7,0	30,5 ± 7,5	NS
BMI (Kg/m²)	26,0 ± 4,1	26,0 ± 3,8	NS
pH	8,4 ± 0,2	8,4 ± 0,3	NS
Semen Volume (ml)	3,3 ± 1,6	3,1 ± 1,6	NS
Sperm Concentration (nx10⁶/ml)	35,4 ± 26,3	32,2 ± 26,6	NS
Sperm Total Count (nx10⁶/ejaculate)	113,5 ± 101,5	92,8 ± 85,1	p<0,05
Total Sperm Motility (%)	57,9 ± 15,9	56,4 ± 16,2	NS
Progressive Sperm Motility (%)	51,8 ± 16,4	49,6 ± 16,9	NS
Normal Sperm Morphology (%)	8,2 ± 3,9	7,3 ± 3,7	p<0,05
Abnormal Sperm Morphology (%)	91,8 ± 3,9	92,7 ± 3,7	p<0,05

Table 11: *Characteristics and seminal parameters of C2 cohort, grouped as semen cadmium (Cd) below or above limit of detection (LoD) cutoff (0,20 µg/l). Data expressed as mean ± SD.*

	Undetectable semen Cd (N=257)	Detectable semen Cd (N=129)	p
Normozoospermia (%)	72,4	59,7	p<0,05
Pathological semen parameters (%) any type	27,6	40,3	p<0,05
Oligozoospermia (%)	12,2	21,7	NS
Asthenozoospermia (%)	2,0	4,7	NS
Teratozoospermia (%)	0,0	0,8	NS
Oligo_Asthenozoospermia (%)	3,1	2,3	NS
Oligo_Teratozoospermia (%)	2,8	3,1	NS
Astheno_Teratozoospermia (%)	1,2	0,8	NS
Oligo_Astheno_Teratozoospermia (%)	5,1	7,8	NS
Azoospermia (%)	0,8	0,0	NS

Table 12: *Prevalence of normozoospermia and pathological seminal parameters in C2 cohort, grouped as semen cadmium (Cd) below or above limit of detection (LoD) cutoff (0,20 µg/l).*

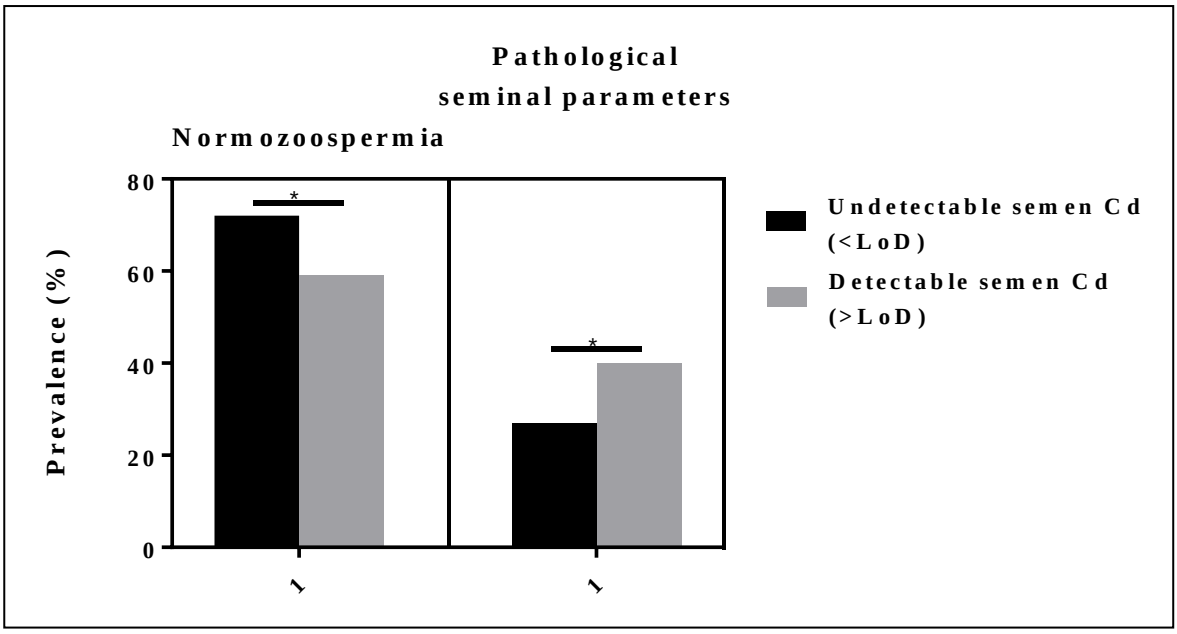

Figure 11: *Prevalence of normozoospermia and pathological seminal parameters in C2 cohort, grouped as semen cadmium (Cd) below or above limit of detection (LoD) cutoff (0,20 µg/l). Normozoospermia was significantly less prevalent (59,7% vs 72,4%), whereas, as expected, pathological seminal parameters were significantly more prevalent (40,3% vs 27,6%) in detectable, compared to undetectable samples; *p<0,05.*

6.3.4 Assessment of confounders

Study results were modeled to address the relative contribution of smoking habit, or potential environmental exposure, as a main source of Cd burden in semen, and to further depict the relationship between semen Cd and semen quality. Given the low prevalence of past-smokers in C2 cohort, analysis was limited to smokers vs non smokers, the latter group being composed by never-smokers and past-smokers pooled together; moreover analysis was carried considering the entire C2 cohort as a whole, rather than single municipalities.

In participants with available data on both smoking habits and semen Cd burden (N=364), smokers (N=161) had detectable concentrations of semen

Cd in 27,3% (44/161), and undetectable concentrations in 72,7% (117/161), whereas non smokers (N=203) had detectable concentrations of semen Cd in 37,4% (76/203), and undetectable concentrations in 62,6% (127/203); prevalence of detectable concentration between groups was significantly different, with smokers displaying the lower prevalence of participants with semen Cd concentration above LoD ($p<0,05$), although mean Cd concentration did not differ between groups (0,9 µg/l vs 1,1 µg/l). In regards to semen quality, no differences were found between smokers and non smokers, in seminal parameters, nor prevalence of normozoospermia and pathological seminal parameters; moreover, semen Cd concentration was no longer correlated to any of the assessed seminal parameters, in either group, when analyzing smokers and non smokers separately. In order to further investigate the higher prevalence of semen Cd concentrations above LoD in non smokers, groups were analyzed for potential confounders; no differences were detected in smokers, compared to non smokers, concerning alcohol consumption, self-reported occupational exposure to toxic chemicals (17,6% vs 12,0%), mean residence period in a HI area (26,3 vs 25,8 years), and mean time spent/24 hours in a HI area (16,3 vs 16,8 hours).

Therefore, smokers and non smokers were analyzed by stratifying participants based on smoking habit plus relative residential distribution within HI municipalities.

When addressing mean semen Cd concentrations and prevalence of semen Cd above LoD in smokers vs non-smokers, stratifying by single municipality, a significantly higher prevalence of semen Cd above LoD in the non smokers group (6,9% vs 30,8%; $p<0,05$) was detected in Afragola, therefore probably explaining the higher prevalence of detectable semen Cd in the entire group of non-smokers. A graphical summary of cumulative semen Cd concentrations at each municipality is depicted in Figure 12.

In regards to semen quality, no differences were detected between smokers and non-smokers resident in Afragola.

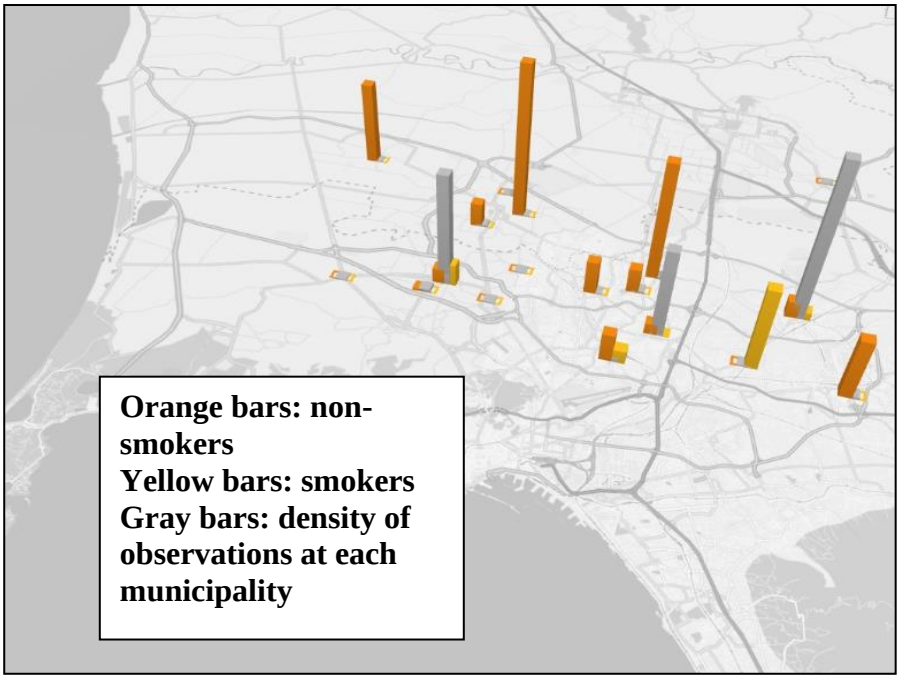

Figure 12: *Geographical distribution of semen Cd detection by municipality. Bars represent cumulative semen Cd burden (pooled values) in smokers (yellow bars) vs non-smokers (orange bars); gray bars indicate the number of samples assayed at each municipality, therefore, the higher the bar, the stronger the statistical power of observations. The three main municipalities of interest are characterized by the higher gray bars; from the left to the right Giugliano in Campania (smokers N=56, non-smokers N=57), Afragola (smokers N=29 vs non-smokers N=52) and Acerra (smokers N=70, non-smokers N=76). When addressing mean semen Cd concentrations and prevalence of semen Cd above LoD in smokers vs non-smokers, stratifying by single municipality, no differences were detected in Acerra (0,9 µg/l vs 1,1 µg/l; 28,6% vs 39,5%) and Giugliano in Campania (1,0 µg/l vs 1,3 µg/l; 35,7% vs 35,1%). Conversely, although there was no difference in mean semen Cd concentration in smokers vs non-smokers (0,6 µg/l vs 0,8 µg/l), the analysis detected a significantly higher prevalence of semen Cd above LoD in the latter group (6,9% vs 30,8%; $p<0,05$), in Afragola.*

6.3.5 SGDM in semen of humans

BMI and age of the analysed dogs' owners were 25,5 ± 2,7 Kg/m² and 22,9 ± 2,5 years, respectively. Mean values for qualitative and quantitative seminal parameters were as follow: ph 8,2± 0,3; semen volume 3,1±1,3 ml; sperm concentration 34,5 ± 27,1 x10^6/ml; total sperm count 139,1± 121 x10^6/ejaculate; total sperm motility 56,8 ± 11,5%; progressive sperm motility 48,9 ± 10,4%; normal sperm morphology 10,1 ± 5,8%; abnormal sperm morphology 89,9 ± 5,8% (table 13). Prevalence of oligozoospermia, asthenozoospermia and teratozoospermia were 20% (6/30), 3,3% (1/30) and 0% (0/30), respectively. When evaluating SGDM we found a value of 3,3 ± 2,6% (mean ± SD), ranging from 0,5 and 11,2%. The Skewness test, Kurtosis test and, Zvalue and Shapiro test showed that data were non-parametric distributed.

