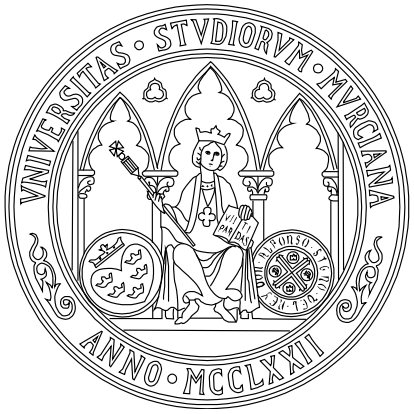

UNIVERSIDAD DE MURCIA

FACULTAD DE MEDICINA

TESIS DOCTORAL

"RISK FACTORS ASSOCIATED WITH THE ALTERATION OF HUMAN SEMEN QUALITY"

"FACTORES DE RIESGO ASOCIADOS A LA ALTERACIÓN DE LA CALIDAD SEMINAL HUMANA"

MEMORIA QUE PRESENTA PARA LA OBTENCIÓN DEL GRADO DE DOCTOR, LA LICENCIADA EN FARMACIA,

LIDIA MÍNGUEZ ALARCÓN

Murcia, Abril de 2012

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A mis padres

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Resumen

En la bibliografía científica se ha descrito que la función reproductiva masculina se ha deteriorado considerablemente durante los últimos 50 años (Carlsen *et al.,* 1992). Una exhaustiva revisión incluyendo 47 estudios adicionales, confirmó el descenso de la concentración espermática, siendo la tasa de descenso más pronunciada en Europa (-2.3%), respecto a los Estados Unidos (-0.8%) u otros países (-0.2%) (Swan *et al.,* 2000).

Sin embargo, incluso dentro de una misma región geográfica existen importantes diferencias entre países. En un estudio de prevalencia, hombres finlandeses y estonianos presentaron mayor recuento total espermático y porcentajes de espermatozoides morfológicamente normales, comparados con hombres noruegos y daneses (Jorgensen *et al*., 2002). Esta variación se ha visto apoyada por otros estudios llevados a cabo con hombres fértiles (Jorgensen *et al*., 2001; Punab *et al*., 2002; Richthoff *et al*., 2002; Paasch *et al*., 2008). Además, también pueden existir diferencias significativas dentro de un mismo país. Swan y colaboradores sugirieron en 2003 que la concentración espermática y la movilidad podrían estar disminuidas en zonas semirurales y agrícolas en relación con zonas más urbanizadas y menos expuestas a la agricultura (Swan *et al*., 2003).

El descenso de la calidad seminal se ha asociado paralelamente al aumento de los desórdenes en el tracto reproductivo masculino (Jacobsen *et al.,* 2006; Jorgensen *et al*., 2011). Estos desórdenes incluyen un incremento de la incidencia del cáncer testicular (Chia *et al.,* 2010; Engholm *et al*., 2010), así como de hipospadias (Paulozzi *et al.,* 1997; Boinsen *et al.,* 2005; Nassar *et al*., 2007) y criptorquidismo (Buenmann *et al.,* 1961; Ansell *et al*., 1992; Boinsen *et al.,* 2004;

Bray *et al.*, 2006). En un artículo publicado por Bray y colaboradores, se encontró que la incidencia en la tasa de cáncer testicular fue entre 8 y 10 veces mayor en hombres nacidos sobre 1980 comparados con hombres nacidos alrededor de 1950 en Finlandia (Bray *et al*., 2006).

Además, el descenso de la calidad espermática se ha relacionado con estilos de vida (Belcheva *et al*., 2004; Agarwal *et al*., 2008), exposiciones ocupacionales y ambientales (Wagner *et al.*, 1999; Benoff *et al*., 2000; Jensen *et al*., 2006) y exposiciones prenatales (Skakkebaek *et al.*, 2001; Ramlan-Hansen *et al.*, 2007).

Los estudios para investigar los determinantes de la calidad seminal se han llevado a cabo en varones de diferentes tipos poblacionales. Sin embargo, hombres jóvenes y sanos o subfértiles que acuden a clínicas de infertilidad, podrían representar alteraciones seminales debido a diferentes factores de exposición o de riesgo. Los hombres jóvenes y sanos se encuentran en una edad reproductiva óptima y la afectación de la calidad seminal se podría atribuir en mayor medida a factores de riesgo relacionados con exposiciones prenatales.

Entre 2010 y 2011 se llevó a cabo un estudio con jóvenes universitarios en Murcia con el propósito de estudiar la epidemiología de la calidad seminal humana. Esta tesis presenta los primeros resultados de ese proyecto (Capítulo 1 y 2). Debido a que la exposición a metales pesados no pudo ser medida en el presente proyecto, se utilizaron además datos previos de un estudio de casos y controles para explorar la asociación entre los metales pesados y la calidad seminal en hombres que acudían a clínicas de infertilidad (Capítulo 3).

FOREWORD

Foreword

There is evidence in the literature that male reproductive function has deteriorated considerably in the past 50 years (Carlsen *et al.*, 1992). A later review including 47 additional studies confirmed that sperm concentration has declined, being the role of decline more pronounced in Europe (-2.3%) than in the United States (-0.8%) or other countries (-0.2%) (Swan *et al*., 2000).

However, even within geographical Regions there are important intercountry differences. In a cross-sectional study, the Finnish and Estonian men had higher total sperm counts, sperm concentrations and frequencies of morphologically normal sperm, than the Norwegian and Danish men (Jorgensen *et al*., 2002). This variation has been supported by other studies with fertile men (Jorgensen *et al*., 2001; Punab *et al*., 2002; Richthoff *et al*., 2002; Paasch *et al*., 2008). Besides, there are significant intracountry variations. Swan *et al*. suggested in 2003 that sperm concentration and motility might be reduced in semirural and agricultural areas relative to more urban and less agriculturally exposed areas (Swan *et al*., 2003).

The decline in semen quality has been associated with a parallel increase of reproductive male disorders (Jacobsen *et al.,* 2006; Jorgensen *et al*., 2011). These disorders include an increase of the incidence of testicular cancer (Chia *et al.,* 2010; Engholm *et al*., 2010), as well as hypospadias (Paulozzi *et al.,* 1997; Boinsen *et al.,* 2005; Nassar *et al*., 2007) and cryptorchidism (Buenmann *et al.,* 1961; Ansell *et al*., 1992; Boinsen *et al.,* 2004; Bray *et al.,* 2006). In an article published by Bray *et al*., it was found that the incidence rate of testicular cancer was 8-10 times higher in

men born around 1980 compared with men born in 1950 in Finland (Bray *et al*., 2006).

The decline of sperm quality has been related to lifestyle factors (Belcheva *et al*., 2004; Agarwal *et al*., 2008), occupational and environmental exposures (Wagner *et al.,* 1999; Benoff *et al*., 2000; Jensen *et al*., 2006, and prenatal exposures (Skakkebaek *et al.,* 2001; Ramlan-Hansen *et al.,* 2007).

Studies have been conducted in men of different kinds of population to investigate determinants of semen quality. However, selected or unselected population, young healthy men or subfertile men in infertility clinics might represent alterations in semen quality due to different factors or exposures. Young healthy men are in an optimal reproductive age and their semen quality could be attributed most likely to prenatal exposures or strong effects coming from lifestyle or environmental exposures.

Between 2010 and 2011, a study with young university students with the purpose to study the epidemiology of human semen quality was conducted in Murcia. This thesis presents the first results of that project (Chapter 1 and 2). As exposure to heavy metals could not be addressed in that study, data from a previous case-control study was used to explore the association between heavy metals and semen quality in men attending infertility clinics (Chapter 3).

INTRODUCTION

2.1. Development and maturation of the male reproductive system.

To ensure fertility, each component of the male reproductive system, including the testis, internal reproductive tract or accessory organs, the penis and the brain, has to work satisfactorily at different ages or periods.

The development of the normal male reproductive system could be divided in five periods (fetal, neonatal, infancy or childhood, puberty and adulthood) defined by changes in activity of the reproductive hormone axis and testosterone levels (Fig. 1) (Woodruf *et al.,* 2010).

The importance and contribution of each of these periods for normal reproductive function in adults should be noted. For example, the penis grows during the fetal and neonatal periods, but, especially, during puberty (George *et al.,* 1994; Brown *et al.,* 1999). This growth depends on and coincides with high testosterone levels. An investigating hypothesis based on clinical experience suggests that in normal males, androgen is required in all three periods to confirm a normal size to the penis, although, early deficiencies in growth may be correctable during puberty (Bin-Abbas *et al.,* 1999).

