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The Environment and Male Fertility: observational study evaluating semen quality in a large cohort of young men living in the «Land of Fires»

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INDEX

Abstract……p. 3

Introduction……p. 5

Temporal and geographic trends in semen quality……p. 5

Male fertility as a target of environmental exposures: heavy metals ……p. 10

The "Land of Fires" phenomenon……p. 15

Aim of the research study……p. 17

Participants and methods……..p. 17

Recruitment and enrolment of participants……p. 17

Clinical procedures and blood collection……p. 19

Semen analysis……p. 21

Trace elements analysis……p. 22

Participants data collection……p. 23

Statistical analysis……p. 23

Study design…….. p. 24

Results…… p. 26

Participants characteristics and semen quality……p. 26

Trace elements burden in serum and semen and semen quality……p. 33

Assessment of confounders……p. 42

Discussion……...p. 47

Bibliography……..p. 52

Abstract

Campania Region has been characterized by a waste management crisis since 1980, resulting in the largely documented illegal disposal of urban, toxic and industrial waste, and diffuse practice of illegal waste burning set up by residents, which by time determined a significant increase in local environmental pressure. Despite mounting media attention and pervasive concern among residents in regards of potential waste exposure-induced health problems, no large investigations have been performed so far, addressing semen quality of young men living within the “Land of “Fires” (LF), and/or the potential implication of heavy metals burden, quantitatively determined in a large number of semen samples. The aim of the current research study was 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 between non-occupational exposure to non-essential trace elements, particularly cadmium (Cd), and seminal parameters, by evaluating a large cohort of healthy men with at least 10 years of residence within the LF, exhaustively controlled for potential confounders. 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.

Within the first cohort (C1), 730 participants were enrolled: 544 healthy males from HI areas belonging to the LF, and 186 healthy males resident in a low environmental impact (LI) area not belonging to the LF. Within the second cohort (C2) 512 participants were enrolled, resident in the HI municipalities of Acerra (N=197), Afragola (N=117), and Giugliano in Campania (N=162), belonging to the LF. Participants were interviewed on medical history and underwent a complete physical examination including evaluation of anthropometric characteristics, urogenital examination, and scrotal and prostate transrectal ultrasonography, and semen analysis according to the 2010 World Health Organization laboratory manual guidelines. Trace

elements quantitative determination by ICP-MS was performed in serum and semen of C2. 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 HI areas of Campania Region, did not differ from age- and BMI-matched controls from LI areas 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. The current study also demonstrated that Cd, a known reproductive toxicant, might be detected in semen at significantly higher concentrations than serum, suggesting that seminal Cd determination might serve as a sensitive earlier marker of exposure, particularly in non-occupationally exposed men, and that 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, therefore supporting the assumption that even micro-doses of metal may have effects on semen quality. Concurrent factors potentially implicated in semen Cd burden might be snatched from additional observations deriving from the current study, including the potential presence of isolated sources of Cd in specific areas within the HI target of the study, or the potential contribution of the balance between seminal concentrations of essential and non-essential trace elements to the individual susceptibility to Cd accumulation within the testis.

1. Introduction

1.1 Temporal and geographic trends in semen quality

Couple infertility is defined as the failure to establish a clinical pregnancy after 12 months of regular unprotected sexual intercourses [1]. Female factors contribute to about 37% of infertility problems, whereas male factors account for about 29%, and combined female and male factors for about 18% of causes; the remaining 16% is due to genetic factors (1%), or to unexplained, or idiopathic, infertility, which is diagnosed in absence of a specific etiological determinant [2].

Worldwide decline in human fertility has been recorded since the early 1950s, and reported by the international literature [3], and a steady decay of birth rates in all European countries was reported by several demographic surveys [4]. Such a consistent, progressive, a widespread deterioration of couple fertility is not likely justified exclusively by changes in lifestyle (increased women occupational rate, increased maternal age at pregnancy), prevention of undesired parenthood, or increased socio-economic burden of parenthood (Figures 1, 2).

Providing proper definition of male infertility is challenging; diagnosis is traditionally based on semen analysis, according to the 2010 World Health Organization laboratory manual guidelines [5]. Worldwide sperm counts have been reported to 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 [6]; in particular, average sperm counts of 100 million, 75 million, and 50 million/ml were reported in 1950, 1970, and 1990, respectively, in western countries [7]. The 1992 metanalysis raised much attention as well as controversies and animated debate, since historical data on semen quality collected for different aims were obviously poorly homogeneous, mainly due to selection bias

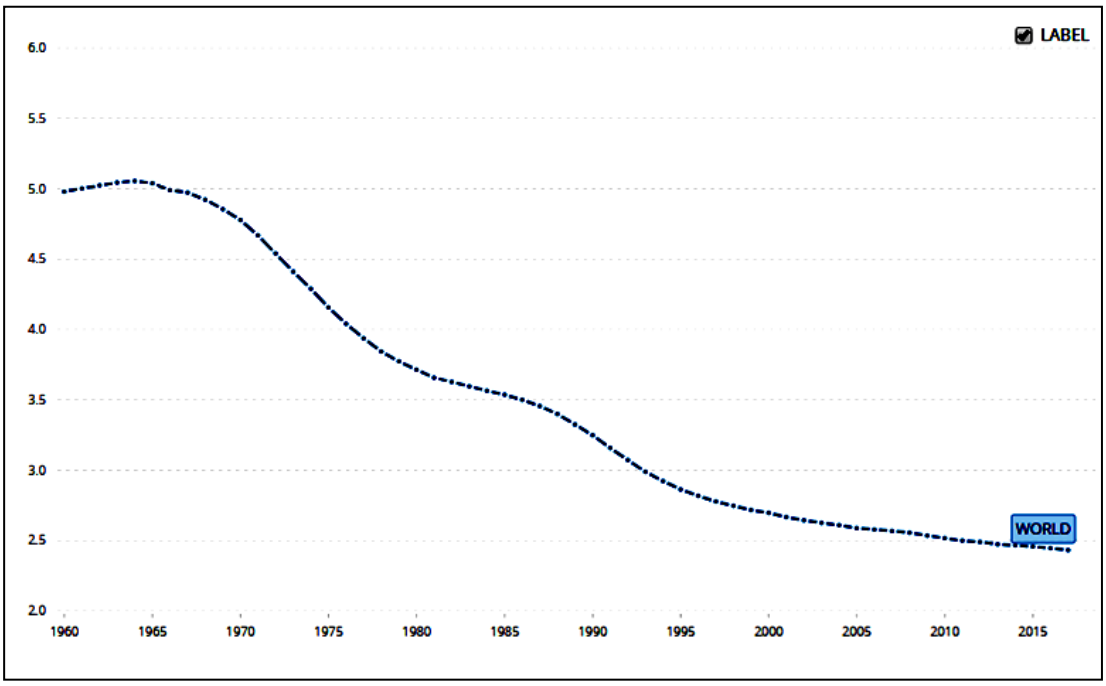

Figure 1. Fertility rate, total (births per woman, weighted average). Trends in birth rate from 1960 to 2015, showing a progressive decline in worldwide total fertility rate. Total fertility rate represents the number of children that would be born to a woman if she were to live to the end of her childbearing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