PARAMETER	**MEAN ± SD**	**N°**
BMI Kg/m²	25.5 ± 2.7	30
Age (years)	22.9 ± 2.5	30
Ph	8.2 ± 0.3	30
Semen volume (ml)	3.1 ± 1.3	30
Sperm concentration (x10^6/ml)	34.5 ± 27.1	30
Sperm total count (x10^6/ejaculate)	139.1 ± 121	30
Total sperm motility (%)	56.8 ± 11.5	30
Progressive sperm motility (%)	48.9 ± 10.4	30
Normal sperm morphology (%)	10.1 ± 5.8	30
Abnormal sperm morphology (%)	89.9 ± 5.8	30
SGDM (%)	3.3 ± 2.6	30

Table 13: *Dogs owners semen quality and quantity parameters.*

According to Spearman's rank coefficient a regular positive correlation was found between %5-mc and sperm total count (r= 0,4; p<0,05), respectively (Figure 13).

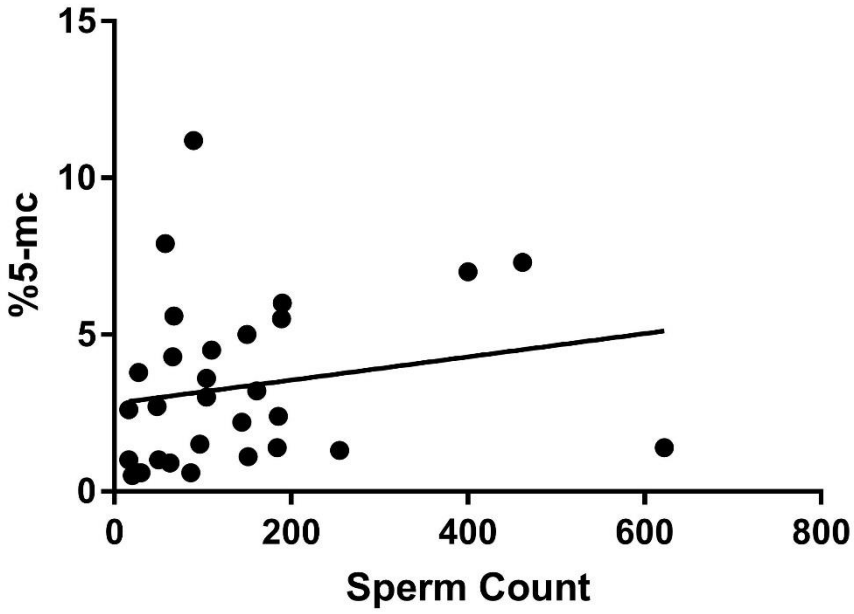

Figure 13: *Relationship between %5-mc and and sperm total count in dogs owners population. Spearman's correlation analysis shows a significant regular positive correlation between %5-mc a sperm total count (r = 0,4; p <0,05).*

SGDM percentage was significantly lower in oligozoospermic dogs owners when compared to those with sperm total counts above normality threshold (10,1% vs 20,1%; p<0,005), whereas no significant difference was found in SGDM percentage when stratifying subjectsbased on progressive sperm motility or sperm morphology normality thresholds (Figure 14).

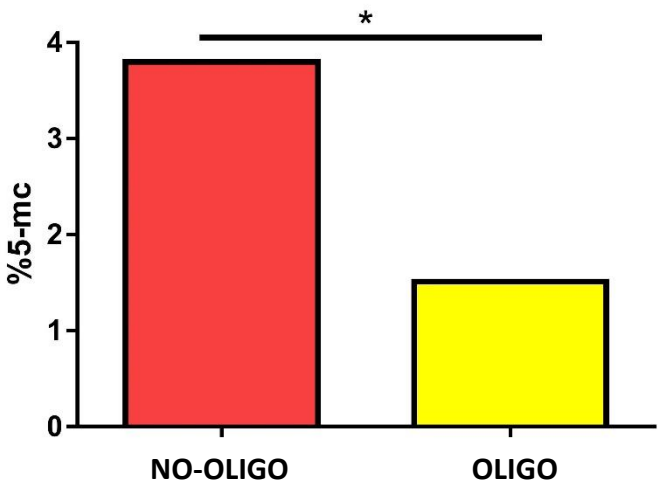

Figure 14: *Histogram showing the SGDM (%5-mc) in non oligozooospermic/oligozoospermic dogs owners. NO-OLIGO: Non Oligozoospermic; OLIGO: Oligozoospermic. A significant difference was found *p < 0,05.*

6.3.6 SGDM in semen of dogs

Mean body weight and age of the analysed dogs were 41,8 ± 14,7 kg and 3,5 ± 2,2 years, respectively. Mean values for qualitative and quantitative seminal parameters were as follow: semen volume 7 ± 3 ml; sperm concentration 57,3 ± 39,1 $x10^6$/ml; sperm total count 356,7 ± 226,3 $x10^6$/ejaculate; progressive sperm motility 68,9 ± 20,5%; normal sperm morphology 7,1 ± 10,8%; sperm head area 16,5 ± 1,7µm²; SDF 3,3 ± 2,7% (table 14). Prevalence of oligozoospermia, asthenozoospermia and teratozoospermia were 46% (14/30), 34% (10/29) and 20% (6/29), respectively. When evaluating SGDM we found a value of 7,8 ± 6,4% (mean ± SD), ranging from 1,2 and 30,5%. The Skewness test, Kurtosis test and, Zvalue and Shapiro test showed that data were non-parametric distributed.

PARAMETER	MEAN ± SD	N°
Body weight (kg)	41.8±14.7	30
Age (years)	3.5±2.2	30
Semen volume (ml)	7±3	30
Sperm concentration ($x10^6$/ml)	57.3±39.1	30
Sperm total count ($x10^6$/ejaculate)	356.7±226.3	30
Progressive sperm motility (%)	68.9±20.5	29
Normal sperm morphology (%)	71.1±10.8	29
Sperm head area (µm²)	16.5±1.7	30
SDF (%)	3.3±2.7	12
SGDM (%)	7.8± 6.4	30

Table 14: *Dogs semen quality and quantity parameters.*

According to Spearman's rank coefficient a regular positive correlation was found between body weight and sperm concentration (r = 0,5; p<0,005) and between body weight and sperm total count (r = 0,56; p<0,05) (Figure 15) while a regular positive correlation and a strong positive correlation were found between %5-mc and sperm concentration (r= 0,41; p<0,05), and sperm total count (r= 0,61; p<0,001), respectively (Figure 16).

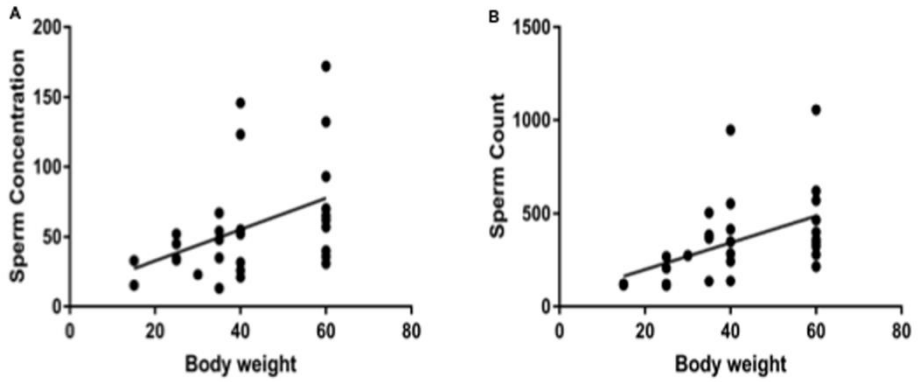

Figure 15: *Relationship between body weight and sperm concentration (A), and sperm total count (B). Spearman's correlation analysis shows a significant regular positive correlation between body weight and sperm concentration (r = 0,5; p <0,005) (A) and a significant regular positive correlation between body weight and sperm total count (r = 0,5; p <0,05) (B).*

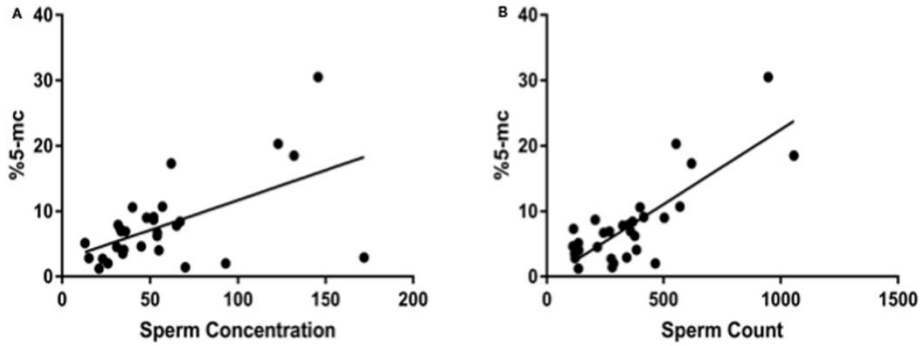

Figure 16: *Relationship between %5-mc and sperm concentration (A) and sperm total count (B) in dogs population. Spearman's correlation analysis shows a significant regular positive correlation between %5-mc and sperm concentration (r = 0,4; p <0,05) (A) and a significant strong positive correlation between %5-mc and sperm total count (r = 0,6; p <0,001) (B).*

SGDM percentage was significantly lower in oligozoospermic dogs when compared to those with sperm total counts above normality threshold (10,1% vs 20,1%; p<0,005) (Figure 17), whereas no significant difference was found in SGDM percentage when stratifying animals based on progressive sperm motility or sperm morphology normality thresholds.

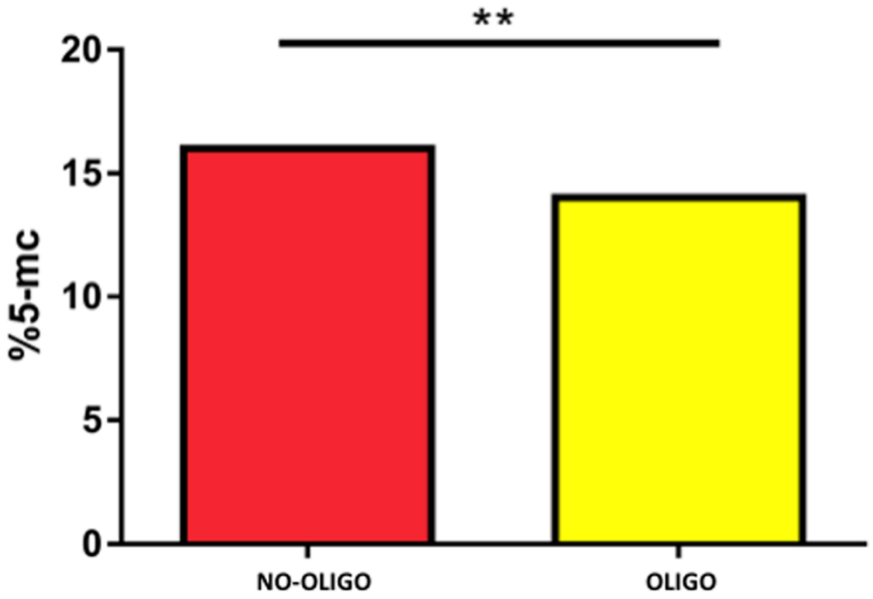

Figure 17: *Histogram showing the SGDM (%5-mc) in non oligozooospermic/oligozoospermic dogs, in the total study population. NO-OLIGO: Non Oligozoospermic; OLIGO: Oligozoospermic. A significant difference was found **p < 0,005.*

A further analysis was performed dividing the samples according to dog's size in medium (n=11), large (n=9) and giant sized (n=10). When analysing data by dividing dogs according to their size (medium, large and giant) a significant differencewas observed across medium, large and giant cathegories in observed across medium, large and giant cathegories in body weight (27,3 ± 7,5 vs 39,4 ± 1,7 vs 60,0 ± 1,5; p<0,0001), sperm concentration (40,0 ± 14,8 vs 57,9 ± 46,2 vs 75,8 ± 45,1; p<0,05) and sperm total count (259,9 ± 136,0 vs 356,3 ± 226,0 vs 463,7 ± 242,6; p<0,05), as we expected. No difference observed across medium, large and giant cathegories for age (4,1 ± 2,4 vs 4,5 ± 2,3 vs 2,8 ± 2,4); semen volume (6,9 ± 3,3 vs 7,2 ± 3,1 vs 7,1 ± 2,9); progressive sperm motility (70,9 ± 15,4 vs 71,3 ± 25,9 vs 64,7 ± 21,3); normal sperm morphology (71,5 ± 9,9 vs 72,5 ± 12,1 vs 69,3 ± 11,5); sperm head area (16,7 ± 1,5 vs 16,4 ± 1,9 vs 16,6 ± 1,7);

SDF (3,7 ± 3,4 vs 2,8 ± 1,3 vs absence of data) and SGDM (5,8 ± 2,4 vs 9,6 ± 9,6 vs 8,3 ± 6,0) Table 15.