Masculinization of the reproductive tract takes place earlier in fetal life than in the brain (Cohen-Bendahan *et al.,* 2005; Welsh *et al.,* 2008), which gives an idea of the long time necessary for the brain to develop the complex process of masculinization.

Germ cell development occurs in fetal and puberty periods, although the processes are not similar in both periods (Gaskell *et al.,* 2004; Mitchell *et al.,* 2008). There are three main stages in the development of the male reproductive system. Firstly, the organization of the reproductive system which takes place in

fetal life but extends into the neonatal period. Secondly, the development and activation of the reproductive system that occurs during puberty. And finally, full activation and maintenance of the reproductive system during the rest of adulthood.

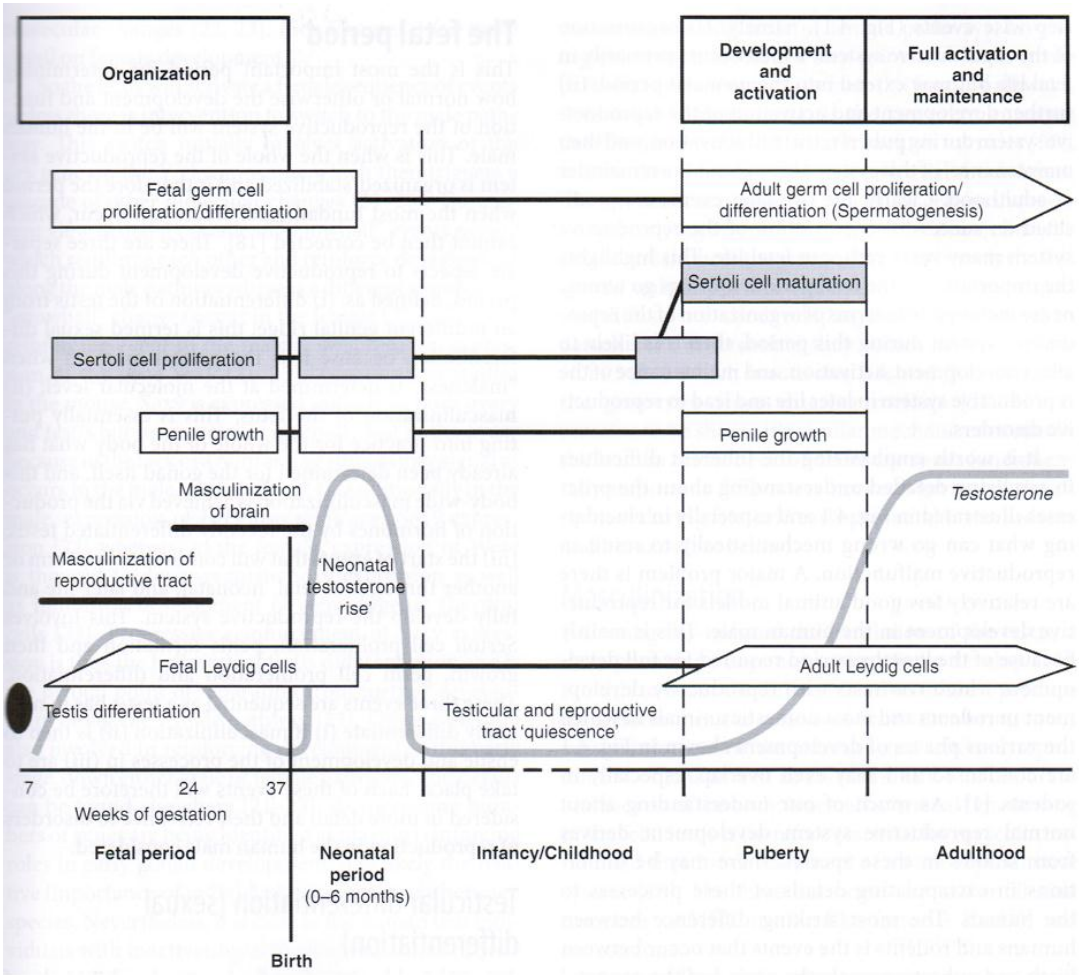

Figure 1. Summary of the main events of the human male reproductive development and function, from fetal period to adulthood. Reproduced from Woodruff *et al.* (2010)

2.1.1 Fetal period.

This is the most important stage determining the reproductive development and function that the human male will have in the future. It is the period when the most fundamental errors can occur because the reproductive system is being organized and stabilized (Sharpe *et al.,* 2006). If organization is incomplete during this period, development, activation and maintenance of the reproductive system may be affected in later life.

There are three processes in this stage: testicular or sexual differentiation, masculinization of the fetus and testicular development.

Testicular differentiation

Sexual differentiation begins with a recognizable first testis at approximately 7 weeks of gestation (Krone *et al.,* 2007). Before that, both males and females possess Müllerian ducts, Wolffian ducts and a genital tubercle, and so cannot be distinguishes on the basis of their reproductive systems.

The fetus develops as a female activating a female sequence of events, unless there is intervention to change to the male way (Sharpe *et al.,* 2006). This change triggers the activation of the SRY gene on the Y chromosome, which causes a cascade of molecular reactions (Brennan *et al.,* 2004). SOX9 is one of the main genes that collaborate in the change to the male way. In studies with mouse, this gene is expressed initially in both ovary and testis, however, its expression is later lost in the ovary when SRY has been expressed (Kim *et al.,* 2006). Another gene, FGF9, also reinforces SOX9 expression in males, while in females, WNT4 down-

regulates SOX9 expression and inhibits the development of Leydig cells (DiNapoli *et al.*, 2008).

Masculinization

The differentiation of Sertoli cells indicates the formation of the testis (Sharpe *et al.*, 2006). However, a male phenotype does not automatically develop. The differentiated testis must produce three hormones which will induce masculinization through the internal and external reproductive organs, the brain and the rest of the body. These are anti-Müllerian hormone (AMH), insulin-like factor 3 (INSL3) and testosterone.

In Sertoli cells and immediately after their differentiation, it is produced the synthesis and secretion of the AMH occurs (Sharpe *et al.*, 2006). Later it is transported to the Müllerian conducts, where it induces their regression. Anti-Müllerian hormone-induced degeneration of the Müllerian conducts is an early event in masculinization (Klatigg *et al.*, 2007). The role of INSL3 is to regulate the postnatal trans-abdominal testicular descent, although this has only been confirmed in studies with rodents (Adhamie *et al.*, 2004; Kawamura *et al.*, 2004).

Testosterone is the most important hormone produced by the fetal testis, and it is this hormone that causes body-wide masculinization (Fig. 2) (Sharpe *et al.*, 2006).

Within the epididymis, vas deferens and seminal vesicles, testosterone produces its effects directly linking with the androgen receptor (AR) (Welsh *et al.*, 2008). However, it is metabolized to a more potent androgen, dihydrotestosterone (DHT), which develops its actions in the rest of the body (Sultan *et al.*, 2001).