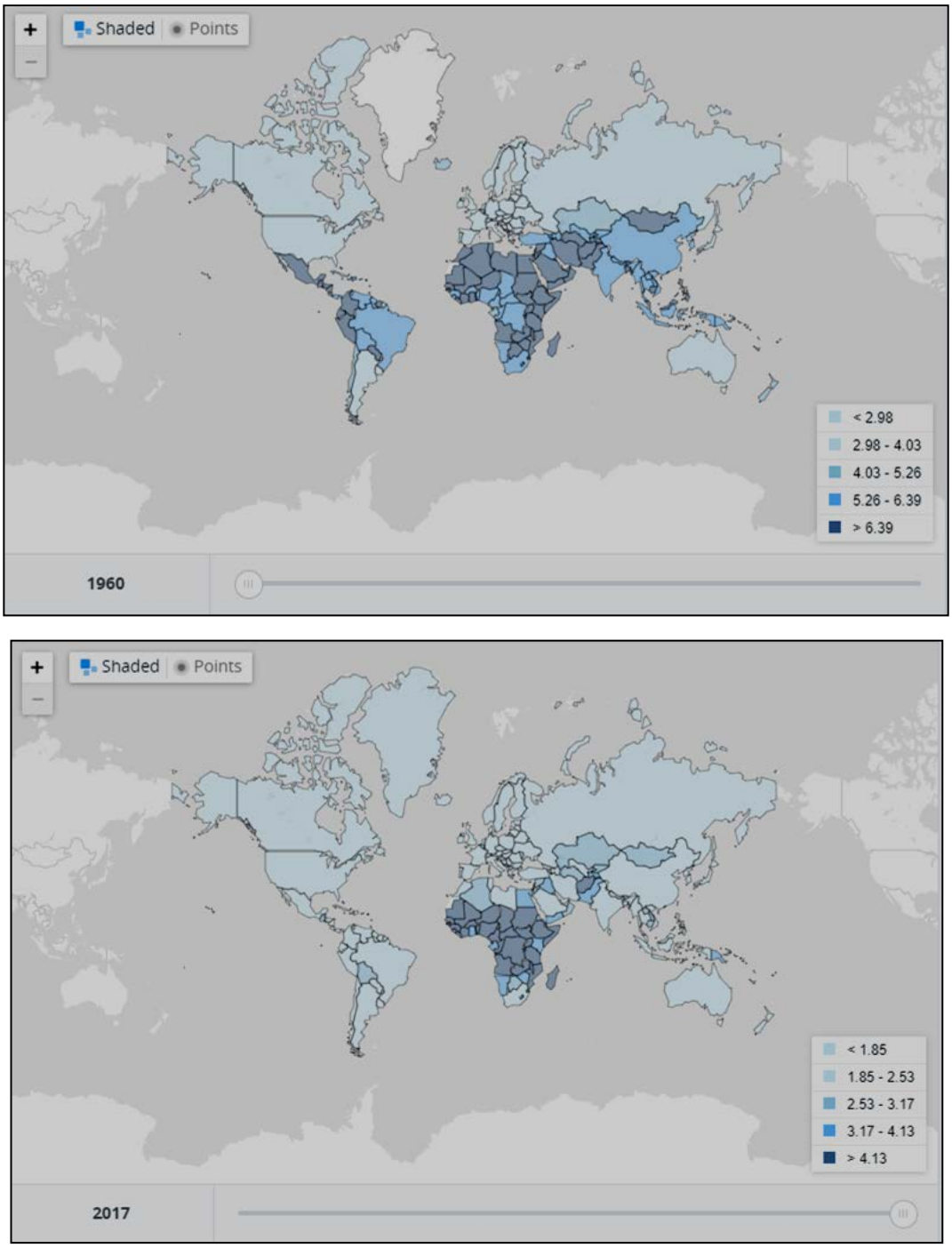

Figure 2. Fertility rate, total (births per woman, weighted average). Map showing trends in birth rate from 1960 (upper panel) to 2015 (lower panel), in relation to geographical setting https://data.worldbank.org/indicator/SP.DYN.TFRT.IN?view=map.

and differences in study design, which was predominantly retrospective; moreover, it was argued that the observed decline could mirror heterogeneous geographical setting, rather than temporal variations in sperm concentration [8]. A scrupulous re-analysis of 1992 metanalysis included 56 out of 61 studies and modeled results adjusting for confounders and bias sources such as age, abstinence, proven fertility, method of analysis, and main aim of the individual studies; when stratified for geographical location, estimated decline in mean sperm concentration was 1.5% per year in the United States (1938–1988) and 3.1% per year in Europe (1971–1990) [9]; declining semen quality was afterwards confirmed by an extended metanalysis of 101 studies (1934-1996) [10]. As a matter of fact, even 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 [11]. Several more 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 [12] (Figure 3). Therefore, the contribution of concurrent, global and local factors should be taken into account to explain differences among studies.

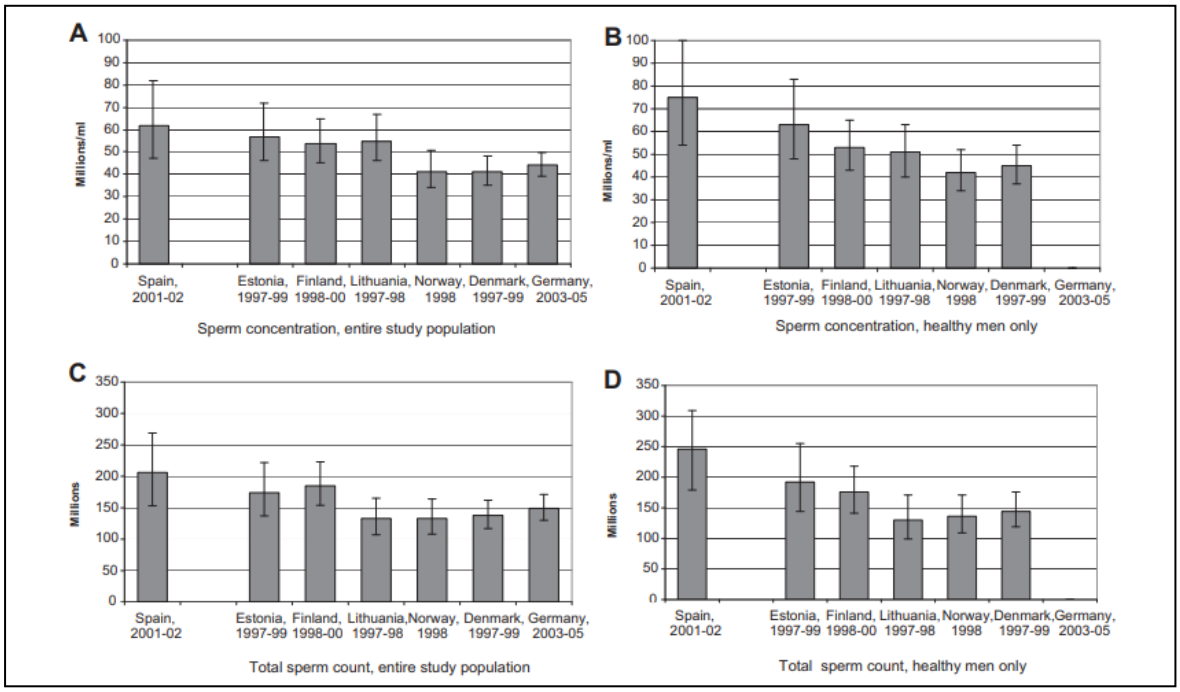

Figure 3. Sperm concentrations and sperm total count of young men from seven European countries. The bars show the adjusted median sperm concentration (nx106/ml) and sperm total count (nx106/ejaculate) in the entire study populations (A and C) and in subgroups of men without any recent use of medicine or known andrological disease (B and D) [12].