For medium sized dogs a strong positive correlation was found between SGDM percentage and sperm concentration ($r= 0,6$; $p<0,05$). For large sized dogs a strong positive correlation was found between SGDM percentage and sperm concentration ($r= 0,7$; $p<0,05$) and sperm total count ($r= 0,8$; $p<0,005$), respectively. For giant sized dogs a strong positive correlation was found between SGDM percentage and sperm total count ($r= 0,7$; $p<0,05$). Furthermore, when grouping large and giant dogs (n=19), a strong positive correlation was found SGDM percentage and sperm total count ($r= 0,7$; $p<0,0001$) (Figure 18). Interestingly when grouping large and giant dogs the correlation between SGDM percentage and total sperm count is more evident. Based on the same dog's size grouping, SGDM has assessed in oligozospemic and non-oligozospemic dogs. The prevalence of oligozoospermia in medium sized, large sized, giant sized, and large and giant dogs grouped together was 63% (7/11), 55% (5/9), 20% (2/10), and 36% (7/19), respectively. The SGDM percentage was overall lower in oligozoospermic dogs compared to those with sperm total counts above normality threshold in all groups, with a statistically significant difference in large sized dogs (3% vs 7,5%; $p<0,05$), and in large and giant dogs grouped together (5,2% vs 12,8%; $p<0,005$) (Figure 19).

PARAMETER	Medium sized	Large sized	Giant sized
Body weight (kg)	27.3 ± 7.5[a]	39.4 ± 1.7[ab]	60.0 ± 1.5[b]
Age (years)	4.1 ± 2.4	4.5 ± 2.3	2.8 ± 2.4
Semen volume (ml)	6.9 ± 3.3	7.2 ± 3.1	7.1 ± 2.9
Sperm concentration (x10^6/ml)	40.0 ± 14.8[c]	57.9 ± 46.2	75.8 ± 45.1[d]
Sperm total count (x10^6/ejaculate)	259.9 ± 136.0[c]	356.3 ± 226.0	463.7 ± 242.6[d]
Progressive sperm motility (%)	70.9 ± 15.4	71.3 ± 25.9	64.7 ± 21.3
Normal sperm morphology (%)	71.5 ± 9.9	72.5 ± 12.1	69.3 ± 11.5
Sperm head area (µm²)	16.7 ± 1.5	16.4 ± 1.9	16.6 ± 1.7
SDF (%)	3.7 ± 3.4	2.8 ± 1.3	n.d.
SGDM (%)	5.8 ± 2.4	9.6 ± 9.6	8.3 ± 6.0

Table 15: *Dogs semen quality and quantity parameters in medium, large and giant sized. A value of p < 0,05 indicates a statistically significant difference ([ab]p<0,0001; [cd]p<0,05).*

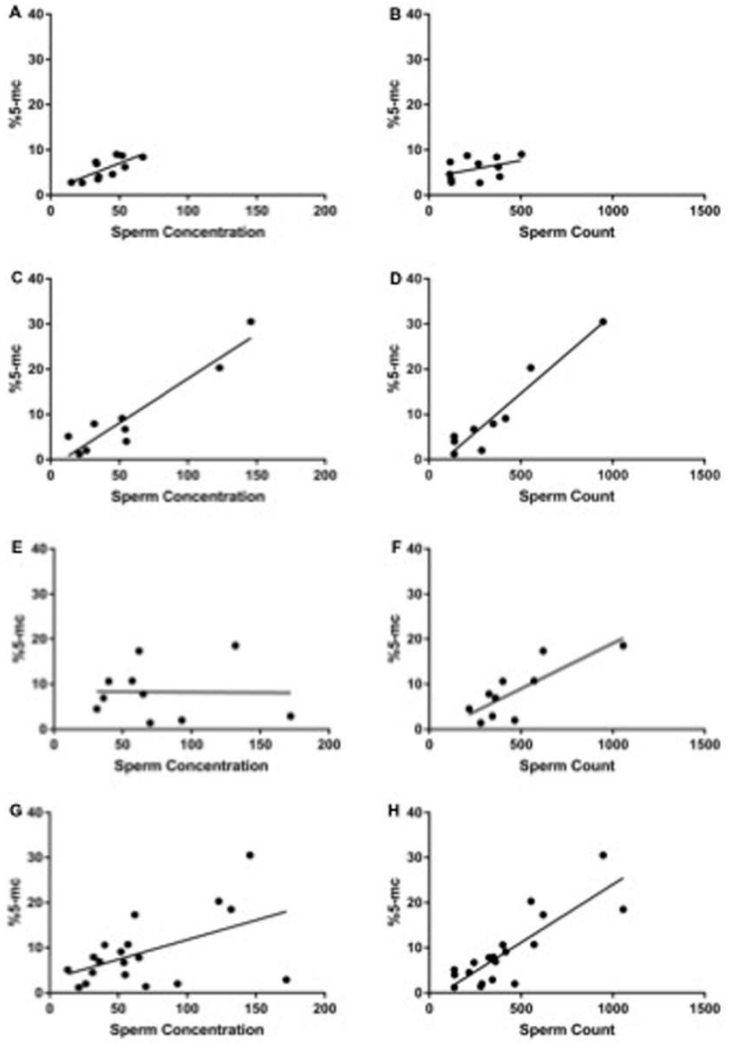

Figure 18: *Relationship between %5-mc and sperm concentration (A,C,E,G) and total sperm count (B,D,F,H) dividing the samples according to dog's size: medium (A, B); large (C,D); giant (E,F) and grouped large and giant sized dogs (G,F).Spearman's correlation analysis shows a significant strong positive correlation between %5-mc and sperm concentration in medium sized dogs (r = 0,6; p <0,05)(A) and large sized dogs (r=0,7; p<0,05) (C). A significant strong positive correlation between %5-mc and sperm total*

count was found in large (r = 0,8; p <0,005) (D), giant (r=0,7; p<0,05) (F) and grouped large and giant sized dogs (r=0,7; p<0,0001) (H).

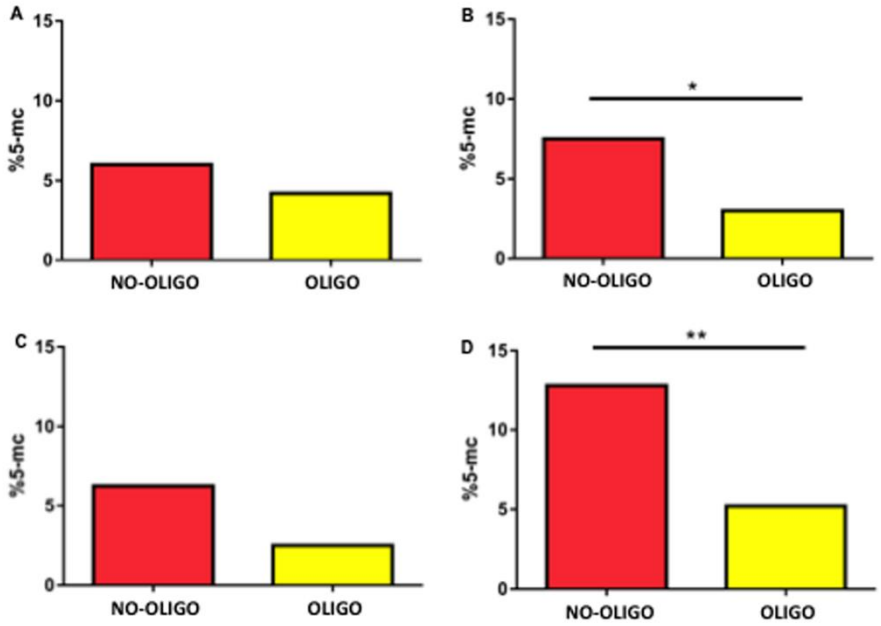

Figure 19: *Histogram showing the SGDM(%5-mc) in non oligozooospermic/oligozoospermic dogs, dividing the samples according to dog's size. NO-OLIGIO: Non Oligozoospermic; OLIGO: Oligozoospermic. Medium sized (A) and giant sized dogs (C) show no significant differences. Large sized dogs (B) and grouped large and giant dogs (D) show a significant difference: (B) *p<0,05 and (D) **p<0,005.*

6.4 Discussion

6.4.1 Enviroment and male fertility

The current research study on clinically healthy young men of reproductive age, non-occupationally exposed to toxic chemicals, demonstrated that seminal parameters of men who had lived for at least 10 years within the LF, a HI area of Campania Region, did not differ from age- and BMI-matched controls with similar anthropometric characteristics, dietary habits, and physical activity. Moreover, no difference in the prevalence of below-reference values for any seminal parameter was detected, between HI and LI men, in both preliminary and andrological disorders-deprived analysis. Results were partially in line with a previous smaller study with a similar design (*Bergamo et al., 2016*), showing no significant differences in semen quality, except for an increased percentage of immotile spermatozoa, which was not detected by the current investigation on a much larger cohort.

The current study also demonstrated that Cd, a known reproductive toxicant, might be detected in semen at significantly higher concentrations, compared to serum, suggesting that seminal Cd determination might serve as a sensitive earlier marker of exposure, particularly in non-occupationally exposed men; indeed, Cd concentrations in semen were frequently below the LoD (0,20 µg/l) and mean semen Cd concentration was 1,0 µg/l, suggesting an overall low Cd burden in the studied cohort. Moreover, semen Cd did nor correlate to serum Cd, therefore confirming the widely accepted notion that Cd specifically accumulates within the human testis, and more precisely mirrors local testicular exposure. Noteworthy, semen Cd, but not serum Cd burden, was negatively correlated to sperm concentration and sperm total count and this finding was consistent across different statistical modeling strategies; indeed, participants with detectable semen Cd concentrations had significantly reduced sperm total count and normal sperm morphology, and increased abnormal sperm morphology, along with a higher prevalence of poor semen quality. As confirmatory result, semen Cd concentration was significantly higher in participants belonging to the below-reference values group, compared to normozoospermic group. The robust association of semen Cd with poorer

semen quality found in a HI area of Campania Region underpins the majority of clinical studies demonstrating an inverse relationship with seminal parameters in environmentally exposed men de (*Angelis et al., 2017*), and supports the assumption that even micro-doses of metal may have effects on semen quality; nevertheless, these results also highlight a limitation of the study, which is the lack of a control group for semen Cd quantification in men from LI areas.