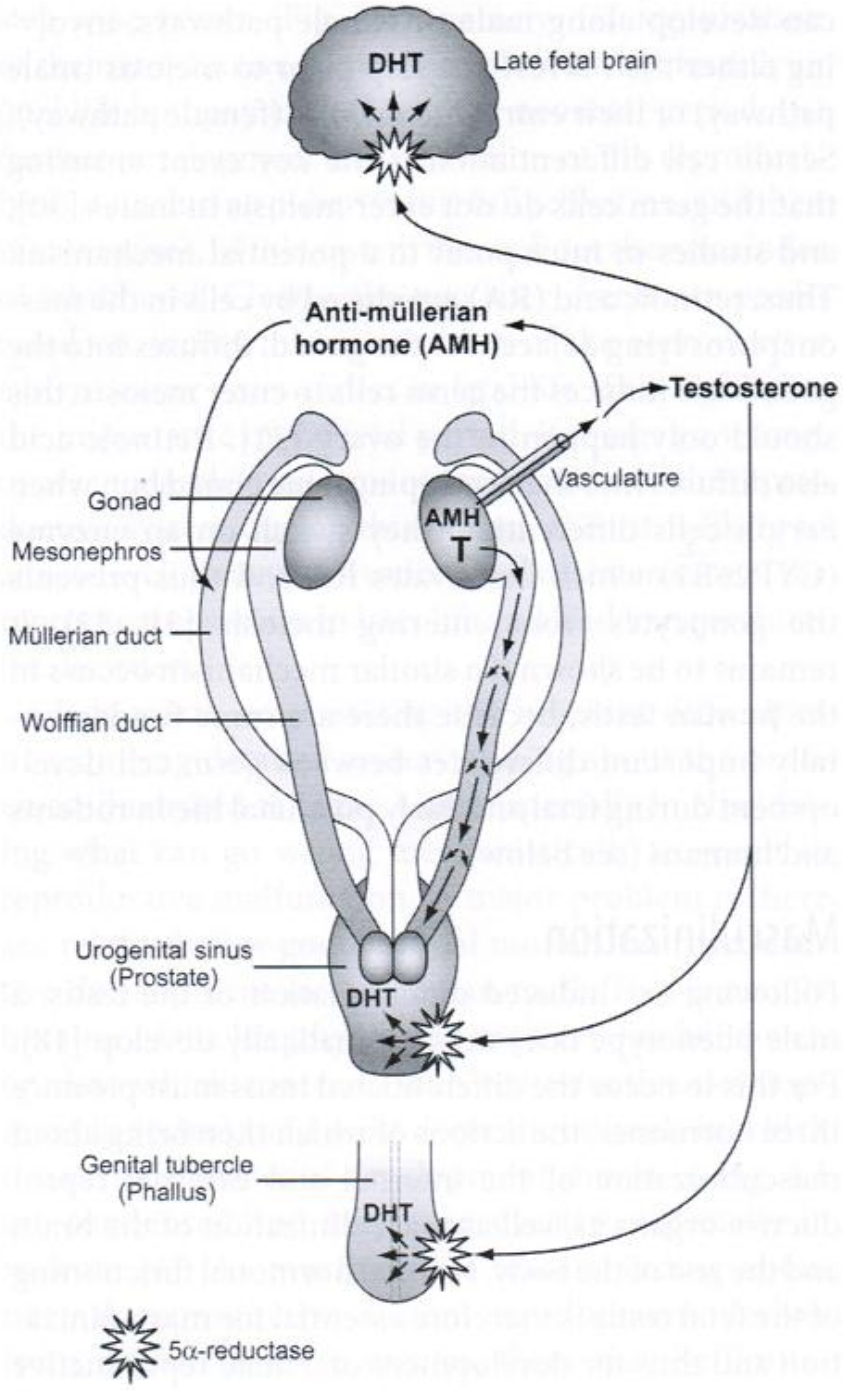

Figure 2. The main hormonal events in human male masculinization. Reproduced from Woodruff *et al.* (2010).

The enzyme 5α-reductase produces the conversion of testosterone to DHT. There are two types of this enzyme. Type 1 is the form expressed mainly in the brain and skin, while type 2 is the form expressed in the urogenital sinus and genital tubercle (Sultan *et al.,* 2001). Testosterone is also converted to estradiol by the enzyme aromatase linking with the estrogen receptors (ERs). However, this conversion is less important than the conversion to DHT (Schwarz *et al.,* 2008).

Male impairment of masculinization is common (Toppari *et al.,* 2001; Boisen *et al.,* 2005). The most important congenital disorders are cryptorchidism (the failure of testis descent into the scrotum) and hypospadias (when the urethral meatus does not open in the middle of the tip of the penis) (Wang *et al.,* 2008). Both are considered potential manifestations of testicular dysgenesis syndrome (TDS), which includes adult disorders such as low sperm counts and testicular germ cell cancer (Skakkeabek *et al.,* 2001). All these disorders have a common fetal origin that manifests themselves at birth or during puberty.

Masculinization programming window

The male programming window is a recent concept that has been developed in rodents to better understand the fetal origin of TDS disorders and the malfunctioning of masculinization (Fig. 3) (Welsh *et al*., 2008).

Masculinization is not an instantaneous process. The differentiation of reproductive structures by the action of the androgens occurs within the male programming window probably at 8-12 week of gestation in humans (Welsh *et al.,* 2008). An important implication is that cryptorchidism and hypospadias, as well as the size of accessory sex organs and final penile length, result from deficient

androgen action within this programming window. Ano-genital distance (AGD) is also programmed by androgen action within the male programming window. This explains why smaller AGD is associated with cryptorchidism and hypospadias in rats (Welsh *et al.,* 2006), and with poor semen quality in humans (Mendiola *et al.,* 2011).

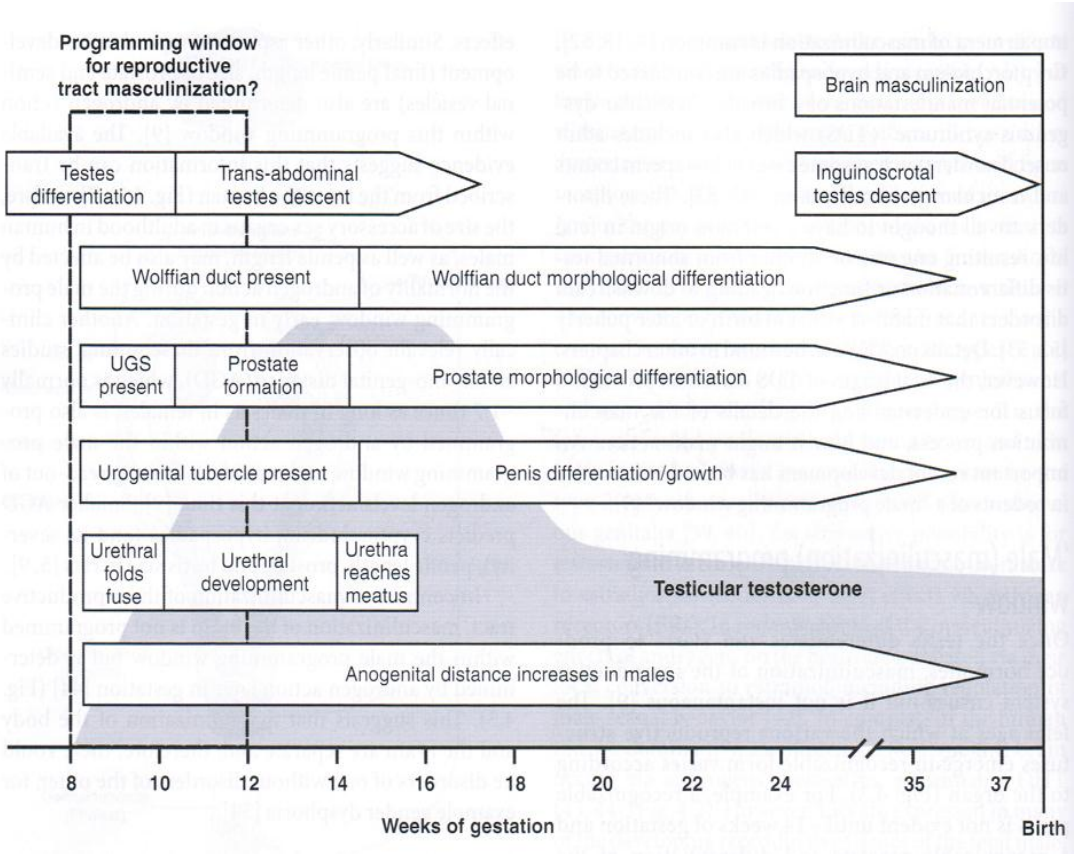

Figure 3. Chronology of reproductive tract and genital development in the human male fetus in relation to testicular differentiation and descent, the testicular levels of testosterone and brain masculinization. The postulated "male reproductive window" is also presented. Reproducted from Woodruff *et al.* (2010).

However, masculinization of the brain follows its own path separate from rest of the body, and there could be disorders in one organ with no association with disorders of the other (Gooren *et al.*, 2002).

Testicular development

The testes develop in fetal life after their differentiation. The most important changes are the increase in Sertoli cell numbers, the expansion and differentiation of the germ cell population, and descent of the testes through the abdomen and pelvis to situate finally in the base of the scrotum. The proliferation of Sertoli and germ cells occurs as soon as testicular differentiation takes place and continues into the neonatal period (Sharpe *et al.,* 2003).

For germ cells, changes in this same period include the loss of pluripotency expression factors and processes associated with the maturation of germ cells (Gaskell *et al.,* 2004). It is believed that noxas in the period might be related to the possibility of developing testicular germ cell cancer (TGCT) (Rajpert *et al.,* 2006).

Subnormal androgen production by the human fetal testis may also lead to reduced numbers of Sertoli cells and, consequently, a lower sperm count, if during other periods it is not compensated with the extra production of these cells. Subnormal testosterone production by the fetal testis could also lead to TDH (Sharpe *et al.,* 2008).