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

Human semen contains various 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 [13]. Physiological concentrations of Zn, Mn, Se and Cu in human sperm and seminal plasma are positively correlated with anti-oxidants concentration and negatively correlated with lipid peroxidation; high concentrations of K and Na can be found in human seminal plasma, and these elements exert an important role in acrosome reaction; Mg and Ca maintain the osmotic balance and are essential for sperm capacitation, acrosome reaction and spermatozoa hyperactive motility [13]. 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 [13]. On the other hand, seminal parameters exhibit a biphasic response to some trace elements such as Mn and Cu, and hormesis has been described, with excessively increased concentrations of these elements being negatively correlated with semen quality [13] (Figure 4). Contrary to essential trace elements, 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 raise of environmental pollutants, particularly in countries with poorer surveillance and regulations [14, 15]. Accumulating 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, there is growing concern for the effects of heavy metals on semen quality and male fertility, even at low environmental, non-occupational, level of exposure [16]. Nevertheless, although the effects of high level exposure to several non-essential heavy metals of concern, particularly cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), and mercury

Elements	Study models	Findings
Ca deficiency	Human seminal	↓Sperm motility
Ca deficiency	Human seminal	↓Steroidogenesis; ↓testosterone
Ca deficiency	Human seminal	↓Sperm chemotaxis
Ca deficiency	Human seminal	↓Sperm acrosome reaction
Ca deficiency	Human seminal	↓Fertilization process
Ca deficiency	Human seminal	↓Semen volume, ↓sperm counts; ↓sperm motility
Na & K deficiency	Human seminal	↓Fertilization rate
Na & K deficiency	Human seminal	↓Sperm quality
Na & K deficiency	Human seminal	↓Semen volume
Na deficiency	Intracellular	↓Sperm capacitation
K deficiency	Human seminal	↓Testosterone
Na deficiency	Human seminal	↓Progesterone; ↓sperm acrosome reaction
Mg deficiency	Human seminal	↓Premature ejaculation
Mg deficiency	Human seminal	↓Sperm motility
Zn deficiency	Human seminal	↓Sperm quality
Zn deficiency	Body	↓Testicular development & function
Zn deficiency	Human seminal	↓Sexual maturation; ↑hypogonadism; ↑gonad dysfunction; ↓testicular weight; ↑Leydig cells damage; ↑testicular atrophy; ↑seminiferous tubules damage
Zn deficiency	Human seminal	↓Steroidogenesis; ↓testosterone; ↓spermatogenesis
Zn deficiency	Human seminal	↓Sperm capacitation; ↓acrosome reaction
Zn deficiency	Human seminal	↓Sperm quality; ↑ROS; ↑oxidative stress; ↑lipid peroxidation; ↓sperm membrane fluidity; ↓sperm–egg interaction; ↓fertilization; ↓antioxidant capacity
Se deficiency	Dietary intake	↑Oxidative stress; ↓spermatogenesis; ↓sperm quality
Se deficiency	Human seminal	↓Secretion of testosterone; ↓spermatogenesis
Se deficiency	Human seminal	↓Sperm count; ↓motility; ↓normal morphology; ↓vitality
Mn deficiency	Human seminal	↓Sperm quality
Mn deficiency	Human seminal	↓Seminal fluid volume; ↓sperm normal morphology
Increased Mn level	Human seminal	↓Sperm motility; ↓sperm count
Cu deficiency	Human seminal	↓Sperm quality
Cu deficiency	Human seminal	↑Oxidative stress; ↓SOD activity
Increased Cu level	Human seminal	↓Sperm motility

Figure 4. Overview of the effect of some trace element deficiency and/or excess on semen quality and male reproductive function [13].

(Hg), on male reproductive function have been clearly demonstrated by experimental studies in animal models and/or in human occupational exposure studies [17-21], there is often lack of strong confirmatory clinical studies concerning low environmental level of exposure, and evidences are 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 [16, 22].

The presence of Cd and Cd 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; 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 [21, 23]. Testis is particularly susceptible to Cd poisoning, and Cd has been repeatedly shown, in experimental studies in animal models and human spermatozoa, 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 [23] (Figure 5). Epidemiological studies addressing the effect of environmental Cd exposure on semen quality have reported conflicting results, although the majority suggested a negative correlation between Cd burden and most seminal parameters [23]; inconsistencies among studies might be addressed by discrepancies in cohorts selection, statistical drawbacks, and the inappropriate choice of the biological matrix for Cd burden

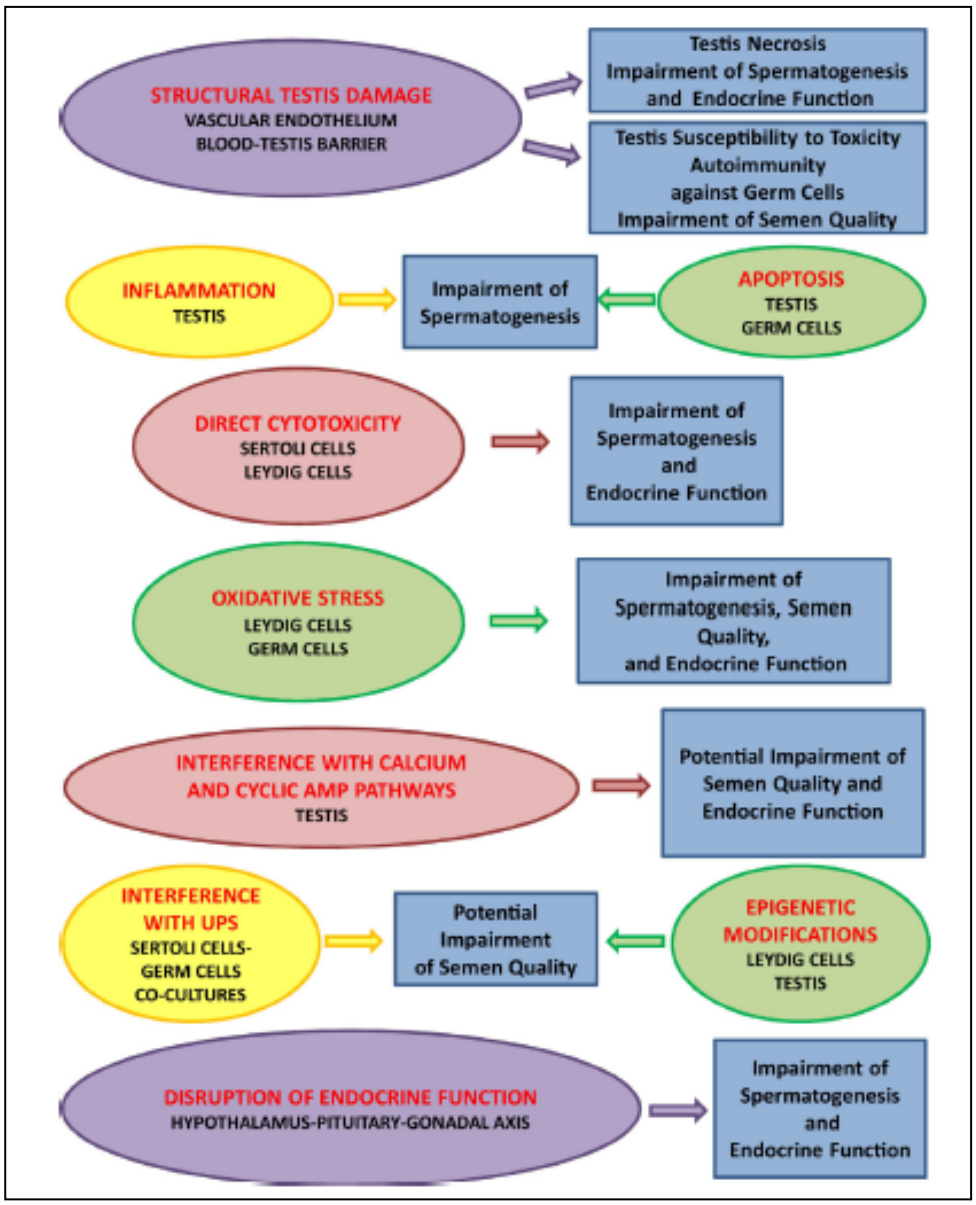

Figure 5. Overview of the proposed pathogenetic mechanisms of cadmium reproductive toxicity. Colored circles report the main pathogenetic mechanisms of cadmium reproductive toxicity,along with the corresponding target organ or cell. The word “testis” refers to pathogenetic mechanisms demonstrated by experiments *in vivo*, or on whole testis homogenates. Light blue squares represent the proposed or hypothesized final effect on male reproductive function [23].

quantification. It is now largely demonstrated that heavy metals have a heterogeneous distribution within human body fluids and/or compartments, and that specific metals, such as Cd, Pb and Zn, display preferential accumulation in male reproductive organs [24-26]; therefore, biological matrices commonly used for metal burden determination may not precisely reflect the real local exposure of the male reproductive tract, whereas determination in semen certainly represent a more suitable substrate [24-26]. 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 [22].