Among environmentally (non-occupationally) exposed population, tobacco smoke is the main source of Cd, since tobacco leaves accumulate large amounts of Cd; it has been estimated that smokers inhale about 1–3 μg Cd from smoking one pack of cigarettes per day yielding approximately a double Cd burden, compared to non-smokers (*ATSDR, 2012*). Non-smokers are exposed to Cd by dietary intake of contaminated food (particularly cereals and grains, leafy vegetables, potatoes and offal) and contaminated water, and vegetarians intake of Cd from food is almost double, compared to non-vegetarians; in most countries, the average daily intake of Cd from food is between 0.1–0.4 μg/kg body weight. Cd may also be perfused in alcoholic beverages, although alcohol consumption represents a significant source of metals only in heavy drinkers (*ATSDR, 2012; Angelis et al., 2017)*. In the general population, blood plasma Cd concentration is within the range of 0.4-1 μg/l in non-smokers and 1.4-4 μg/l in smokers; nevertheless, higher concentrations have been reported in highly contaminated areas (>10 μg/l)[21]. Gastrointestinal absorption of dietary Cd (about 5% in men) varies among individuals and is influenced by dietary intake of essential nutrients, including Fe, Zn and Se (*Minguez et al., 2012*). The estimated biological half-life of Cd is very long, ranging from 10 to 40 years in humans, and the clearance is very low, since about 0,007% and 0,009% is excreted in urine and feces, respectively, per day *(ATSDR, 2012)*; consequently, Cd progressively accumulates in the liver and in kidney, primary targets of Cd toxicity showing the earliest effects of intoxication, but also in ovaries and placenta in women, as well as in testis, epididymis and, consequently, semen, in men (*Angelis et al., 2017*). Taking into account that smoke is the primary source of Cd in environmentally exposed men, study results were repeatedly analyzed to address for this important confounder. First, participants with available data on both smoking habits and semen Cd burden were studied separately, and grouped as smokers or non-smokers;

surprisingly, no differences were found between smokers and non-smokers in seminal parameters nor prevalence of normozoospermia and pathological seminal parameters, which was also reflected by no different mean of semen Cd. Moreover, semen Cd concentration was no longer correlated to seminal parameters in either group, a result which was however explained by the paucity of samples with detectable Cd concentrations per group. Lastly, and again surprisingly, a higher prevalence of semen samples with detectable Cd was found in non-smokers. Interestingly, when recalculating mean semen Cd concentrations and prevalence of semen Cd above LoD in smokers vs non-smokers, stratifying by single municipality, a significantly higher prevalence of detectable semen Cd persisted only in Afragola municipality. A possible explanation of this result might include proxy reporting of smoking habit, in particular for occasional smokers, which might have been incorrectly classified as non-smokers; indeed, we had to rely on self-reported data and misclassification might have occurred. Nevertheless, overall completion of questionnaire was adequate, and the face-to-face interview modality with a trained biologist should have minimized the risk of collecting false figures. Another explanation might rely on the fact that non-smokers and past-smokers were pooled together in the final analysis, which might have generated “false negatives”. These considerations are of utmost importance considering the high rate of Cd burden deriving from smoking, and the long-term persistence in the human body; to address this, however, questionnaire was properly structured to collect information on time elapsed from the last smoked cigarette, in past-smokers.

Smokers and non-smokers did not differ in other potential lifestyle-related confounders; it cannot be ascertained whether the different prevalence of above LoD semen Cd concentrations between smokers and non-smokers, in particular in Afragola, might reflect a different environmental exposure or whether it is a consequence of other factors of susceptibility determining cumulative risk of accumulating higher amounts of Cd. Protective effects of Zn and Se from Cd-induced testis damage were steadily proven by a number of experimental studies in animals (*Angelis et al., 2017*); mimicry and interaction between Cd and Zn and Se, and competition for transporters, enzymes, and molecules involved in important essential ion-mediated biological processes, could partially

account for the different response or susceptibility thresholds to Cd (*Angelis et al., 2017*). In particular, two mechanisms of Cd accumulation within the testis are worth mention: ionic mimicry at the transporters belonging to the ZIP family of Zn transporters, which might favor Cd uptake within the testis in case of Cd excess or Zn deficiency; prevention of Cd-induced testis toxicity by immobilization of Cd in Cd-Se protein complexes (*Angelis et al., 2017*). These evidences suggest that the maintenance of adequate concentrations of trace essential ions and their dietary supplementation could contribute to protect testis and reproductive function from Cd toxicity, and suggest further investigation of the inverse correlation between semen Cd and Se which was demonstrated by the current study.

6.4.2 SGDM in human-dog study

Defects of SGDM level have been related in humans and in model animal species (like mouse and rat) to sperm DNA damages and defective spermatogenesis. Sperm Global DNA hypomethilation, in particular, is associated with fertility alterations in humans with normal and abnormal seminal parameters (*Montjean et al., 2015*). Up to now no data are available about SGDM in dog, a species whose breeding is of strong interest and which at the same time is considered by all an important study model for human with which often shares domestic environment, life style and exposure to pollutants. The first main data is the absence of statistical differences among average age of the dogs when grouped according to their size indicating that the groups are homogeneous among themselves by age. This prevents that aging could impact the differences eventually observed among groups in SGDM percentage. Data reported in this study show a relationship between dog size and sperm concentration and sperm total count, thus the larger is the dog and the higher is the number of produced spermatozoa. This is in line with previous observation and it is due to the higher volume of testes of the larger dogs (*Root Kustritz, 2007*). Moreover, this study shows, for the first time, a relationship between SGDM measured as % 5-mc and seminal parameters in healthy dogs which had fathered at least one litter. According to statistical analysis overall levels of SGDM correlated positively to sperm concentration and sperm

total count, both in the general population and in dogs grouped according to size. Also, in the dog owners sperm total count was positively correlated to % 5-mc, but not the sperm concentration. SGDM was significantly lower in oligozoospermic dogs, compared to those with sperm total counts above normality threshold (>300x10^6), in the general population, as well as in both the groups of large sized dogs and large and giant dogs grouped together. Interestingly, despite it is evident a lower level of SGDM percentage in oligozoospermic medium sized dogs vs no- oligozoospermic medium sized dogs.

Same result was obtained in the population of dog owners by dividing them into oligozoospermics and non-oligozoopsemers, showing a significantly a lower level of SGDM in oligozoospermic men.

These results suggest that epigenetic changes, specifically SGDM, might be used as a marker of testis function and spermatogenesis in dogs and humans. It can be hypothesized that higher SGDM levels might correspond to improved spermatogenesis. This is in line with data reported in human, showing a lower level of SGDM in oligozoospermic men (*Marques et al., 2004; Marques et al., 2008; Montjean et al., 2015; Benchaib et al., 2005; El Hajj et al., 2011*). The positive correlation, found in this study, between SGDM and sperm concentration and sperm total count suggest that improper DNA methylation might be associated with spermatogenesis alterations that reduce the number of spermatozoa. This is also confirmed by the differences in methylation percentage observed between oligozoospermic and non-oligozoospermic.

CHAPTER 7
Genetic and food: maca supplementation

7.1 Material and methods

7.1.1 Animals

Eight healthy Italian thoroughbred stallions (four treated and four controls), aged between 9 and 16 years, were selected for this study. All stallions were evaluated cytogenetically to exclude chromosome abnormalities (*Macrì et al., 2014*) that could affect sperm production (*Albarella et al., 2013*) according to the protocols described in Ciotola et al. (*2012*). All stallions were housed at a farm located in Teggiano (Salerno, Italy) under the same breeding conditions and used for AI. The animals were fed twice daily with hay and concentrates, and they had water provided ad libitum.

7.1.2 Source and supplementation of maca

Yellow maca (Figure 20) hypocotyls used for this experiment were harvested in the Junín district, in the Andean highlands of Peru (4100 m above sea level), and exposed for 2 months to extreme temperature cycles, strong light conditions and atmospheric pressures typical of a high-altitude environment (>3500 m), therefore reproducing traditional open-field drying. Hypocotyls were then selected, washed, milled to a powder with a particle size of 0,8 mm and packaged to be used. Each stallion received a daily dosage of 4 g of maca/100 kg body weight. The dose was chosen according to that found to show beneficial effects on spermatogenesis in humans and rats (*Zheng et al., 2000; Gonzales et al., 2001; Cicero et al., 2002*). Lepidium meyenii Walp. improves sexual behaviour in male rats independent of its action on spontaneous locomotor activity (Cicero et al., 2002; Gonzales et al., 2004). Total glucosinolates content of dry extract from maca powder used for this work was 6,67% of which 3,33% was benzyl glucosinolate, 0,34% was m-methoxybenzyl glucosinolate and 3% was 3-oxo-2-(2-entenyl) cyclopentane octanoic acid.

Figure 20: Peruvian Maca.

7.1.3 Ejaculate and semen processing

Immediately after collection, the total amount of ejaculate (semen and gel) was established using a graduated laboratory bottle (Sigma, Italy), the gel fraction was removed using a nylon semen filter (Minitube, Germany), semen was filtered through a semen filter pouch (Minitube, Germany) and the quantity was measured. Sperm motility was visually assessed under a phase contrast microscope (Nikon Eclipse 80i) at ×100 and ×200 magnification. Sperm count was determined using a biophotometer (Eppendorf), sperm total count (TSC) was calculated based on Jasko (*1992*) and Juhász et al. (*2000*). For morphometric evaluation, fresh semen samples were washed by centrifugation in physiological saline (0,9% NaCl) at 1000 g for 5 min, and then re-extended to a concentration of 100 × 10^6 cells/ml. Amounts measuring 10 µl of the sperm suspension were fixed on slides and stained with a modified haematoxylin standard protocol: 10 min in Mayer's haematoxylin (code no. 05-M06002, Bio-Optica, Milano, Italy), after removing the excess of stain with water the slides were immersed in distilled water for 2 min and then for 5 min in eosin Y, 1% solution (code no. BP2419, Fisher Scientific, Geel, Belgium). Slides were then immersed in distilled water for 5 min and rinsed twice. Serial passages

in ethanol (50% to absolute) were performed. Then slides were treated with Xilolo and mounted with Eukitt (code no. CL04.0503.0500, Chem-Lab NV, Zedelgem, Belgium).

7.1.4 Morphometric analysis

At least 200 spermatozoa (50 per slide) from each ejaculate were observed in a bright field under a Nikon Eclipse 80i microscope (×100 magnification), captured with a digital camera (Nikon DS-Ri1) and analyzed with Nis Elements Imaging Software 4.00.02 (Nikon). Morphometric parameters of the head measured for each spermatozoon were length (L), width (W), perimeter (P), area (A), shape factor (SF) and roughness (R) (Figure 21).

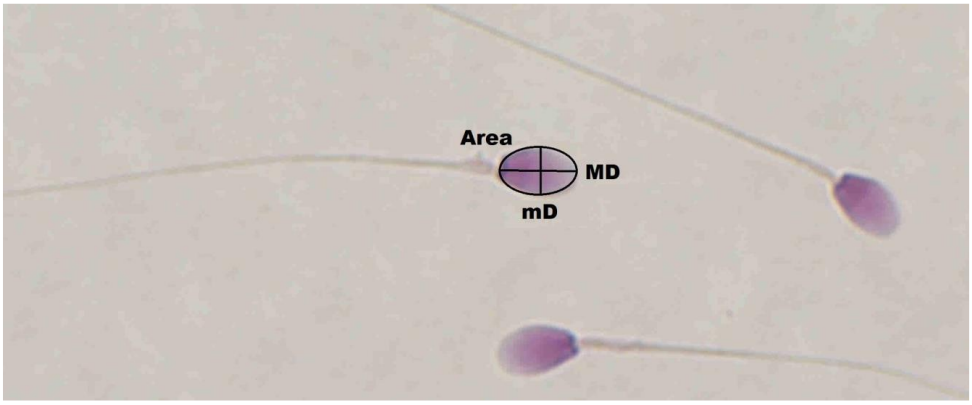

Figure 21: *Example of measures performed on the spermatozoon.*

7.1.5 SDF analysis

SDF was evaluated for each semen sample only on treated stallions using a Halomax kit for *Equus caballus* (Halotech® DNA) according to the user manual. Slides were observed in a bright field under a Nikon Eclipse 80i microscope (×20 magnification), and 300 spermatozoa from each semen sample were captured using a digital camera (Nikon DS-Ri1) and analyzed with Nis Elements Imaging Software 4.00.02 (Nikon) (Figure 22).