The descent of the testis is a two-stage important fetal event, which places the testes in the scrotum, as required for normal spermatogenesis (Fig. 4) (Amann *et al.,* 2007). In the trans-abdominal phase, the testis goes from the kidney to the inguinal region regulated by INSL3 and androgens. In the trans-inguinal phase,

testis goes through the pelvis into the bottom of the scrotum being regulated only by androgens. It has been suggested that an impairment of androgen production/action in the second phase might cause cryptorchidism. In addition, it has been established that, in rat, this second phase is programmed by androgens in the male programming window, in spite of the considerable time elapsing between this window and the phase.

2.1.2. Neonatal period and childhood.

Neonatal period (0-6 months)

In this period the hypothalamic-pituitary axis is activated, increasing circulating levels of luteinizing (LH) and follicle-stimulating (FSH) hormone (Mann *et al.*, 1996). In male babies, it is associated with the stimulation of Leydig cells and increased testosterone levels. The proliferation of the Sertoli cells continues as well as the growth in penile length. It is unknown whether the masculinizing effects of testosterone affect the brain in this period.

Infancy and childhood (6 months – 12 years)

This is the period of testicular and reproductive quiescence or the quietly active period, because germ cell proliferation and development, as well as testosterone secretion especially during the night-time when pulsatile LH secretion is activated, occur during this period but a low level, (Chemes *et al.*, 2001; Grumbach *et al.*, 2002). Sertoli cells proliferate again in this period, although it is not known exactly when.

Harm to germinal cells when a child requires therapy or cancer, can result in complete loss of germ cells and consequent sterility in adulthood (Brougham *et al.*, 2003).

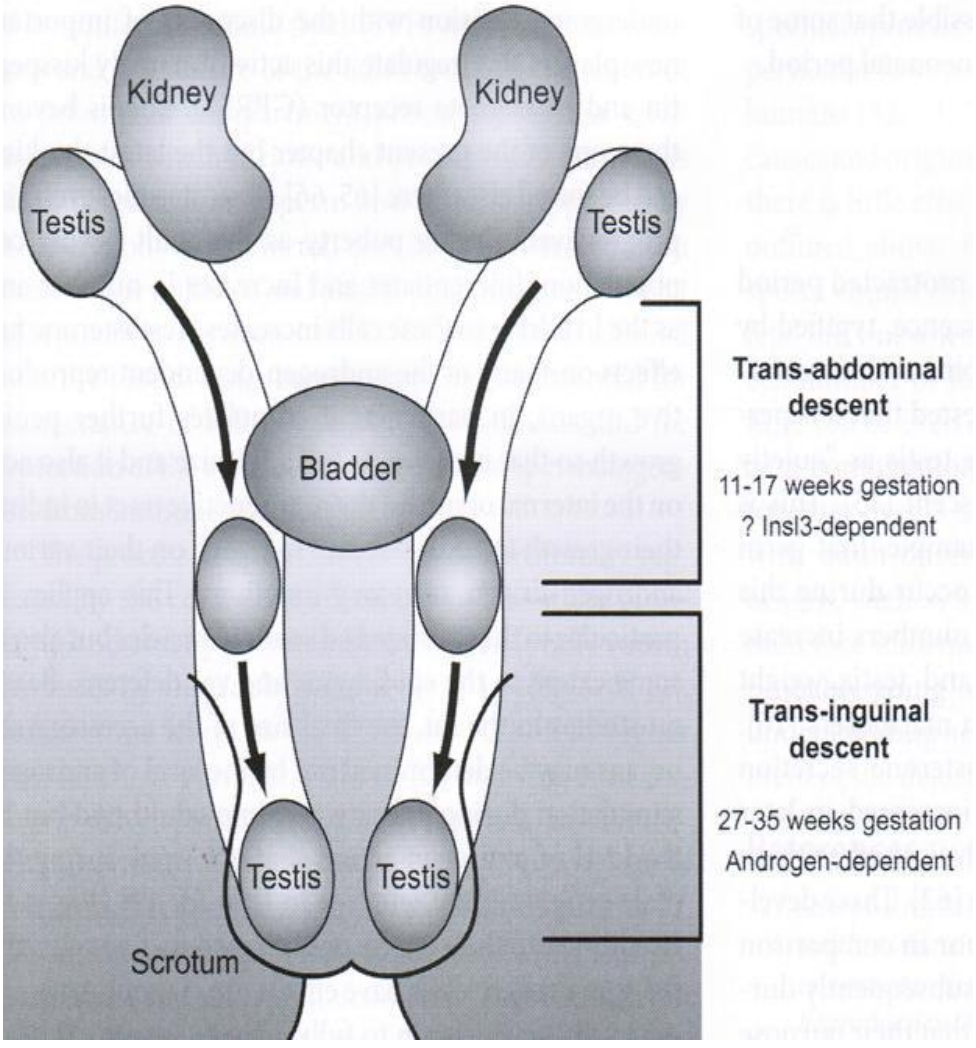

Figure 4. Development and hormone regulation of the two phases of testicular descent in the human male fetus. Reproduced from Woodruff *et al*., (2010).

2.1.3. Puberty and adulthood.

The period of puberty is defined by higher hypothalamic-pituitary (HP) axis activity than in childhood and an increase in LH secretion (Grumbach *et al.*, 2002).

Testosterone levels play an important role in this stage to differentiate and to increase the Leydig cell population, to stimulate further penile growth and to induce organ growth (the prostate, seminal vesicles, the epididymis and vas deferens). In addition, androgen levels have an effect throughout the body, especially in the brain, activating libido and the sexual behavior (Gooren *et al.*, 2002).

However, the most important event in the puberty period is the effect of testosterone levels on the Sertoli cells that lead to spermatogenesis. The descent of the testis into the bottom of the scrotum is essential for spermatogenesis and hormonal regulation, as if the testes are not in the correct location, the production of spermatozoon will fail.

Androgen levels regulate the proliferation of Sertoli cells during the perinatal period. However, during puberty, Sertoli cells start expressing androgen receptors, which is considered one sign of maturation of these cells (Sharpe *et al.*, 2003). Essential for carrying out spermatogenesis, these differentiated cells develop a junction or barrier, creating the adluminal compartment within the seminiferous tubules (Sharpe *et al.*, 1994). This also enables the formation of a lumen through which spermatozoa can be transported out of the testis to the epididymis. The creation of this compartment leads to the meiotic and post-meiotic differentiation of germ cells, and provides protection against immune-attack.

Spermatogenesis is regulated by FSH produced in the pituitary gland and by testosterone produced in the testes by Leydig cells (Sharpe *et al.*, 1994). To complete the process it is necessary for testosterone levels to be higher in the testis than in the blood.

Adulthood begins when puberty finishes. In adulthood, to consolidate fertility and the normal reproductive health of the individual for the rest of his reproductive cycle, it is important to maintain the support and activity of the testis and the other reproductive organs (Sharpe *et al.*, 2008).

2.2. Anatomy and physiology of testicular function.

In order to understand male infertility, a basic literature review on testicular function is presented, including the anatomical and physiological basis of the dual function of the testes or male gonads: the production and maturation of male gametes, and the synthesis and secretion of sexual hormones.

2.2.1 Structure and supporting cells.

The testes are glandular organs enveloped by the tunica albuginea, a strong covering connective tissue. The normal testes lie in the scrotum. Spermatozoa are produced in the testis and later stored in the epididymis to be carried away by the vas deferens (Fig. 5). In the wall of the epididymis there are smooth muscles that contract to thrust the spermatozoa forward into the prostatic urethra. Here, sperms mix with secretions from accessory glands including the prostate, seminal vesicles and bulbourethral gland (de Krester *et al.,* 1998; Elzanaty *et al.,* 2002).

Spermatogenesis is the production of gametes, while steroidogenesis is the series of enzymatic reactions that lead to the production of sexual homones. The two main processes occur in different compartments (tubular and intersticial), but are interconnected.

The interstitial compartment contains one of the most important testis cells, the Leydig cells. The secretion of androgens, including testosterone (the primary male sex hormone) is mainly produced by the Leydig cells (Akhmerova *et al.*, 2006). Luteinizing hormone (LH) is secreted by the pituitary and, stimulates the

Leyding cells to produce testosterone, which is accumulated in the interstitial and seminiferous tubules.

Sertoli cells take up much of the tubular compartment and have several roles in spermatogenesis. Their main function is to nurture the developing germ cells during various stages of the spermatogenesis process (Johnson *et al.,* 1998).