Most of the evidences provided by animal studies significantly contributed to the identification of the specific Cd targets, and to 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.

1.3 The "Land of Fires" phenomenon

Campania Region has been characterized by a waste management crisis since 1980, resulting in the largely documented illegal disposal of urban, toxic and industrial waste, which include several types of substances such as heavy metals, polychlorinated biphenyls, hydrocarbons etc. [27]; illegal burying of waste in not legally identified sites such as rural or agricultural areas, roads and buildings and construction yards, along with the diffuse practice of illegal waste burning and the fires of garbage disposed in the streets set up by residents, determined a significant increase in local environmental pressure [27]. 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 [27]. 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 (law n.6, 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 within 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 nitrogen oxide and Pb exposure, 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 [28, 29]. 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 [30]. In addition, a more recent pilot human biomonitoring study on trace elements 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 index, and reduced redox biomarkers and antioxidant enzymes activity, in semen from LF, compared to a low environmental pressure area [31]. No large investigations have been performed so far, addressing semen quality of young men living within the LF, and/or the potential implication of heavy metals burden, quantitatively determined in a large number of semen samples.

2. Aim of the research study

The aim of the current research study was 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 between non-occupational exposure to non-essential trace elements, particularly Cd, and seminal parameters, by evaluating a large cohort of healthy men with at least 10 years of residence within the LF, exhaustively controlled for potential confounders.

3. Participants and methods

3.1 Recruitment and enrolment of participants

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 [32] (Figure 6). Awareness campaigns were promoted by Centro 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; subjects were also actively recruited by systematic direct phone call based on municipality resident lists and with the support of general practitioners and local pharmacists.

Subjects from both the awareness campaign attending to FERTISEXCARES centre had to meet the following inclusion criteria to be eligible for enrolment in study cohorts: residence for at least 10 years in Campania Region; no known chronic diseases (diabetes, endocrine disease,

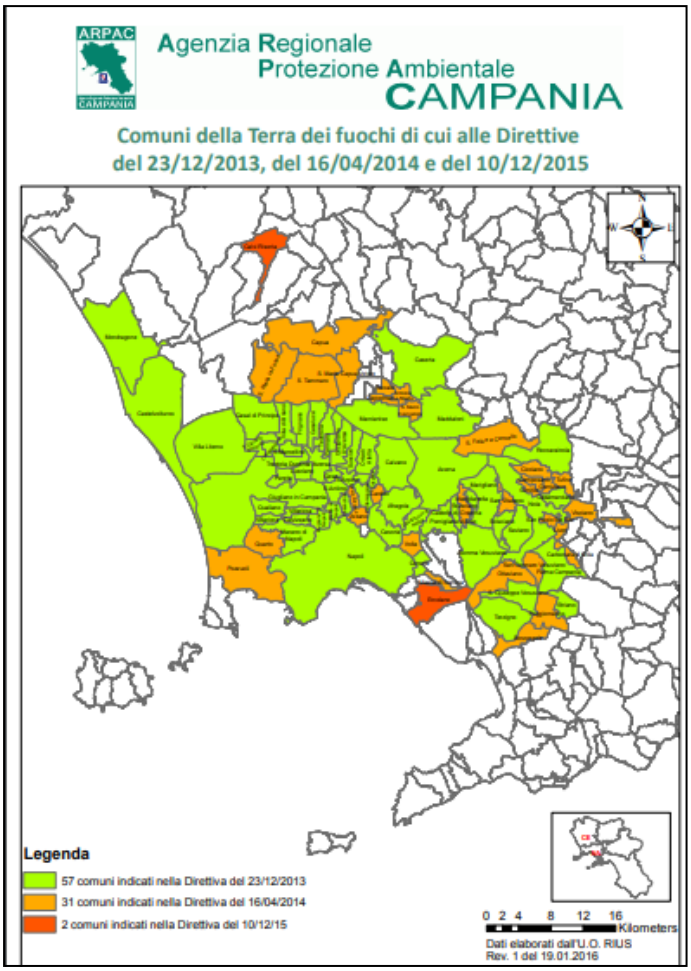

Figure 6. Map depicting the 90 municipalities of the province of Naples and Caserta belonging to the "Land of Fires", an area officially recognized as a high environmental impact area on the basis of the Campania Region Environmental Protection Agency reports (2013-2014-2015), identifying the "Land of 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.

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 uncontrolled waste incineration [32], 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 7. 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.

3.2 Clinical procedures and blood collection

Participants were interviewed on medical history and underwent a complete physical examination including evaluation of anthropometric characteristics, weight, height, body mass

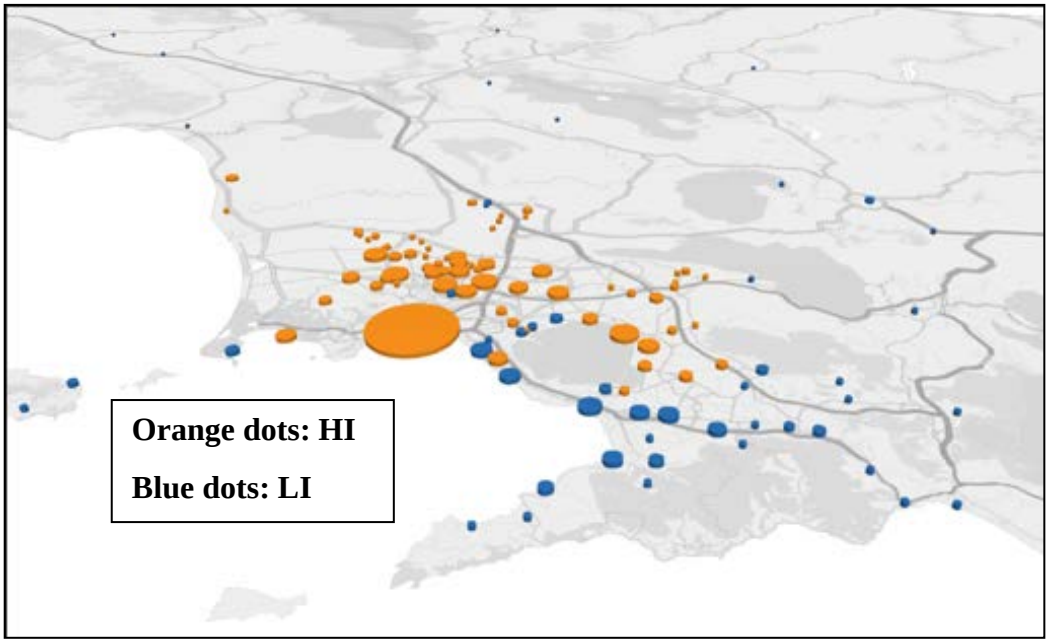

Figure 7. Relative geographical distribution of research study participants residential municipalities within Campania Region, referred to the entire C1 cohort: 544 males resident in a high environmental impact (HI) area comprising municipalities within the Province of Naples and Caserta belonging to 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.