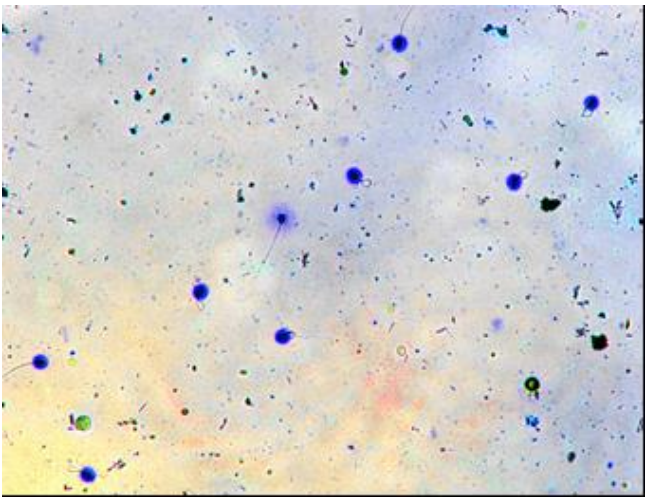

Figure 22: *Example of SDF.*

7.1.6 Statistical analysis

For statistical analysis, data for each sample were grouped into four time periods: T0 + T1, T2 + T3, T4 + T5 and T6, corresponding to before the maca effect (P1), starting maca effects (P2), maca effect (P3) and resting period (P4), respectively. The effects of time of maca supplementation on quality, SDF data and morphometric semen parameters were analyzed by a repeated measurements procedures using mixed-effects models including the random animal effect (SAS PROC MIXED 8.02; SAS Institute, Cary, NC). The level of significance was fixed at $p < 0,05$.

7.2 Study design

The study was planned so that maca administration was performed for one full horse spermatogenic cycle of 57 days (spermatocytogenesis, meiosis and spermiogenesis) (*Johnson et al., 1997*), and its effects were controlled for the next two cycles. Oral maca administration was performed for 60 days starting in April 2016, and sampling continued for 5 months after the end of treatment (October 2016). The first semen collection was planned 1 week before the beginning of maca administration, and its parameters were used as baseline control (T0); the subsequent collections were planned for 15 (T1), 35 (T2), 60 (T3), 75 (T4), 90 (T5) and 180 (T6) days after

the first maca administration, for 28 samples. The experiment was carried out in accordance with the code of ethics (D.lgs. 26-04/03/2014) and the Ethics Committee of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Italy (protocol no. 0003909), approved the protocol and procedures

7.3 Results

7.3.1 Semen quantity and quality

Table 16 shows the data on semen quantity and quality parameters of the T0 group in all the stallions used in this study. According to the data reported the groups were similar as regard semen parameters. Table 17 shows mean values (±SD) of all the parameters for semen quality and quantity in the four periods. Total volume of ejaculate increased gradually from P1 to P3 and then it decreases in P4 in treated stallions while it remained similar to P3 in control group. Semen gel-free volume and TSC increased from P1 to P2, then slightly decreased in P3 and increased again during the resting period (P4) in both groups. TSC shows statistically significant differences in the treated group when comparing P1 with P4 and between the groups only in the P4 period ($p < 0,05$). Sperm concentration showed an increase during the whole period under examination in both groups, however statistically significant differences were observed only when comparing P1 and P4 ($p < 0,05$) and when comparing the treated group with control one, starting from the P2 period. In particular, in P4, the difference was highly significant ($p < 0,01$). For sperm motility there were no statistically significant differences during the whole time period analyzed and between the two groups, while SDF gradually decreased from P2 to P4, showing a statistically significant difference ($p < 0,05$).

Parameter	Treated				Control			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Ejaculate volume (ml)	25	25	47	50	30	27	50	55
Gel-free semen volume (ml)	10	15	25	24	20	14	25	20
Sperm concentration ($x10^6$/ml)	164	98	133	155	160	120	100	143
TSC ($x10^9$)	1.64	1.47	3.32	3.72	3.20	1.68	2.50	2.86
Motility (%)	90	30	70	75	80	60	80	70
SDF (%)	8.38	12.10	7.40	7.90	n.d	n.d	n.d	n.d

Table 16: *Stallions semen quality and quantity parameters at T0 collection time*

Parameter	***Group***	***P1***	***P2***	***P3***	***P4***
Ejaculate volume (ml)	***Tratt***	*36.75±10.67*	*45.42±17.50*	*60.88±29.09^{e}*	*47.5±23.63*
	Contr	*38.32±2.89*	*49.69±8.05*	*48.84±3.67^{f}*	*48.09±1.05*
Semen gel-free vol. (ml)	***Tratt***	*18.50±5.25*	*26.50±3.03*	*25.13±3.72*	*35.00±5.25*
	Contr	*21.68±2.88*	*32.03±7.21*	*29.15±2.44*	*36.67±1.55*
Sperm conc. ($x10^6$/m)	***Tratt***	*137.50±45.80^{a}*	*183.75±26.44^{e}*	*178.88±32.39^{e}*	*279.00±45.80^{bE}*
	Contr	*126.66±5.78*	*153.30±20.82^{f}*	*155.82±29.21^{f}*	*181.67±32.50^{F}*
TSC ($x10^9$)	***Tratt***	*2.54±1.97^{a}*	*5.34±1.14*	*5.27±1.39*	*8.83±1.97^{be}*
	Contr	*2.71±0.23*	*4.81±0.38*	*4.55±0.98*	*6.69±1.47^{f}*
Motility (%)	***Tratt***	*66.25±11.70*	*70.00±6.75*	*78.75±8.27*	*67.50±11.70*
	Contr	*73.33±11.57*	*63.34±12.60*	*72.50±14.07*	*70.01±17.31*
SDF (%)	***Tratt***	*8.18±2.13*	*11.57±4.11^{a}*	*8.57±3.15*	*5.64±2.99^{b}*

Table 17: *Mean values (±SD) of stallions' semen quantity and quality parameters in the four periods (P1, before maca effect; P2, starting maca effect; P3, maca effect; P4, resting period). In the row:* $^{a,b}p < 0,05$ *(significant differences for different letters). In the column:* $^{e,f}p < 0,05$; $^{E,F}p < 0,01$ *(significant differences for different letters).*

7.3.2 Morphometric analysis

Mean values (±SD) of all the parameters for semen morphometry of the four periods are shown in Table 18. Values of spermatozoa L, W, P and A increased significantly from P1 to P3 ($p < 0,01$) and then they decreased in P4 ($p < 0,01$). This trend was observed in both groups, but was greater in the treated subjects than in the control.
SF showed a statistically significant difference in P1 to P2 and P3 ($p < 0,05$) and in P2 and P3 to P4 ($p < 0,01$). R showed a statistically significant difference in P1, P2 and P3 versus P4 ($p < 0,05$).

Parameter	**Group**	**P1**	**P2**	**P3**	**P4**
Length (L)	**Tratt**	5.73 ± 0.01^{A}	5.87 ± 0.01^{B}	6.17 ± 0.01^{CE}	5.95 ± 0.01^{DE}
	Contr	5.73 ± 0.01	5.85 ± 0.01	5.94 ± 0.01^{F}	5.62 ± 0.01^{F}
Width (W)	**Tratt**	3.08 ± 0.01^{A}	3.09 ± 0.01^{AE}	3.17 ± 0.01^{BE}	3.01 ± 0.01^{CE}
	Contr	3.07 ± 0.01	2.99 ± 0.01^{F}	3.07 ± 0.01^{F}	2.79 ± 0.01^{F}
Perimeter (P)	**Tratt**	14.32 ± 0.07^{AD}	14.88 ± 0.04^{A}	15.26 ± 0.05^{CE}	14.40 ± 0.07^{De}
	Contr	14.33 ± 0.04	14.66 ± 0.04	14.26 ± 0.04^{F}	13.99 ± 0.04^{f}
Area (A)	**Tratt**	12.13 ± 0.05^{A}	12.45 ± 0.03^{Be}	13.23 ± 0.04^{CDe}	13.03 ± 0.05^{DE}
	Contr	12.14 ± 0.05	12.92 ± 0.05^{f}	12.95 ± 0.05^{f}	12.30 ± 0.05^{F}
Shape Factor (SF)	**Tratt**	0.79 ± 0.01^{a}	0.77 ± 0.01^{Abe}	0.77 ± 0.01^{AbE}	0.81 ± 0.01^{B}
	Contr	0.79 ± 0.01	0.80 ± 0.01^{f}	0.82 ± 0.01^{F}	0.82 ± 0.01
Roughness (R)	**Tratt**	1.02 ± 0.01^{A}	1.03 ± 0.01^{A}	1.03 ± 0.01^{AE}	1.01 ± 0.01^{BE}
	Contr	1.02 ± 0.01	1.03 ± 0.01	1.01 ± 0.01^{F}	1.02 ± 0.01^{F}

Table 18: *Mean values (±SD) of the parameters for stallions' semen morphometry in the four periods. In the row:* $^{a,b}p< 0,05$; $^{A,B,C,D}p < 0,01$.

In the column: $^{e,f}p < 0,05$; $^{E,F}p < 0,01$.

7.4 Discussion

The mean values obtained in this study for quantity and quality parameters conventionally used to evaluate stallion semen (gel-free semen volume, sperm concentration, TSC and sperm motility) confirm results already reported in the literature: maca food supplementation improves semen in horses (*Del Prete et al., 2018a; Tafuri et al., 2019b*).
SDF progressively decreased from P2 to P4, which could indicate that active substances in maca prevented spermatozoa from DNA damage due to ageing after the second meiotic division has settled, and during the period they stand in the genital tract. In particular, the decrease in percentage of spermatozoa with fragmented DNA may indicate that DNA spermatozoa is more fragile during the first stages of gametogenesis. Therefore, it is conceivable that maca components are more effective in DNA damage prevention if they are present in the seminal tract from the first stages of the differentiation of the gametes. Unfortunately, it was not possible to carry out this same test on control horses, therefore this hypothesis needs be confirmed in the future with appropriate experimental tests.
According to Waheed et al. (*2015*), stallion spermatozoa L and W increases from spring to summer and then decrease in autumn in a not statistically significant manner. The same trend could be observed in the eight stallions studied in this work, however the differences observed in this case were statistically significant and were greater in the subjects treated with maca. All this was then reflected on the measurements relating to P and A (Table 18).
Among all the morphometric parameters measured, L was the one that mainly increased (Table 18), indicating an elongation of the sperm head, which is a shape that can positively affect sperm fluidodynamic behaviour, making it move more efficiently. In fact, a recent study of Iberian reed deer (*Ramón et al., 2013*) showed that spermatozoa with rapid and linear movements were strongly correlated with spermatozoa having a small and elongated head; both subpopulations occur more frequent in high-fertility males. Sperm morphometry has also been successfully used in sperm competition studies. Sperm competition has been associated with an

increase in total sperm dimensions and in sperm head elongation, and both aspects have been related to an improved sperm migratory efficiency (*Sánchez et al., 2013*). When spermatozoa increase in size, all sperm components increase in size simultaneously (*Tourmente et al., 2011*), and are able to produce more energy and swim faster (Sánchez et al., 2013). TSC, motility and sperm L, W, P and A, even when analyzed separately for the four stallions, improved when associated with maca food integration. Figure 23 shows how improvements in the quantity and quality parameters of sperm were different in the four stallions indicating a marked individual response to this food supplement. However, in all the animals the highest values were in P3, corresponding to the maca effect period.