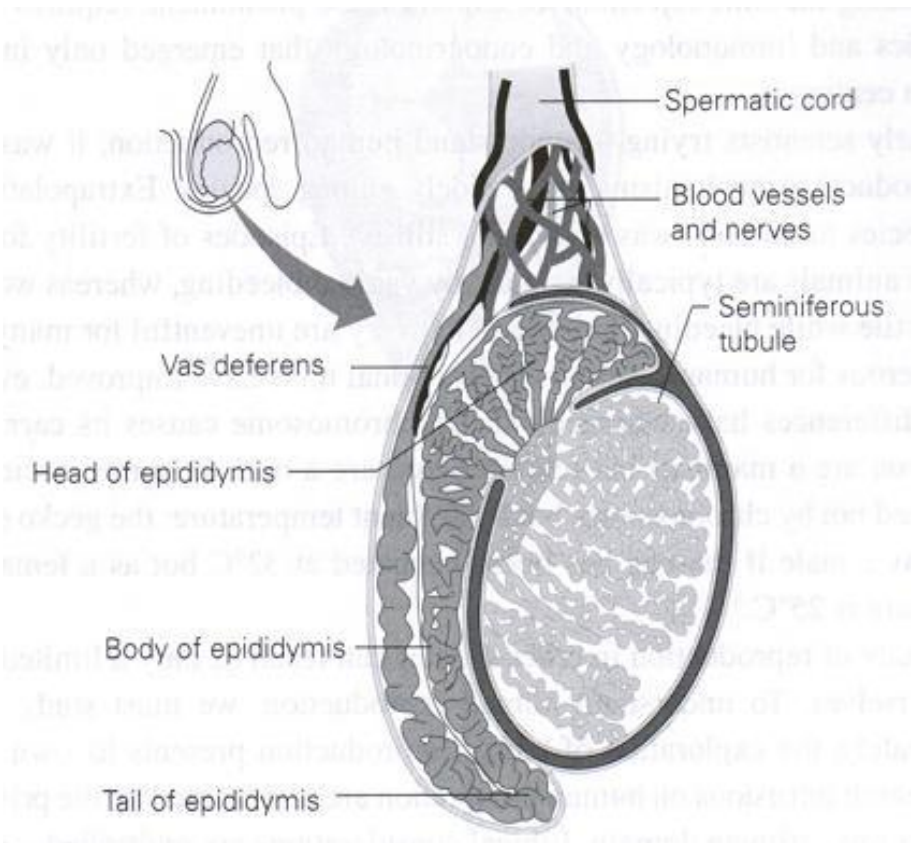

Figure 5. Testis structures. Reproduced from Wilcow *et al.* (2010).

Sertoli cells, which are regulated by the pituitary gland, also provide the signals that initiate spermatogenesis and sustain spermatid development. Sertoli cells control spermatogenesis and secrete liquids to aid sperm transport. In addition, the Sertoli cells divide the seminiferous tubules into two compartments (basal and adluminal) for spermatozoa development.

In the basal compartment, which is in contact with the circulatory system, spermatogonia develop into primary spermatocytes. In the adluminal compartment, meiosis is finished and blood-testis barrier made by connections between the Sertoli cells protects spermatocytes as well.

2.2.2. Spermatogenesis.

The process by which male spermatogonia develop into mature spermatozoa is called spermatogenesis (Fig. 6). In this complex process, primitive totipotent stem cells divide to produce daughter cells, which, in approximately 70 days, mature into spermatids. Spermatogenesis involves both mitosis and meiosis and is regulated by Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) from the anterior pituitary (Karpenko *et al.,* 2007.

The process of spermatogenesis includes several stages. In the first stage, spermatocytogenesis (Fig. 7), stem cells divide to produce a population of cells that later become mature sperm cells, during a process that occurs in the basal compartment. There are three functional types of spermatogonium; types Ad (dark), Ap (pale) and B.

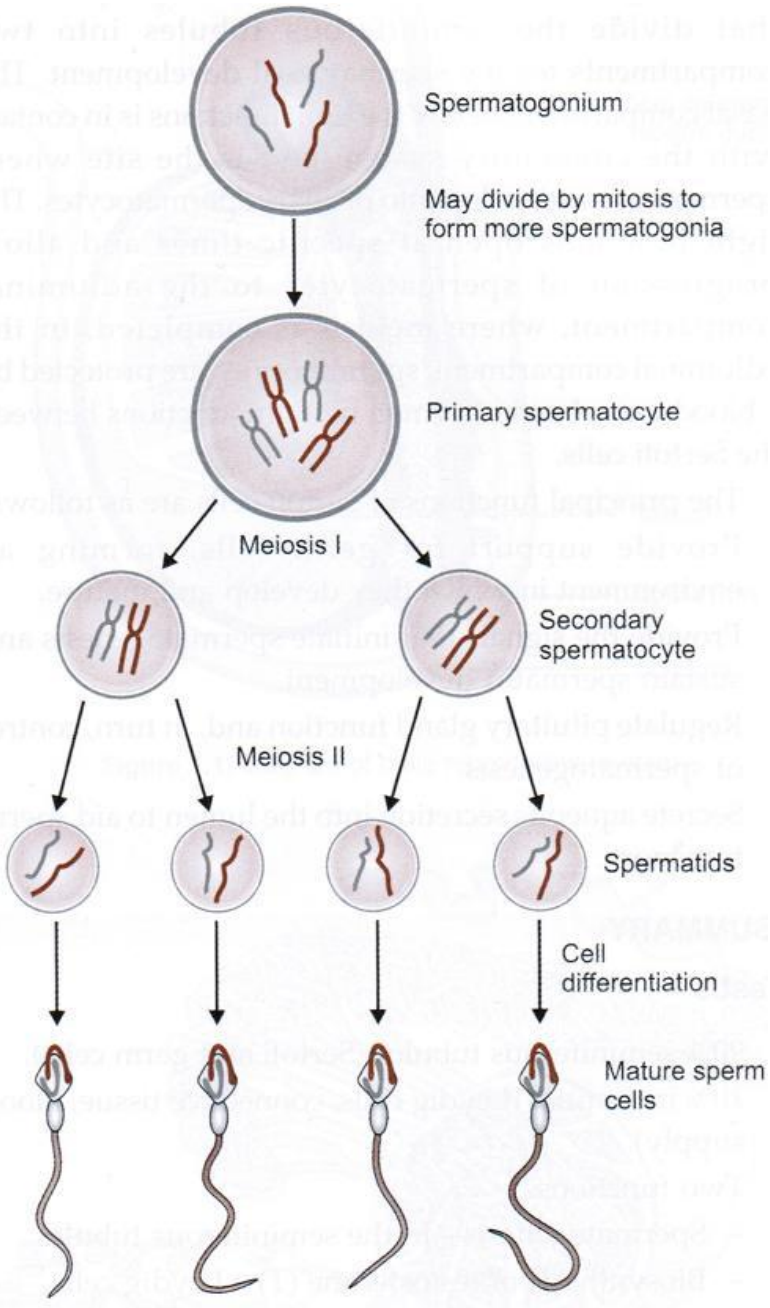

Figure 6. The process of spermatogenesis. Reproduced from Kao *et al.* (2010).