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.

3.3 Semen analysis

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 [5]. 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 (40X) 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% [5]. 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.

3.4 Trace elements analysis

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 [33][REF]. 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%.

3.5 Participants data collection

Participants were interviewed face-to-face by a specifically trained biologist using a structured questionnaire to collect data concerning lifestyle, dietary information, smoking habit and alcohol consumption, physical activity, occupational history with self-reported occupational exposure to toxic chemicals. The interview also investigated parental residential information, occupational exposure to toxic chemicals, smoking habit and alcohol consumption in the peri-conceptional and prenatal period, mother's age at birth, breastfeeding.

3.6 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 [5]; 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.

4. Study design

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. C2 participants were categorized into three groups, according to residential address being comprised within HI areas, namely, municipality of Acerra, Afragola, or Giugliano in Campania; a preliminary descriptive analysis of seminal parameters was performed and prevalence of pathological seminal parameters was determined in all groups, for comparison. 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 8.

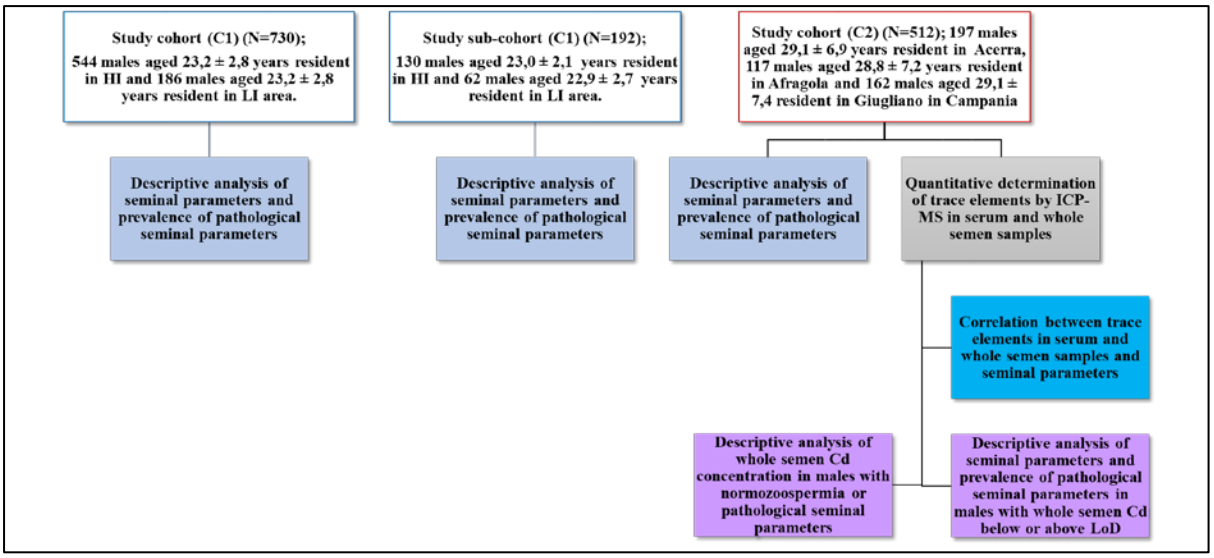

Figure 8. Flow diagram of research study. Research study included Caucasian healthy males of reproductive age (13-50 years old) resident in Campania Region. Two different cohorts of subjects (C1, C2) were recruited within two awareness and prevention campaigns on infertility and testis cancer in high 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.

5. Results

5.1 Participants characteristics and semen quality

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 1.

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 2.

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,

	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 1. C1 cohort characteristics and seminal parameters expressed as mean ± SD.

	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 2. Prevalence of normozoospermia and pathological seminal parameters in C1 cohort.

prostatitis, other urogenital infections, testicular trauma, history of testicular injury or surgery, Klinefelter Syndrome (Table X).

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 3.

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 4.

In C2 cohort (N=512), mean residence period in a HI areas 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%. Participants from Acerra (N=197), Afragola (N=117), and

	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 3. 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 4. Prevalence of normozoospermia and pathological seminal parameters in C1 sub-cohort.

Giugliano in Campania (N=162) groups did not differ for age (29,1 ± 6,9 vs 28,8 ± 7,2 vs 29,1 ± 7,4), BMI (25,9 ± 3,7 vs 25,3 ± 4,7 vs 26,0 ± 3,7), diet, physical activity or self-reported occupational exposure to toxic chemicals (13,3% vs 9% vs 11,6%). Prevalence of never-, past-, and current-smokers was not different among municipalities: Acerra 38,4%, 12,4%, and 49,7%; Afragola 52,0%, 7,0%, and 41,0%; Giugliano in Campania 42,3% 6,3%, and 50,7%.

All groups showed similar values for pH (8,3 ± 0,3 vs 8,3 ± 0,3 vs 8,3 ± 0,3), semen volume (3,0 ± 1,5 vs 3,2 ± 1,6 vs 3,3 ± 1,9), sperm concentration (36,9 ± 31,7 vs 39,5 ± 31,3 vs 37,0 ± 27,2) sperm total count (106,5 ± 112,2 vs 119,5 ± 105,8 vs 113,9 ± 94,6), total sperm motility (55,9 ± 16,1 vs 57,2 ± 15,7 vs 57,8 ± 16,6), progressive sperm motility (49,3 ± 17,1 vs 50,2 ± 16,5 vs 51,5 ± 16,4), normal sperm morphology (7,5 ± 4,1 vs 8,2 ± 4,1 vs 8,2 ± 3,8) and abnormal sperm morphology (92,5 ± 4,1 vs 91,8 ± 4,1 vs 91,8 ± 3,8). C2 cohort characteristics and seminal parameters are shown in Table 5.

Prevalence of normozoospermia and pathological seminal parameters did not differ among Acerra (65,1% and 34,9%), Afragola (65,2% and 34,8%), and Giugliano in Campania (769,7% and 30,3%). No significant differences were detected among municipalities, concerning prevalence of oligozoospermia (14,6% vs 16,1% vs 12,3%); teratozoospermia (0,5% vs 0,9% vs 0,7%); oligo-asthenozoospermia (4,1% vs 1,8% vs 4,9%); oligo-teratozoospermia (3,1% vs 0% vs 2,6%); astheno-teratozoospermia (1,6% vs 0,9% vs 1,3%); oligo-astheno-teratozoospermia (8,3% vs 5,4% vs 4,5%), and azoospermia (2,1% vs 2,7% vs 1,9%), although a significantly higher prevalence of asthenozoospermia was detected in Afragola, compared to Acerra (6,3% vs 1,0%). Prevalence of normozoospermia and pathological seminal parameters in C2 cohort is shown in Table 6.

	Total C2 cohort (N=512)	Acerra (N=197)	Afragola (N=117)	Giugliano in Campania (N=162)	p
Age (years)	29,1 ± 7,2	29,1 ± 6,9	28,8 ± 7,2	29,1 ± 7,4	NS
BMI (Kg/m^2)	25,8 ± 4,1	25,9 ± 3,7	25,3 ± 4,7	26,0 ± 3,7	NS
pH	8,3 ± 0,3	8,3 ± 0,3	8,3 ± 0,3	8,3 ± 0,3	NS
Semen Volume (ml)	3,1 ± 1,7	3,0 ± 1,5	3,2 ± 1,6	3,3 ± 1,9	NS
Sperm Concentration (nx10^6/ml)	37,5 ± 30,2	36,9 ± 31,7	39,5 ± 31,3	37,0 ± 27,2	NS
Sperm Total Count (nx10^6/ejaculate)	111,2 ± 104,0	106,5 ± 112,2	119,5 ± 105,8	113,9 ± 94,6	NS
Total Sperm Motility (%)	56,8 ± 16,1	55,9 ± 16,1	57,2 ± 15,7	57,8 ± 16,6	NS
Progressive Sperm Motility (%)	50,2 ± 16,6	49,3 ± 17,1	50,2 ± 16,5	51,5 ± 16,4	NS
Normal Sperm Morphology (%)	8,0 ± 4,0	7,5 ± 4,1	8,2 ± 4,1	8,2 ± 3,8	NS
Abnormal Sperm Morphology (%)	92,0 ± 4,0	92,5 ± 4,1	91,8 ± 4,1	91,8 ± 3,8	NS

Table 5. C2 cohort characteristics and seminal parameters expressed as mean ± SD.