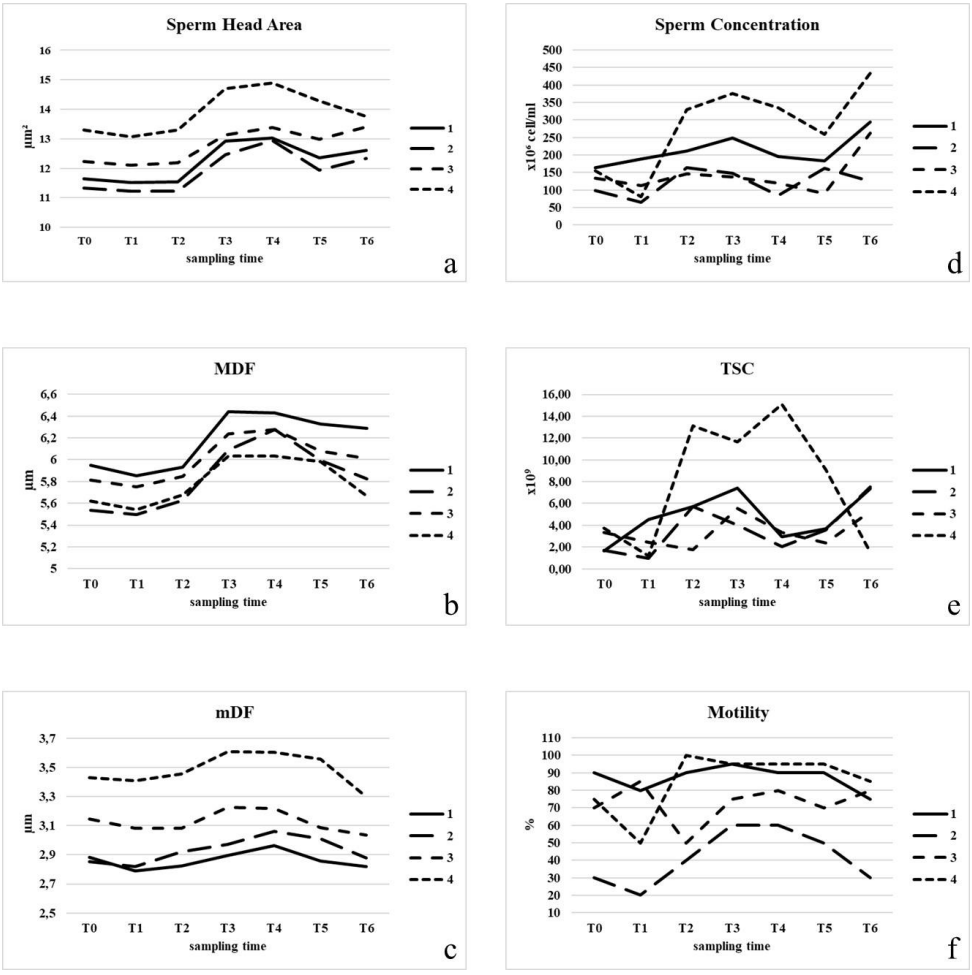

Figure 23: *Spermatozoa morphometry and quality parameters of the four stallions treated with maca at each sampling time. (a) Sperm head area (µm^2). (b) MFD, maximum Feret's diameter (µm). (c) mFD, minimum Feret's diameter (µm). (d) Sperm concentration (×10^6). (e) TSC sperm total count (×10^9). (f) Motility (%). T0, 1 week before maca administration; T1, Day 15 of maca administration (m.a.); T2, Day 35 of m.a.; T3 = Day 60 of m.a.; T4, Day 75 of m.a.; T5, Day 90 of m.a.; T6, Day 180 of m.a.*

CHAPTER 8

Conclusion

The aim of this doctorate research project has been to investigate the interplay between environmental pollution, nutraceutical, genetics and epigenetics, and their relative impacts of seminal parameters and semen quality in mammalian species, whit particular references to humans, dogs and horses. This aim was achieved by connecting expertise and methodological skills from two research areas: human reproduction and the genetic improvement of livestock species.

The studies here reported tried to bridge the existing gap of information concerning semen quality of men living in the high environmental pressure area of the LF, and to investigate the potential association with objectively evaluated non-occupational exposure to non-essential trace elements with known reproductive toxicity, such as Cd. To the best of our knowledge, this is the largest study focused on these outcomes in young healthy men, exhaustively adjusted for lifestyle factors, i.e. smoking habits, otherwise disregarded by other studies. In study subjects a strict correlation was showed between seminal parameters, particularly sperm total count, and Cd burden in semen samples, testifying a potential harmful effect of both environmental ad lifestyle-related sources of this toxicant on reproductive health in humans. Moreover, reduced SDGM was associated to reduced semen quality, particularly sperm total count, in a selected dog/owner pairs group within the LF, demonstrating an overall similar relationship between epigenetic changes and seminal parameters in men and their dog in an area with high environmental pressure. Unfortunately, the lack of particular interest, motivation or compliance by the dog owners and the strict dog selection criteria (having produced at least one litter, to live with their owners, with no drug treatments in place at the moment of the collection, to live in LF) has determined that very few dogs were enrolled in three years thus it was impossible to achieve an adequate sample size for Cd burden assessment in dog semen; this piece of information collected also in an animal model plausibly exposed to a set of common pollutants/environmental factors with respect to their owners, would have been helpful in a more detailed comprehension of the specific contribution of environmental pollution or lifestyle components to the observed epigenetic and seminal impairment, a specific aim which will be pursued in the next future by this still ongoing study. In this scenario, seminal Cd determination in dogs might serve as a sensitive earlier marker of Cd

exposure, particularly in non-occupationally exposed men; another future direction of the present reaserch will indeed comprise trace elements determination in the semen of selected human-dog pairs from LI areas, therefore adding further strength to the provided results. Although longitudinal data might be required, and the cross-sectional design of the study restricts the feasibility of establishing causal relationships between exposure and outcome, active biomonitoring with follow up visits and semen analysis is already planned, to accomplish the goal. Moreover, this is the first report in which a correlation of SGDM percentage with conventional semen parameters in dogs is proved. This epigenetic finding could be of considerable interest also in the zootechnical field, due to the possible reproductive and economic repercussions. Indeed, it is evident that a critical phase for animal reproduction is represented by the triggering of epigenetic mechanisms during pregnancy, a period in which the mother, the fetus, and the fecundating potential of the progeny, may be affected by deleterious effects deriving from exposure to epigenetic factors. It would be therefore interesting to verify the potential correlation between SGDM percentage and assisted reproduction outcomes, recurrent pregnancy loss etc., also in this species. Future studies should be aimed at evaluating the relationship between SGDM and other factors potentially affecting dog semen quality and fertility, to verify whether SGDM could be used as an additional analysis, along with conventional seminal parameters, in order to increase the predictive value on reproductive potential. Lastly, considering that aberrant DNA methylation and chromatin compaction may result in inherited genomic errors, a wider implication of study results might comprise the role of epigenetic changes in congenital diseases affecting the offspring.

Alongside the mentioned implications of the study findings, the evidence of the efficacy of maca as a natural food supplement to improve stallion convention seminal parameters as well as morphometric measures and SDF during a stressful period, such as the reproductive season, offers a new tempting potential approach to counteract the damaging effect of ROS on seminal parameters. Future trials could be aimed to assess pregnancy rate of mares sired with stallions supplemented orally with maca, or artificially inseminated with their cryopreserved semen, to confirm its usefulness as a dietary supplement in horse reproduction. The innovative data provided

by the current study concerning the use of maca held a potential translational value for its applicability in the human reproduction field, as both an in vivo (on patients) intervention in environmentally exposed men with a potentially elevated ROS seminal content and increased risk of sperm DNA damage, or in cases of nutritionally-caused impairment of semen quality, and in vitro (on gametes) treatment before AI. Further in dept investigation is at present still required to deepen this intruiguing theoretical framework.

Bibliography

1. Agarwal, Ashok et al. “New semen quality scores developed by principal component analysis of semen characteristics.” *Journal of andrology* vol. 24,3 (2003): 343-52. doi:10.1002/j.1939-4640.2003.tb02681.x
2. Agarwal, Ashok, and Lucky H Sekhon. “The role of antioxidant therapy in the treatment of male infertility.” Human fertility (Cambridge, England) vol. 13,4 (2010): 217-25. doi:10.3109/14647273.2010.532279
3. Agarwal, Ashok et al. “Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios.” *Translational andrology and urology* vol. 5,6 (2016): 935-950. doi:10.21037/tau.2016.10.03
4. Agarwal, Ashok et al. “Male Oxidative Stress Infertility (MOSI): Proposed Terminology and Clinical Practice Guidelines for Management of Idiopathic Male Infertility.” The world journal of men's health vol. 37,3 (2019): 296-312. doi:10.5534/wjmh.190055
5. Albarella, S et al. “A new translocation t(1p;18) in an Italian Mediterranean river buffalo (Bubalus bubalis, 2n = 50) bull: cytogenetic, fertility and inheritance studies.” *Cytogenetic and genome research* vol. 139,1 (2013): 17-21. doi:10.1159/000342360
6. Albarella, S et al.” Diagnosis of XX/XY blood cell chimerism at a low percentage in horses”. J Equine Vet Sci (2018)69, 129–35.
7. Albarella, S et al. “Cytogenetic Analyses in Ewes with Congenital Abnormalities of the Genital Apparatus.” *Animals : an open access journal from MDPI* vol. 9,10 776. 10 Oct. 2019, doi:10.3390/ani9100776
8. Albarella, Sara et al. “Analysis of XX *SRY*-Negative Sex Reversal Dogs.” *Animals : an open access journal from MDPI* vol. 10,9 1667. 16 Sep. 2020, doi:10.3390/ani10091667
9. Ali, Ahmed et al. “Management strategies, reproductive performance and causes of infertility in sheep flocks in the central region of Saudi Arabia.” *Tropical animal health and production* vol. 52,4 (2020): 1691-1697. doi:10.1007/s11250-019-02182-9

10. Aliarabi, Hassan et al. “The Effect of Maternal Supplementation of Zinc, Selenium, and Cobalt as Slow-Release Ruminal Bolus in Late Pregnancy on Some Blood Metabolites and Performance of Ewes and Their Lambs.” Biological trace element research vol. 187,2 (2019): 403-410. doi:10.1007/s12011-018-1409-8
11. Almeida, A M et all. “Greyling Scrotal, testicular and semen characteristics of young Boer bucks fed winter veld hay: the effect of nutritional supplementation”. *Small Rum. Res* (2007):73, pp. 216-220
12. Amann RP. “A critical review of methods for evaluation of spermatogenesis from seminal characteristics”. *J Androl* (1981):2:37–58.
13. (Arpac), L.A.r.p.a.C. Comuni della Terra dei fuochi di cui alle Direttive del 23/12/2013, del 16/04/2014 e del 10/12/2015. 2016; Available from: http://www.arpacampania.it/documents/30626/0/TDF_tutti%20i%20comuni%20delle%20due%20Direttive%20rev%201%20def.pdf
14. ATSDR, Toxicological profile for Mercury. 1999.
15. ATSDR, Toxicological profile for Nickel. 2005.
16. ATSDR, Toxicological profile for Chromium. 2012.
17. ATSDR, Toxicological profile for Cadmium. 2012.
18. ATSDR, Toxicological profile for lead. 2019.
19. Bank, T.W., Fertility rate, total (births per woman). 2019.
20. BARTLETT, D J. “Studies on dog semen. I. Morphological characteristics.” *Journal of reproduction and fertility* vol. 3 (1962): 173-89. doi:10.1530/jrf.0.0030173
21. Benchaib, M et al. “Influence of global sperm DNA methylation on IVF results.” *Human reproduction (Oxford, England)* vol. 20,3 (2005): 768-73. doi:10.1093/humrep/deh684
22. Bergamo, P et al. “Human semen as an early, sensitive biomarker of highly polluted living environment in healthy men: A pilot biomonitoring study on trace elements in blood and semen and their relationship with sperm quality and RedOx status.” *Reproductive toxicology (Elmsford, N.Y.)* vol. 66 (2016): 1-9. doi:10.1016/j.reprotox.2016.07.018