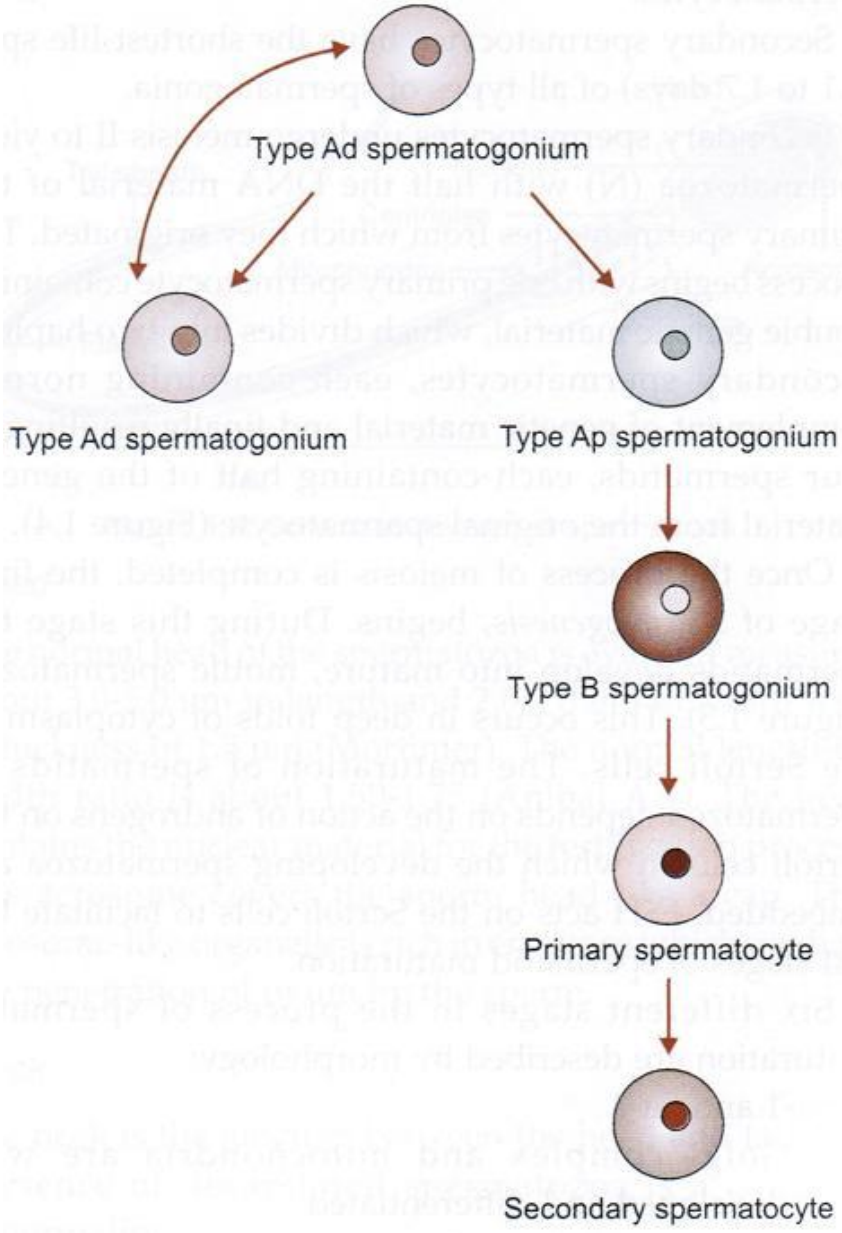

Figure 7. Spermatocytogenesis. Reproduced from Kao *et al.* (2010).

Type Ad cells are divided into type Ap and type Ad itself. They preserve the initial pool of spermatogonia. Type Ap spermatogonia produce clones of themselves by mitotic division, and later, they differentiate into type B

spermatogonia. Type B spermatogonia produce the primary spermatocytes (diploid intermediate cells) by mitosis. During puberty, the diploid (2N) primary spermatocytes enter meiosis I and divide to become haploid (N) secondary spermatocytes. Due to meiosis II, secondary spermatocytes become spermatozoa (N) with half the DNA material of the primary spermatocytes in less than two days.

During spermiogenesis, the acrosomal vesicle and the chromatin body of the Sa-1 and Sa-2 forms, appears opposite each other, and the Golgi complex and mitochondria are well defined. The proximal centriole and axial filaments also appear. In the Sb-1 and Sb-2 forms, the acrosome and intermediate piece are formed. And finally, in the Sc-1 and Sc-2, the development of the tail is completed.

When the process of meiosis is completed, the second process of spermatogenesis begins. In this stage, called spermiogenesis (Fig. 8), FSH acts on the Sertoli cells to facilitate the last stage of spermatid maturation, which includes six stages, and by the end, the spermatids have developed into mature and motile spermatozoa. This process depends on the androgens action in the Sertoli cells and it takes place in the Sertoli cells cytoplasm (Karpenko *et al.*, 2007).

During the post meiotic phase, progressive condensation of the nucleus occurs, with subsequent genome inactivation. The histones convert to transitional proteins, and protamines convert to well developed disulfide bonds.

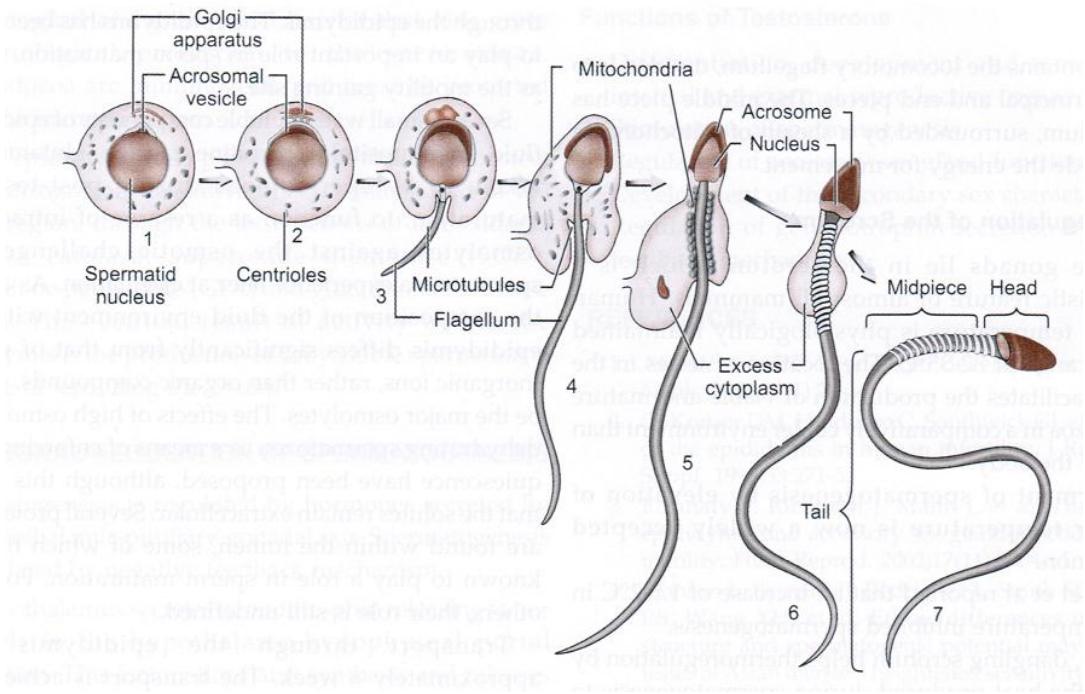

Figure 8. Spermiogenesis. Reproduced from Kao *et al.* (2010).

Later, during spermiation, mature spermatozoa are released from the Sertoli cells and move freely in the lumen of the tubules (Karpenko *et al.*, 2007). Since mature spermatozoa are non-motile, activation of the CatSper protein localized in the sperm tail develops the progressive motility of the sperm, within the epididymis. This protein is like a Ca^{2+} ion channel that leads cAMP-generated Ca^{2+} influx.

The epididymides play an important role in the maturation of the sperm in terms of motility. Small water-soluble components of epididymal fluid are taken up by spermatozoa after maturation. Those components act as a store of intracellular osmolytes to buffer the osmotic challenges that spermatozoa experience later at ejaculation. Motility is attained when spermatozoa pass through the caput of the

epididymis, and the fertilizing ability is acquired when it pass through the caput`s body (Johnson *et al.,* 1998). Spermatozoa are immunogenic and therefore must be protected from the immune system. This immunological protection is provided by the blood-epididymis barrier. The epididymis also has the capability to protect spermatozoa from oxidative attack, because the epididymis stores spermatozoa in the cauda region where antioxidant enzymes like catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD) are found.

2.2.3. Hormonal regulation of spermatogenesis.

Hypothalamus secretes gonadotrophin-releasing factor (GnRH) into the hypothalamo-hypophyseal portal circulation system. This factor stimulates the synthesis and release of the gonadotrophins FSH and LH into the systemic circulation by means of the pituitary gland (Fig. 9). GnRH secretion is pulsatile, therefore, the effect of LH and FSH occurs in discrete peaks (Wu et al., 2007).

GnRH interacts with a specific receptor that is one of the smallest G protein-coupled receptors with 328 amino acids, and its main function is to stimulate LH and FSH secretion through the pituitary gland (Weinbauer *et al.,* 1993; 1996).

The pituitary function is also controlled by gonadal steroids and peptides, which are influenced by the hypothalamus. Very close anatomical and functional connections exist between the pituitary gland and the hypothalamus, so that both are considered a single functional unit.

Hormones secreted by the hypothalamic-pituitary-gonadal axis regulate spermatogenesis by a negative feedback mechanism. Testosterone inhibits the secretion of LH and FSH, whereas LH stimulates testosterone synthesis, and FSH controls spermatogenesis.