	Total C2 cohort (N=512)	Acerra (N=197)	Afragola (N=117)	Giugliano in Campania (N=162)	p
Normozoospermia (%)	66,6	65,1	65,2	69,7	NS
Pathological seminal parameters (%) any type	33,4	34,9	34,8	30,3	NS
Oligozoospermia (%)	14,0	14,6	16,1	12,3	NS
Asthenozoospermia (%)	3,0	1,0*	6,3*	1,9	*p<0,05
Teratozoospermia (%)	0,6	0,5	0,9	0,7	NS
Oligo_Asthenozoospermia (%)	3,4	4,1	1,8	3,9	NS
Oligo_Teratozoospermia (%)	2,4	3,1	0,0	2,6	NS
Astheno_Teratozoospermia (%)	1,2	1,6	0,9	1,3	NS
Oligo_Astheno_Teratozoospermia (%)	6,1	8,3	5,4	4,5	NS
Azoospermia (%)	2,2	2,1	2,7	1,9	NS

Table 6. Prevalence of normozoospermia and pathological seminal parameters in C2 cohort.

5.2 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). Relative distribution of available samples among the three municipalities of interest was as follow: Acerra 106 serum, 153 semen; Afragola 44 serum, 89 semen; Giugliano in Campania 94 serum, 120 semen.

Trace elements burden in serum and semen was correlated to seminal parameters in the entire C2 cohort, and by single municipality. In a preliminary analysis, the most consistent results in terms of statistical significance and available number of samples was found for Cd and Se; therefore, further analyses were performed on these selected trace elements. Both serum and semen burden of Cd and Se did not differ among municipalities of Acerra, Afragola and Giugliano in Campania. For both Cd and Se, serum burden did not correlate to semen burden, in total C2 cohort (Table 7) and in single municipalities. Conversely, a negative correlation was found between Cd and Se burden in semen in total C2 cohort ($r= -0,280$; $p<0,01$) (Table 7), as well as in Acerra ($r= -0,371$; $p<0,01$) and Afragola ($r= -0,597$; $p<0,01$), but not Giugliano in Campania. 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 9), as well as in Acerra (1,0 μg/l vs 0,6 μg/l; $p<0,01$), but not in Afragola and Giugliano in Campania; 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 9), as well as in Acerra (66,9 μg/l vs 55,0 μg/l; $p<0,0001$), Afragola (68,6 μg/l vs 56,8 μg/l; $p<0,0001$) and Giugliano in Campania (67,4 μg/l vs 51,9 μg/l; $p<0,0001$).

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

	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 7. 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.

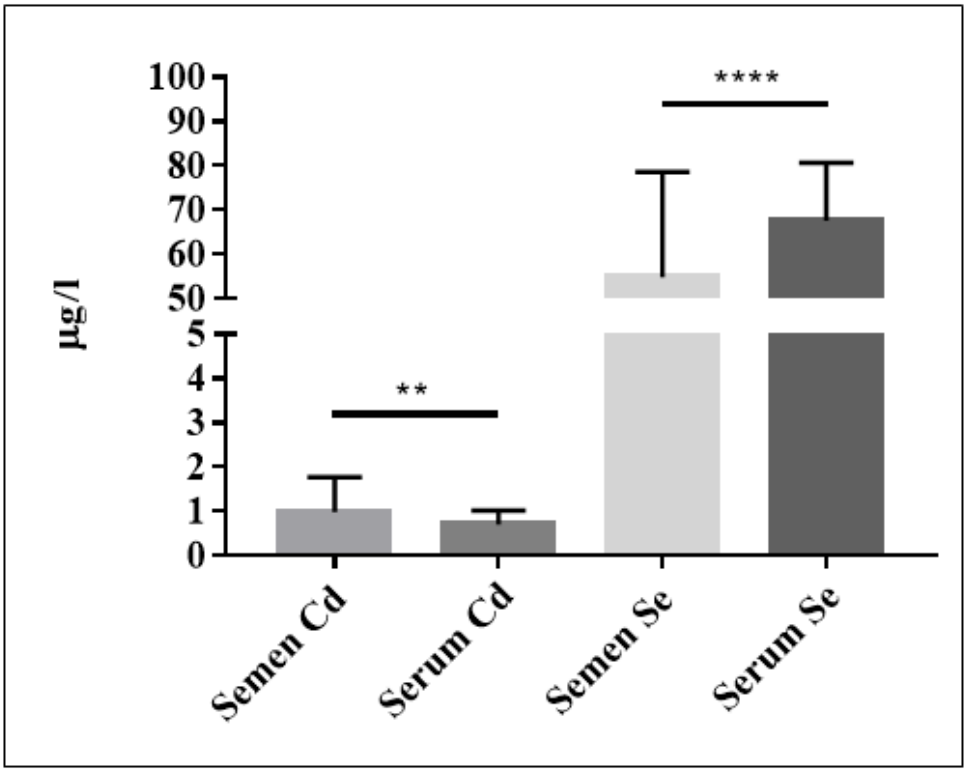

Figure 9. Comparison between semen and serum cadmium (Cd) and selenium (Se) concentrations (µg/l), expressed as mean ± SD, within the same participants in total C2 cohort; mean Cd concentration was significantly higher in semen, compared to serum (1,0 µg/l vs 0,8 µg/l), whereas mean Se concentration was significantly higher in serum, compared to semen (67,6 µg/l vs 54,7 µg/l). **p<0,01, ****p<0,0001.

C2 cohort (Table 8) (Figures 10, 11); no significant correlation was found when single municipalities were considered. 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 8); these correlations were maintained when single municipalities were considered.

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; Acerra had 34,6% (53/153) detectable and 65,4% (100/153) undetectable semen samples; Afragola had 23,6% (21/89) detectable and 76,4% (68/89) undetectable semen samples; Giugliano in Campania had 35,8% (43/120) detectable and 64,2% (77/120) undetectable semen samples (Figure 12).

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

	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 8. 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.

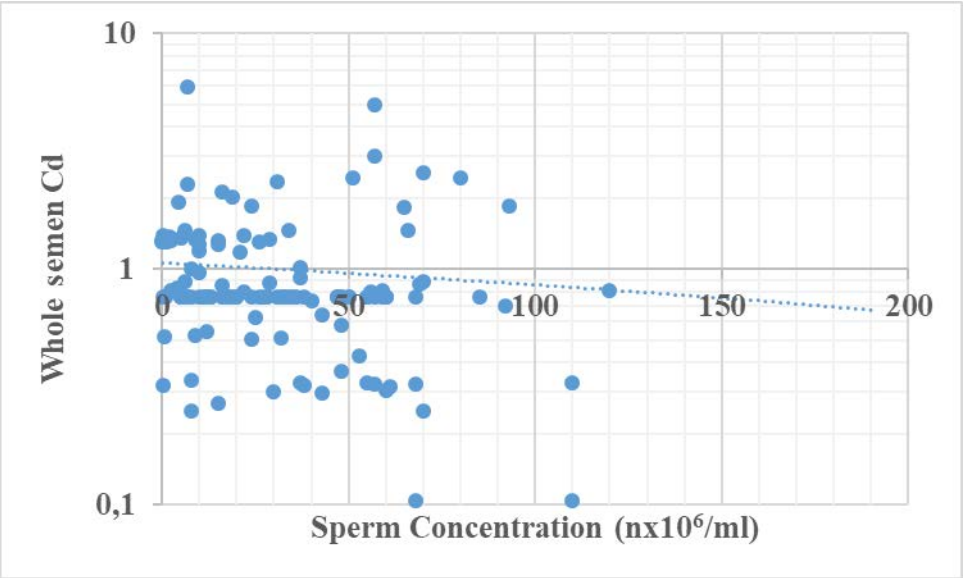

Figure 10. Distribution of semen cadmium (Cd) concentration plotted on a Log10 scale against sperm concentration. Semen Cd was negatively correlated to sperm concentration (r= -0,211; p<0,05), in total C2 cohort.