23. Boggia, B et al. “Effects of working posture and exposure to traffic pollutants on sperm quality.” *Journal of endocrinological investigation* vol. 32,5 (2009): 430-4. doi:10.1007/BF03346481
24. Boissonnas, Céline Chalas et al. “Specific epigenetic alterations of IGF2-H19 locus in spermatozoa from infertile men.” *European journal of human genetics : EJHG* vol. 18,1 (2010): 73-80. doi:10.1038/ejhg.2009.117
25. Brown, B W. “A review of nutritional influences on reproduction in boars, bulls and rams.” *Reproduction, nutrition, development* vol. 34,2 (1994): 89-114. doi:10.1051/rnd:19940201
26. Calogero, A E et al. “The use of nutraceuticals in male sexual and reproductive disturbances: position statement from the Italian Society of Andrology and Sexual Medicine (SIAMS).” Journal of endocrinological investigation vol. 40,12 (2017): 1389-1397. doi:10.1007/s40618-017-0699-6
27. Carlsen, E et al. “Evidence for decreasing quality of semen during past 50 years.” *BMJ (Clinical research ed.)* vol. 305,6854 (1992): 609-13. doi:10.1136/bmj.305.6854.609
28. Carrell, D., T., and Kenneth, I., Aston (eds.). (2013) Spermatogenesis: Methods and Protocols. Methods in Molecular Biology, vol.927. © Springer Science+ Business Media LLC.
29. Carrell, Douglas T, and Saher Sue Hammoud. “The human sperm epigenome and its potential role in embryonic development.” *Molecular human reproduction* vol. 16,1 (2010): 37-47. doi:10.1093/molehr/gap090
30. Casey, P J et al. “Morphometric differences in sperm head dimensions of fertile and subfertile stallions.” *Theriogenology* vol. 47,2 (1997): 575-82. doi:10.1016/s0093-691x(97)00015-0
31. CDC, National Report on Human Exposure to Environmental Chemicals. 2019.
32. CDC, National Report on Human Exposure to Environmental Chemicals. 2019. 2.
33. Cicero, A F G et al. “Hexanic Maca extract improves rat sexual performance more effectively than methanolic and chloroformic Maca extracts.” *Andrologia* vol. 34,3 (2002): 177-9. doi:10.1046/j.1439-0272.2002.00490.x

34. Ciotola, F et al. “Molecular and cytogenetic studies in a case of XX SRY-negative sex reversal in an Arabian horse.” *Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation* vol. 6,1-3 (2012): 104-7. doi:10.1159/000331480
35. Clément, C et al. “The potential influence of plant-based feed supplements on sperm quantity and quality in livestock: a review.” *Animal reproduction science* vol. 132,1-2 (2012): 1-10. doi:10.1016/j.anireprosci.2012.04.002
36. Currie, A L., Detection and quantification limits: origins and historical overview. Analytica Chimica Acta, (1999): 391(2): p. 127-134.
37. de Angelis, C et al. “The environment and male reproduction: The effect of cadmium exposure on reproductive function and its implication in fertility.” *Reproductive toxicology (Elmsford, N.Y.)* vol. 73 (2017): 105-127. doi:10.1016/j.reprotox.2017.07.021
38. Del Prete, C et al. “Influences of dietary supplementation with Lepidium meyenii (Maca) on stallion sperm production and on preservation of sperm quality during storage at 5 °C.” *Andrology* vol. 6,2 (2018): 351-361. doi:10.1111/andr.12463
39. Del Prete, C et al. “Effect of superoxide dismutase, catalase, and glutathione peroxidase supplementation in the extender on chilled semen of fertile and hypofertile dogs.” *Journal of veterinary science* vol. 19,5 (2018): 667-675. doi:10.4142/jvs.2018.19.5.667
40. Del Prete, C et al. “Combined addition of superoxide dismutase, catalase and glutathione peroxidase improves quality of cooled stored stallion semen.” Animal reproduction science vol. 210 (2019): 106195. doi:10.1016/j.anireprosci.2019.106195
41. De Rosa, M et al. “Traffic pollutants affect fertility in men.” *Human reproduction (Oxford,* England) vol. 18,5 (2003): 1055-61. doi:10.1093/humrep/deg226
42. Dindyal, S. “The sperm count has been decreasing steadily for many years in Western industrialized countries: Is there an endocrine basis for this decrease?”. The Internet Journal of Urology (2003): 2(1). doi:10.5580/118d

43. Doerksen, T, and J M Trasler. “Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats.” Biology of reproduction vol. 55,5 (1996): 1155-62. doi:10.1095/biolreprod55.5.1155
44. Egger, Gerda et al. “Epigenetics in human disease and prospects for epigenetic therapy.” Nature vol. 429,6990 (2004): 457-63. doi:10.1038/nature02625
45. EUR-Lex. [(accessed on 20 March 2020)]; Available online: http://data.europa.eu/eli/reg/2006/1924/2012-11-29.
46. El Hajj, N et al. “Methylation status of imprinted genes and repetitive elements in sperm DNA from infertile males.” Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation vol. 5,2 (2011): 60-9. doi:10.1159/000323806
47. England, G C, and W E Allen. “Seminal characteristics and fertility in dogs.” The Veterinary record vol. 125,15 (1989): 399. doi:10.1136/vr.125.15.399
48. Falsig, A-M L et al. “The influence of omega-3 fatty acids on semen quality markers: a systematic PRISMA review.” Andrology vol. 7,6 (2019): 794-803. doi:10.1111/andr.12649
49. Feng, Jian et al. “Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system.” *Journal of neuroscience research* vol. 79,6 (2005): 734-46. doi:10.1002/jnr.2040.
50. Fuente-Lara, Alejandro de la et al. “Effects from aging on semen quality of fresh and cryopreserved semen in Labrador Retrievers.” Theriogenology vol. 132 (2019): 164-171. doi:10.1016/j.theriogenology.2019.04.013
51. Garolla, Andrea et al. “Dietary Supplements for Male Infertility: A Critical Evaluation of Their Composition.” Nutrients vol. 12,5 1472. 19 May. 2020, doi:10.3390/nu12051472
52. Giaccio, L et al. “Does heavy metals pollution affects semen quality in men? A case of study in the metropolitan area of Naples (Italy)”. *Journal of Geochemical Exploration* (2012): 112: p. 218–225.

53. Gonzales, G F et al. “Lepidium meyenii (Maca) improved semen parameters in adult men.” *Asian journal of andrology* vol. 3,4 (2001): 301-3.
54. Gonzales, G F et al. “Effect of Lepidium meyenii (Maca) on spermatogenesis in male rats acutely exposed to high altitude (4340 m).” *The Journal of endocrinology* vol. 180,1 (2004): 87-95. doi:10.1677/joe.0.1800087
55. Gonzales, Gustavo F. “Ethnobiology and Ethnopharmacology of Lepidium meyenii (Maca), a Plant from the Peruvian Highlands.” *Evidence-based complementary and alternative medicine : eCAM* vol. 2012 (2012): 193496. doi:10.1155/2012/193496
56. Goto, K et al. “Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice.” *Differentiation; research in biological diversity* vol. 56,1-2 (1994): 39-44. doi:10.1046/j.1432-0436.1994.56120039.x
57. Graham, J K. “Analysis of stallion semen and its relation to fertility.” *The Veterinary clinics of North America. Equine practice* vol. 12,1 (1996): 119-30. doi:10.1016/s0749-0739(17)30299-7
58. Günzel-Apel, A R et al. “Computer-assisted analysis of motility, velocity and linearity of dog spermatozoa.” *Journal of reproduction and fertility. Supplement* vol. 47 (1993): 271-8.
59. Hammoud, Saher Sue et al. “Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility.” *Fertility and sterility* vol. 94,5 (2010): 1728-33. doi:10.1016/j.fertnstert.2009.09.010
60. Hemingway, R G. “The influences of dietary intakes and supplementation with selenium and vitamin E on reproduction diseases and reproductive efficiency in cattle and sheep.” Veterinary research communications vol. 27,2 (2003): 159-74. doi:10.1023/a:1022871406335
61. Herceg, Zdenko, and Thomas Vaissière. “Epigenetic mechanisms and cancer: an interface between the environment and the genome.” *Epigenetics* vol. 6,7 (2011): 804-19. doi:10.4161/epi.6.7.16262

62. Hirai, M et al. “Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors.” *Journal of andrology* vol. 22,1 (2001): 104-10.
63. Holliday, R. “The inheritance of epigenetic defects.” Science (New York, N.Y.) vol. 238,4824 (1987): 163-70.
64. Holliday, R. “Epigenetics: an overview.” *Developmental genetics* vol. 15,6 (1994): 453-7. doi:10.1002/dvg.1020150602
65. Houshdaran, Sahar et al. “Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm.” *PloS one* vol. 2,12 e1289. 12 Dec. 2007, doi:10.1371/journal.pone.0001289
66. Jasko, D J. “Evaluation of stallion semen.” *The Veterinary clinics of North America. Equine practice* vol. 8,1 (1992): 129-48. doi:10.1016/s0749-0739(17)30471-6
67. Jenuwein, T, and C D Allis. “Translating the histone code.” *Science (New York, N.Y.)* vol. 293,5532 (2001): 1074-80. doi:10.1126/science.1063127
68. Johnson, L et al. “Factors affecting spermatogenesis in the stallion.” *Theriogenology* vol. 48,7 (1997): 1199-216. doi:10.1016/s0093-691x(97)00353-1
69. Juhász, J et al. “Methods for semen and endocrinological evaluation of the stallion: a review”. *Acta Vet Brno* (2000): 69, 247–59.CrossRefGoogle Scholar
70. Kastelic, J P, and J C Thundathil. “Breeding soundness evaluation and semen analysis for predicting bull fertility.” *Reproduction in domestic animals = Zuchthygiene* vol. 43 Suppl 2 (2008): 368-73. doi:10.1111/j.1439-0531.2008.01186.x
71. Kelsey, Gavin, and Robert Feil. “New insights into establishment and maintenance of DNA methylation imprints in mammals.” *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* vol. 368,1609 (2013): 20110336. doi:10.1098/rstb.2011.0336
72. Kendall, N R et al. “The effect of a zinc, cobalt and selenium soluble glass bolus on trace element status and semen quality of ram

lambs." Animal reproduction science vol. 62,4 (2000): 277-83. doi:10.1016/s0378-4320(00)00120-2
73. Kobayashi, Hisato et al. "Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients." *Human molecular genetics* vol. 16,21 (2007): 2542-51. doi:10.1093/hmg/ddm187
74. Kolster, Kara A. "Evaluation of Canine Sperm and Management of Semen Disorders." *The Veterinary clinics of North America. Small animal practice* vol. 48,4 (2018): 533-545. doi:10.1016/j.cvsm.2018.02.003
75. Kutzler, Michelle Anne. "Semen collection in the dog." *Theriogenology* vol. 64,3 (2005): 747-54. doi:10.1016/j.theriogenology.2005.05.023
76. Lanzafame, Francesco M et al. "Oxidative stress and medical antioxidant treatment in male infertility." Reproductive biomedicine online vol. 19,5 (2009): 638-59. doi:10.1016/j.rbmo.2009.09.014
77. Liu, Ling, and Thomas A Rando. "Manifestations and mechanisms of stem cell aging." *The Journal of cell biology* vol. 193,2 (2011): 257-66. doi:10.1083/jcb.201010131
78. Lutz, Wolfgang et al. "Demographics. Europe's population at a turning point." *Science (New York, N.Y.)* vol. 299,5615 (2003): 1991-2. doi:10.1126/science.1080316
79. Macrì, F et al. "A rare case of simple syndactyly in a puppy." *The Journal of small animal* practice vol. 55,3 (2014): 170-3. doi:10.1111/jsap.12165
80. Malo, Aurelio F et al. "Sperm design and sperm function." *Biology letters* vol. 2,2 (2006): 246-9. doi:10.1098/rsbl.2006.0449
81. Marques, Cristina Joana et al. "Genomic imprinting in disruptive spermatogenesis." *Lancet (London, England)* vol. 363,9422 (2004): 1700-2. doi:10.1016/S0140-6736(04)16256-9
82. Marques, C J et al. "Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia." *Molecular human reproduction* vol. 14,2 (2008): 67-74. doi:10.1093/molehr/gam093
83. Martin, G B et al. "Interactions between nutrition and reproduction in the management of the mature male ruminant." *Animal : an*

international journal of animal bioscience vol. 4,7 (2010): 1214-26. doi:10.1017/S1751731109991674