FSH acts in the development of Sertoli cells (Weinbauer *et al.,* 1993; 1996). The effect of FSH results in increased cAMP concentrations, which is counterregulated by phosphatases, phosphodiesterases, and kinase inhibitors. Under the influence of the FSH, Sertoli cells secrete androgen binding protein (ABG), inhibin and plasminogen activator. ABG is necessary to maintain high levels of androgens locally. The plasminogen factor helps in spermiation, and inhibin has a negative feedback effect on FSH secretion through the anterior pituitary gland (Wu *et al.,* 2007).

LH acts on the Leydig cells, stimulating the production of testosterone and is the crucial hormone in the initiation of the spermatogenesis. The activation of protein kinase that follows LH receptor activation in Leydig cells results in the synthesis and secretion of testosterone. The activation of the receptor leads to stimulation of the conversion of cholesterol to prenenolone, the first step of testosterone biosynthesis. Then, LH stimulates the synthesis of RNA and proteins, including the P450 enzymes of the steroidogenic pathway.

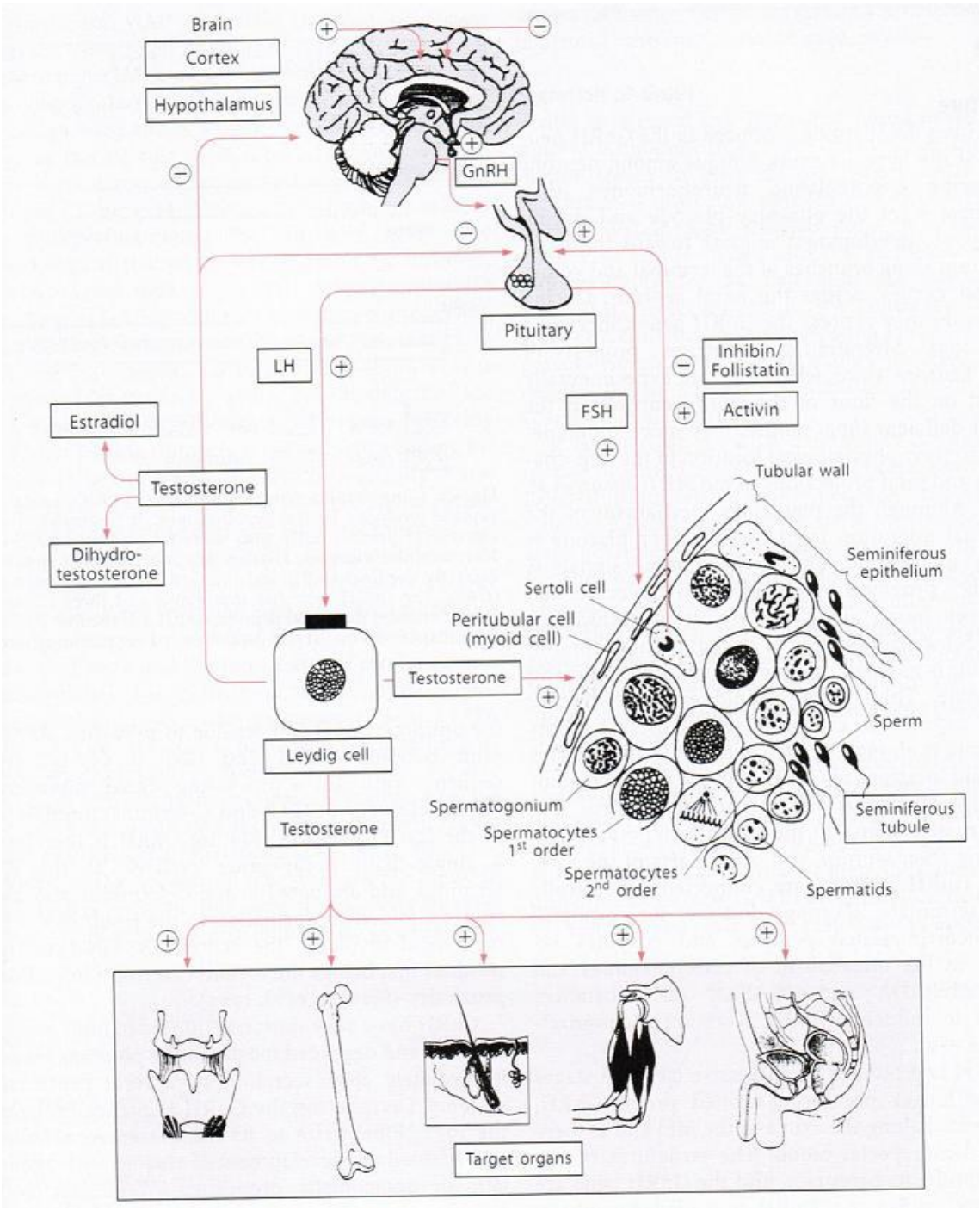

Figure 9. Hormonal regulation of spermatogenesis. Reproduced from Nieschlag *et al.* (1997).

Both LH and FSH are capable of regulating the number of their own receptors. The maximal biological effects are evident even after partial occupation of available receptors. Following hormone-receptor interaction, the complex is internalized and degraded into lysosomes, with very limited recycling.

Under the influence of LH, testosterone is the most important androgen produced by the Leyding cells in the testis. Testosterone has four main functions: the stimulation of spermatogenesis; the regulation of accessory sex gland functions; the development of secondary sex characters and the regulation of gonadotrophin secretion by a negative feedback mechanism. In conclusion, testosterone produces the differentiation, development and maturation of internal and external reproductive organs in males. However, it also has an effect on bone, hair growth, and muscle mass and distribution (Fig. 9).

2.2.4. Human spermatozoa.

Spermatozoa are motile, highly specialized, differentiated and condensed cells that do not divide. An approximately 60 µm long and 1 µm wide, the spermatozoon is composed of a head, neck or midpiece, and tail (Fig. 10). The head contains the chromosomal material for the fertilization process. The acrosome, which is rich in enzymes, covers the sperm head like a cap and mediates the penetration of ovum by the sperm. The neck is the connection between the head and the tail. The tail is divided into middle, principal and end pieces. The middle piece has the motile flagellum, surrounded by a sheath of mitochondria that give them the energy for movement.

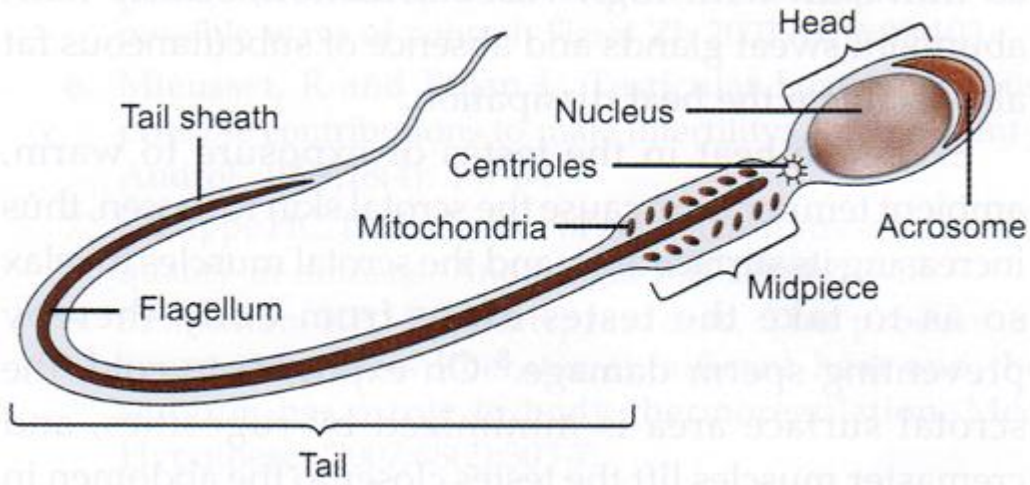

Figure 10. The spermatozoon. Reproduced from Rao *et al.* (2010).

Testis vascularization has two main roles: the regulation of testicular temperature and the transportation and mobilization of endocrine factors and metabolites. Human testicular temperature is physiologically maintained within a range of 32-35 ºC for the survival of the spermatozoa. The location of the testis in the scrotum facilitates the production of viable and mature spermatozoa, cooler than the rest of the body (Mieusset *et al.,* 1995).