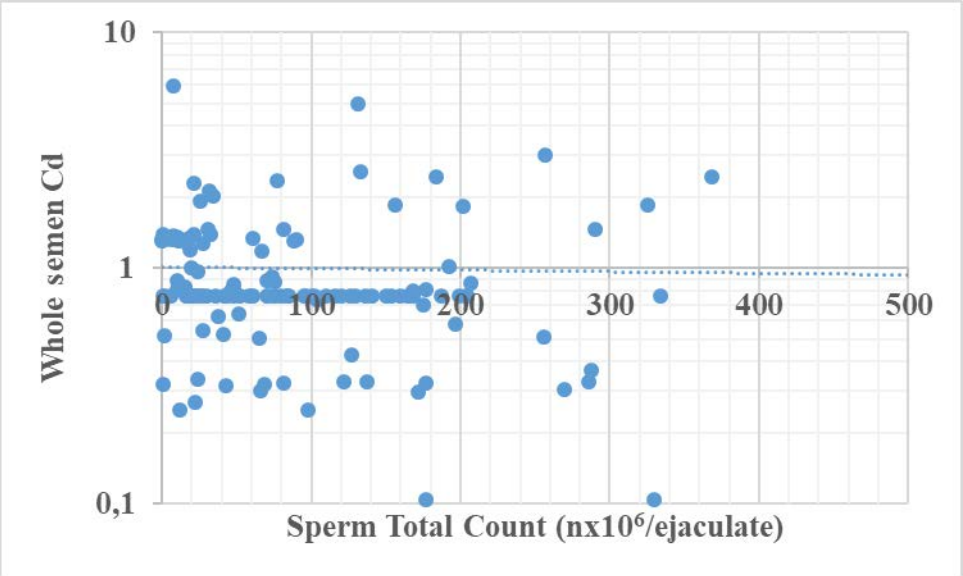

Figure 11. Distribution of semen cadmium (Cd) concentration plotted on a Log10 scale against sperm total count. Semen Cd was negatively correlated to sperm total count (r= -0,177; p<0,05), in total C2 cohort.

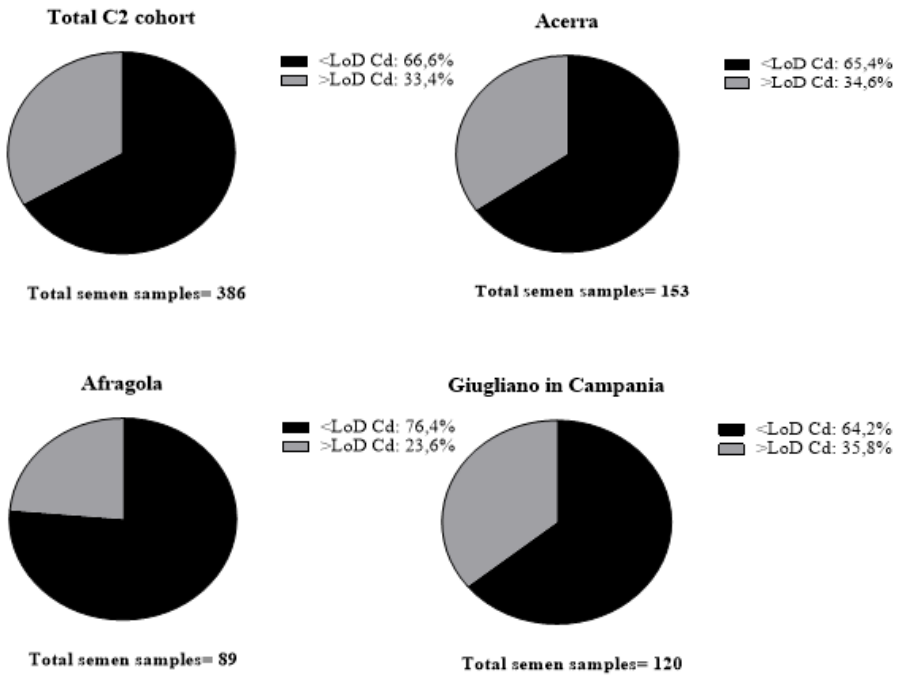

Figure 12. Percentage of semen samples with cadmium (Cd) concentration below or above limit of detection (LoD) cutoff (0,20 µg/l): the total C2 cohort had 33,4% (129/386) detectable and 66,6% (257/386) undetectable semen samples; Acerra had 34,6% (53/153) detectable and 65,4% (100/153) undetectable semen samples; Afragola had 23,6% (21/89) detectable and 76,4% (68/89) undetectable semen samples; Giugliano in Campania had 35,8% (43/120) detectable and 64,2% (77/120) undetectable semen samples.

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 9.

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 10.

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 13).

	Undetectable semen Cd (N=257)	Detectable semen Cd (N=129)	p
Age (years)	29,5 ± 7,0	30,5 ± 7,5	NS
BMI (Kg/m^2)	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 (nx10^6/ml)	35,4 ± 26,3	32,2 ± 26,6	NS
Sperm Total Count (nx10^6/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 9. 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 10. 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).

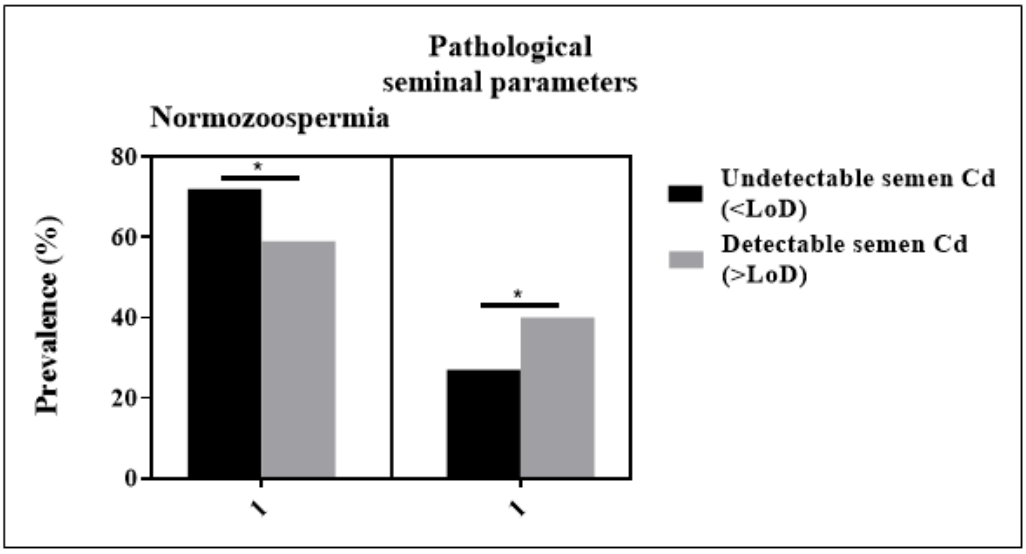

Figure 13. Prevalence of normozoospermia and pathological seminal parameters in C2 cohort, grouped as semen cadmium (Cd) below or above limit of detection (LoD) cutoff (0,20 µg/l). Normozoospermia was significantly less prevalent (59,7% vs 72,4%), whereas, as expected, pathological seminal parameters were significantly more prevalent (40,3% vs 27,6%) in detectable, compared to undetectable samples; *p<0,05.