84. Martínez, A I Peña. “Canine fresh and cryopreserved semen evaluation.” *Animal reproduction science* vol. 82-83 (2004): 209-24. doi:10.1016/j.anireprosci.2004.04.024
85. Melnikovova, Ingrid et al. “Effect of Lepidium meyenii Walp. on Semen Parameters and Serum Hormone Levels in Healthy Adult Men: A Double-Blind, Randomized, Placebo-Controlled Pilot Study.” *Evidence-based complementary and alternative medicine : eCAM* vol. 2015 (2015): 324369. doi:10.1155/2015/324369
86. Mendiola, Jaime et al. “Relationships between heavy metal concentrations in three different body fluids and male reproductive parameters: a pilot study.” *Environmental health : a global access science source* vol. 10,1 6. 19 Jan. 2011, doi:10.1186/1476-069X-10-6
87. Mínguez-Alarcón, Lidia et al. “Correlations between Different Heavy Metals in Diverse Body Fluids: Studies of Human Semen Quality.” *Advances in urology* vol. 2012 (2012): 420893. doi:10.1155/2012/420893
88. Minor, Agata et al. “Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with obstructive azoospermia and undergoing vasectomy reversal.” *Reproduction (Cambridge, England)* vol. 141,6 (2011): 749-57. doi:10.1530/REP-11-0008
89. Mirnamniha, Mahdiyeh et al. “An overview on role of some trace elements in human reproductive health, sperm function and fertilization process.” *Reviews on environmental health* vol. 34,4 (2019): 339-348. doi:10.1515/reveh-2019-0008
90. Montjean, D et al. “Sperm global DNA methylation level: association with semen parameters and genome integrity.” *Andrology* vol. 3,2 (2015): 235-40. doi:10.1111/andr.12001
91. Moore, Lisa D et al. “DNA methylation and its basic function.” *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* vol. 38,1 (2013): 23-38. doi:10.1038/npp.2012.112

92. Nordkap, Loa et al. “Regional differences and temporal trends in male reproductive health disorders: semen quality may be a sensitive marker of environmental exposures.” *Molecular and cellular endocrinology* vol. 355,2 (2012): 221-30. doi:10.1016/j.mce.2011.05.048
93. O'Flynn, N. “Assessment and treatment for people with fertility problems: NICE guideline.” *The British journal of general practice : the journal of the Royal College of General Practitioners* vol. 64,618 (2014): 50-1. doi:10.3399/bjgp14X676609
94. Olar, T T et al. “Relationships among testicular size, daily production and output of spermatozoa, and extragonadal spermatozoal reserves of the dog.” *Biology of reproduction* vol. 29,5 (1983): 1114-20. doi:10.1095/biolreprod29.5.1114
95. Oldereid, N B et al. “Concentrations of lead, cadmium and zinc in the tissues of reproductive organs of men.” *Journal of reproduction and fertility* vol. 99,2 (1993): 421-5. doi:10.1530/jrf.0.0990421
96. Ostrander, E A, and E Giniger. “Semper fidelis: what man's best friend can teach us about human biology and disease.” *American journal of human genetics* vol. 61,3 (1997): 475-80. doi:10.1086/515522
97. Pacheco, Sara E et al. “Integrative DNA methylation and gene expression analyses identify DNA packaging and epigenetic regulatory genes associated with low motility sperm.” *PloS one* vol. 6,6 (2011): e20280. doi:10.1371/journal.pone.0020280
98. Pauciullo, A et al. “X-Y aneuploidy rate in sperm of two "minor" breeds of cattle (Bos taurus) by using dual color fluorescent in situ hybridization (FISH).” Theriogenology vol. 78,3 (2012): 688-95. doi:10.1016/j.theriogenology.2012.03.017
99. Pereira, Ana Margarida et al. “Effect of Zinc Source and Exogenous Enzymes Supplementation on Zinc Status in Dogs Fed High Phytate Diets.” Animals : an open access journal from MDPI vol. 10,3 400. 29 Feb. 2020, doi:10.3390/ani10030400
100. Pitel, Mariya O et al. “Influence of specific management practices on blood selenium, vitamin E, and beta-carotene concentrations in horses and risk of nutritional deficiency.” Journal of veterinary

internal medicine vol. 34,5 (2020): 2132-2141. doi:10.1111/jvim.15862

101. Poplinski, A et al. “Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1.” International journal of andrology vol. 33,4 (2010): 642-9. doi:10.1111/j.1365-2605.2009.01000.x
102. Power JH. “A study of canine semen: physical factors which may effect the electrolyte concentration in the ejaculate”. Irish Vet J (1963):17:226–31.
103. Ramón, Manuel et al. “Sperm population structure and male fertility: an intraspecific study of sperm design and velocity in red deer.” Biology of reproduction vol. 89,5 110. 7 Nov. 2013, doi:10.1095/biolreprod.113.112110
104. Rigau, T et al. “Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates.” Theriogenology vol. 56,5 (2001): 801-15. doi:10.1016/s0093-691x(01)00609-4
105. Robinson, J J et al. “Nutrition and fertility in ruminant livestock”. Anim. Feed Sci. Technol (2006): 126, pp. 259-276
106. Root Kustritz, M V. “The value of canine semen evaluation for practitioners.” Theriogenology vol. 68,3 (2007): 329-37. doi:10.1016/j.theriogenology.2007.04.017
107. Rui, H et al. “Time- and temperature-related alterations in seminal plasma constituents after ejaculation.” *International journal of andrology* vol. 9,3 (1986): 195-200. doi:10.1111/j.1365-2605.1986.tb00882.x
108. Seisenberger, Stefanie et al. “Conceptual links between DNA methylation reprogramming in the early embryo and primordial germ cells.” *Current opinion in cell biology* vol. 25,3 (2013): 281-8. doi:10.1016/j.ceb.2013.02.013
109. Sikka, S C. “Relative impact of oxidative stress on male reproductive function.” Current medicinal chemistry vol. 8,7 (2001): 851-62. doi:10.2174/0929867013373039
110. Slama, Rémy et al. “How would a decline in sperm concentration over time influence the probability of pregnancy?.” *Epidemiology (Cambridge, Mass.)* vol. 15,4 (2004): 458-65. doi:10.1097/01.ede.0000129520.84568.87

111. Sun, Jiantao et al. “Heavy Metal Level in Human Semen with Different Fertility: a Meta-Analysis.” *Biological trace element research* vol. 176,1 (2017): 27-36. doi:10.1007/s12011-016-080
112. Swan, S H et al. “The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996.” Environmental health perspectives vol. 108,10 (2000): 961-6. doi:10.1289/ehp.00108961
113. Varea Sánchez, María et al. “Geometric morphometrics of rodent sperm head shape.” *PloS one* vol. 8,11 e80607. 28 Nov. 2013, doi:10.1371/journal.pone.0080607
114. Viguer, J M. “Reproducción e inseminación artificial en animales” *E.S.E Hafez* (1987): 47
115. Tafuri, Simona et al. “*Lepidium meyenii* (Maca) in male reproduction.” *Natural product research*, 1-10. 5 Dec. 2019, doi:10.1080/14786419.2019.1698572
116. Tafuri, Simona et al. “Chemical Analysis of *Lepidium meyenii* (Maca) and Its Effects on Redox Status and on Reproductive Biology in Stallions †.” *Molecules (Basel, Switzerland)* vol. 24,10 1981. 23 May. 2019, doi:10.3390/molecules24101981
117. Tourmente, Maximiliano et al. “Mass-specific metabolic rate and sperm competition determine sperm size in marsupial mammals.” *PloS one* vol. 6,6 (2011): e21244. doi:10.1371/journal.pone.0021244
118. Triassi, Maria et al. “Environmental pollution from illegal waste disposal and health effects: a review on the "triangle of death".” *International journal of environmental research and public health* vol. 12,2 1216-36. 22 Jan. 2015, doi:10.3390/ijerph120201216
119. Tsai, Kate L et al. “Understanding hereditary diseases using the dog and human as companion model systems.” *Mammalian genome: official journal of the International Mammalian Genome Society* vol. 18,6-7 (2007): 444-51. doi:10.1007/s00335-007-9037-1
120. Turner, B M. “Decoding the nucleosome.” *Cell* vol. 75,1 (1993): 5-8.
121. Urdinguio, Rocío G et al. “Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility.” *Human*

reproduction (Oxford, England) vol. 30,5 (2015): 1014-28. doi:10.1093/humrep/dev053
122. Vicente-Fiel, S et al. “In vitro assessment of sperm quality from rams of high and low field fertility.” *Animal reproduction science* vol. 146,1-2 (2014): 15-20. doi:10.1016/j.anireprosci.2014.02.005
123. Viguer, J M. “Reproducción e inseminación artificial en animales”. *E.S.E Hafez* (1987): 47
124. Waddington CH. The epigenotype. Endeavour. 1942;1:18–20. [Google Scholar]
125. Waheed, M M et al. “Seminal plasma and serum fertility biomarkers in dromedary camels (Camelus dromedarius).” *Theriogenology* vol. 83,4 (2015): 650-4. doi:10.1016/j.theriogenology.2014.10.033
126. WHO. WHO laboratory manual for the Examination and processing of human semen. 2010; Available from: https://apps.who.int/iris/bitstream/handle/10665/44261/9789241547789_eng.pdf;jsessionid=A02692004380B7C87B844A6652346549?sequence=1.
127. Wirth, Julia J, and Renée S Mijal. “Adverse effects of low level heavy metal exposure on male reproductive function.” *Systems biology in reproductive medicine* vol. 56,2 (2010): 147-67. doi:10.3109/19396360903582216
128. Yániz, J L et al. “Computer assisted sperm morphometry in mammals: a review.” *Animal reproduction science* vol. 156 (2015): 1-12. doi:10.1016/j.anireprosci.2015.03.002
129. Zegers-Hochschild, Fernando et al. “The International Glossary on Infertility and Fertility Care, 2017.” *Human reproduction (Oxford, England)* vol. 32,9 (2017): 1786-1801. doi:10.1093/humrep/dex234
130. Zheng, B L et al. “Effect of a lipidic extract from lepidium meyenii on sexual behavior in mice and rats.” *Urology* vol. 55,4 (2000): 598-602. doi:10.1016/s0090-4295(99)00549