5.3 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 out at first by 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. Relative geographical distribution of smokers and non smokers is shown in Figure 14. Specifically, residential municipality percentages for smokers vs non-smokers were as follow: 43,5% from Acerra, 18,0% from Afragola, 34,8% from Giugliano in Campania, 3,7% from other different municipalities, in smokers, vs 37,6% from Acerra, 25,7% from Afragola, 28,2% from Giugliano in Campania, 8,4% from other different municipalities, in non-smokers; proportion of residential distribution among Acerra, Afragola and Giugliano in Campania was not different in the smokers vs non-smokers groups, and smokers and non-smokers were well balanced for each municipality (Acerra: smokers N=70, non-smokers N=76; Giugliano in Campania; smokers N=56, non-smokers N=57), except for Afragola, which had a higher prevalence of non-smokers (N=29 vs N=52).

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, 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 15.

In regards to semen quality, no differences were detected between smokers and non-smokers resident in Acerra and Afragola, whereas smokers resident in Giugliano in Campania had significantly lower sperm concentration (27,9% vs 40,5%; $p<0,05$) and normal sperm

morphology (7,2% vs 8,8%; $p<0,05$), and significantly higher abnormal sperm morphology 92,8% vs 91,2%; $p<0,05$), compared to non-smokers of the same municipality.

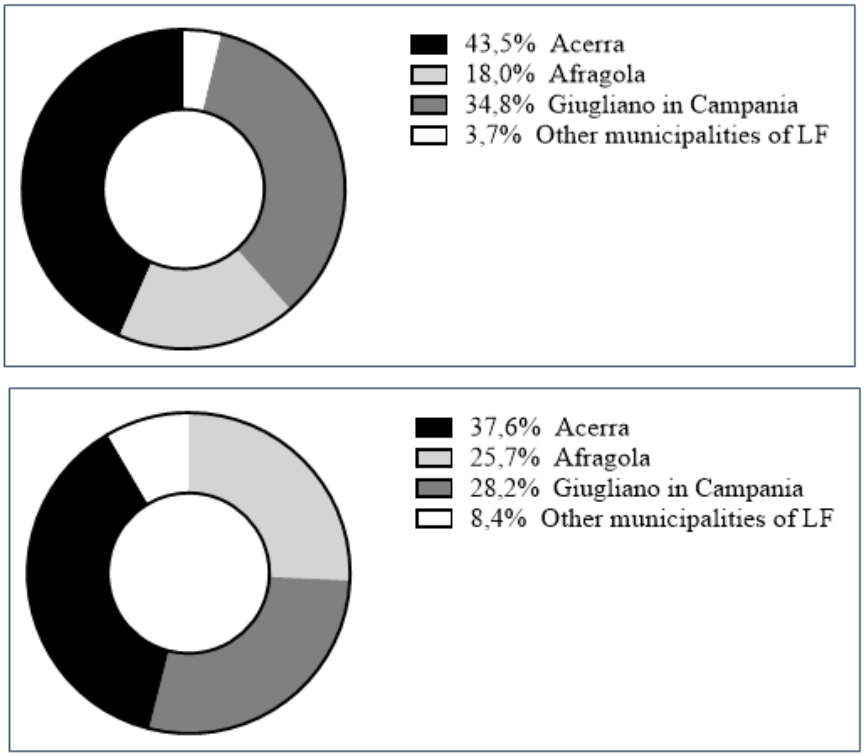

Figure 14. Relative residential distribution of smokers (upper panel) and non-smokers (lower panel) within HI areas.

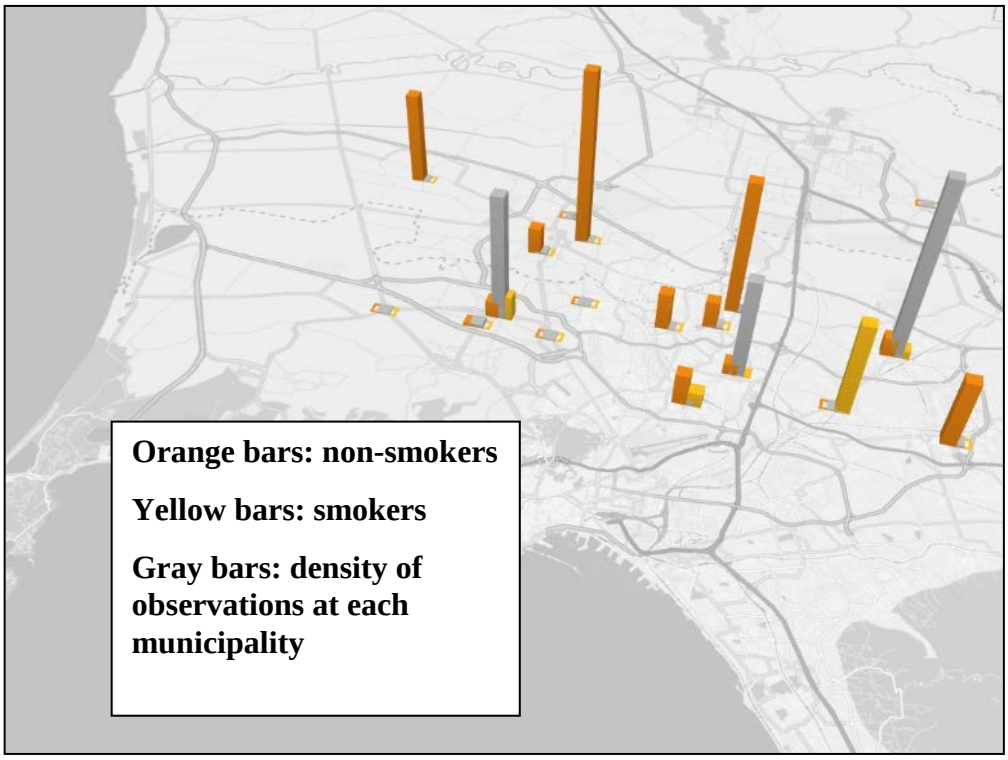

Figure 15. Geographical distribution of semen Cd detection by municipality. Bars represent cumulative semen Cd burden (pooled values) in smokers (yellow bars) vs non-smokers (orange bars); gray bars indicate the number of samples assayed at each municipality, therefore, the higher the bar, the stronger 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. Discussion

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. These findings were corroborated by a more stringent analysis carried out on the same cohort, by excluding participants with positive history of andrological disorders known to affect semen quality, which was performed to deal with the potentially biased occurrence of pathological findings, given that participants were recruited on a voluntary basis within an awareness campaign on male fertility. 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 [31], 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 [23], 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 [21]. 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 [21, 23]. 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)[23]. 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 [26]. 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 [21]; 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 [23]. 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 [23]; 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 [23]. 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 [23]. 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.

To the best of our knowledge, this is the largest study investigating the relationship between high environmental pressure in the LF and semen quality in young healthy men, by encompassing the potential role of a known reproductive toxicant, such as Cd, and adjusting for lifestyle factors otherwise disregarded by other studies. Research study is ongoing, and trace elements determination will be performed also in semen of participants from LI areas, therefore

adding further strength to the provided results; moreover, 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.

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