For the preservation of a physiologically lower temperature the testis relies on two thermoregulatory systems. Heat can be transferred to the external environmental through the scrotal skin, as it possesses no subcutaneous fat tissue. The other regulatory system is the pampiniform plesus. Here, the convoluted testicular artery is surrounded by several veins coiling around the artery several times. In the case of a varicocele, caused by a local disturbance of the venous circulation, there is an increased in scrotal temperature. A raise in testicular

temperature may result in damage to the spermatogenetic function of the testis. However, if the testicular temperature is increased in adults, the spermatogenic damage can be reversible (Skandhan *et al.*, 2007).

2.3. Semen parameters and quality criteria.

Semen is produced during ejaculation, from a concentrated suspension of spermatozoa stored in the epididymides and mixed with fluid secretions from the accessory sex organs. The nature of the spermatozoa (motility and morphology), the sperm numbers (total sperm count and sperm concentration), and the composition of seminal fluid are the key characteristics of sperm (WHO 2010).

The methods for evaluations male infertility have typically been limited to a semen analysis that evaluates mainly sperm numbers, motility and morphology. For an epidemiologist, the semen analysis is the basis for assessing hazards in the environment, occupational exposures, or the effect of drugs and chemicals.

Since 1987, the World Health Organization (WHO) has established parameters to evaluate the quality of human semen. Since then, the normal cut offs for semen parameters have been revised downward three times by the WHO (WHO, 1987; WHO, 1992; WHO, 1999; WHO, 2010).

2.3.1. Sperm morphology.

Observations on spermatozoa recovered from the female reproductive tract, especially in the postcoital endocervical mucus and also from the surface of the zona pellucida, have helped to define the appearance of potentially fertilizing (morphologically normal) spermatozoa (Fig. 11) (WHO, 2010).

For a complete assessment of a semen sample, the evaluation of the morphological characteristics of the spermatozoa consists of the quantitative

evaluation of the percentage of normal and abnormal sperm forms present in an ejaculate.

Papanicolau stain in the most widely used. It is recommended by the WHO laboratory manual since it clearly provides a good staining of spermatozoa and other cells. It disthinguishes basophilic and acidophilic cell components. It also allows a comprehensive examination of the nuclear chromatin pattern.

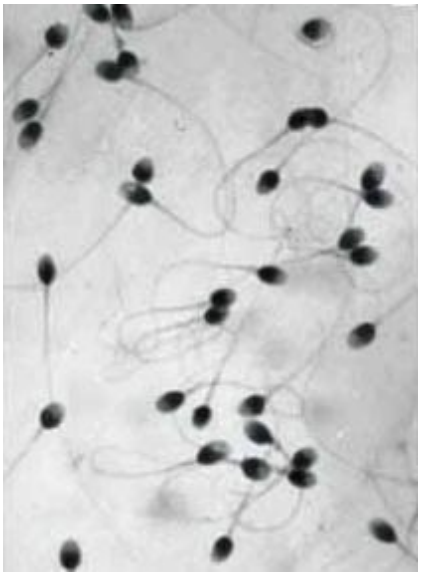

Figure 11. Morphologically normal spermatozoa from endocervical mucus in Papanicolau stained. Reproduced from Menkveld & Kruger (1990).

Morphologically normal spermatozoa must have very few defects on the sperm head, midpiece or tail. The head cannot be large, small, tapered, pyriform, round, amorphous or vacuolated. Neck and midpiece defects include a bent neck, asymmetrical insertion of the midpiece into the head, and irregular or abnormally

thin midpiece. The tail may be curved but not sharply angulated, short, hairpin, broken, bent or kinked.

The lower reference limit for normal morphology is 4% (5th centile, 95% Confidence Interval 3, 5) (WHO, 2010).

2.3.2. Sperm motility.

Sperm motility is the ratio of the number of motile sperm to total number of sperm in a given volume and is expressed as a percentage.

A simple system for grading motility is recommended to distinguish progressive, non-progressive motility and immotile spermatozoa. This provides an assessment of sperm motility without requiring sophisticated equipments. Five microscopic fields are needed to systematically and classify 200 spermatozoa.

WHO classifies motility as: progressive motility (PR), that is spermatozoa moving actively, either linearly or in a large circle, regardless of speed; non-progressive motility (NP) which includes all other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed; and immotility (IM) for spermatozzon with no movement.

The time to start semen analysis must be considered. To interpret the motility in semen samples, studies have shown an inverse relationship between the time to start analysis and the percentage of motile spermatozoa.

The lower reference limit for total motility (PR + NP) is 40% (5th centile, 95% CI 38, 42). The lower reference limit for progressive motility (PR) is 32% (5th centile, 95% CI 31, 34) (WHO, 2010).

2.3.3. Sperm concentration.

The concentration of spermatozoa is calculated from the number of spermatozoa in the ejaculate, which is measured during semen evaluation. Sperm concentration refers to the number of spermatozoa per unit volume of semen.

The most accurate method of determining sperm concentration is volumetric dilution and hemocytometry. In a Neubauer hemocytometer, a fixed volume of a liquefied semen aliquot is used, and fixed sperm are counted. But extreme care must be taken while making dilutions and preparing the hemocytometer. In addition, gently mixing the semen sample using a positive displacement pipette before the volume is withdrawn is essential for an accurate determination of sperm concentration.

Abstinence time must be considereded when interpreting the sperm concentration in the semen sample, since increases in the time of abstinence will result in an increased total sperm concentration.

The lower reference limit for sperm concentration is 15×10^6 spermatozoa per ml (5th centile, 95% CI 12, 16×10^6) (WHO, 2010).

2.3.4. Total sperm count.

Total sperm number refers to the total number of spermatozoa in the total ejaculate, and is obtained by multiplying the sperm concentration by the semen volume. Abstinence time must also be considerer to evaluate the sperm concentration in the semen sample.

The lower reference limit for total sperm number is 39×10^6 spermatozoa per ejaculate (5th centile, 95% CI 33, 46×10^6) (WHO, 2010).

Total motile (progressive or non-progressive) sperm count may also be used to describe semen quality. This is obtained by multiplying the total sperm count and the percentage of motile spermatozoa (progressive or non-progressive).

2.3.5. Semen volume.

The volume of the ejaculate is formed mainly from fluids coming from the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymides. Precise measurement of volume is essential in any evaluation of semen, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated.

The volume of the ejaculate should be measured by transferring the liquefied sample into a graduated 15 mL conical centrifuge tube. Retrograde ejaculation, obstruction of the lower urinary tract like urethra, or the congenital absence of vas deferens or seminal vesicles might yield a low volume. Volume can also be measured by weight as semen has a density close to 1.

Semen volume is differentiated into three categories to facilitate interpretation and diagnosis. Aspermia is when no semen sample is produced after orgasm. Hypospermia is a semen sample <0.5 mL, and hyperspermia is <6.0 mL of semen ejaculated.

The lower reference limit for semen volume is 1.5 mL (5th centile, 95% CI 1.4, 1.7) (WHO, 2010).

Table 1 summarizes the lower reference limits of the WHO for semen characteristics (WHO, 2010).

Parameter	**Lower Reference Limit**	
	5^{th} centiles	**95% CI**
Sperm morphology (%)	4	3, 5
Total sperm motility (%)	40	38, 42
Progressive sperm motility (%)	32	31, 34
Sperm concentration (10^6/mL)	15	12, 16
Total sperm count (10^6)	39	33, 46
Semen volume (mL)	1.5	1.4, 1.7

Table 1: Lower reference limits (5^{th} centiles and their 95% confidence intervals) for semen characteristics (WHO, 2010).

2.4. Trends in semen quality.

2.4.1. Sperm quality decline.

"*Evidence for decreasing quality of semen during the past 50 years*" was published by the British Medical Journal in 1992, and this paper has probably been one of the main references that has stimulated a myriad of articles on the impairment of male reproduction (Fig. 12) (Carlsen *et al*., 1992). In this meta-analysis, the author reviewed 61 papers published between 1938 and 1991 that included in total 14947 men without a history of infertility.

A significant decrease in mean sperm concentration was found. In 1940 men had, on average, 113 x 10^6 cells/mL of sperm concentration, while the mean was 66 x 10^6 cells/mL in 1990 ($p<0.0001$), with a corresponding decline of approximately 1% per year during the period considered.

In spite of the methodological drawbacks of the above study, which received considerable criticism, later, studies have supported the findings (Auger *et al.*, 1995; Irvine *et al*., 1996; Van *et al.*, 1996; Swan *et al*., 1997). For example, Irvine and colleagues analyzed whether semen quality had changed in 500 men born between 1951 and 1973 in Scotland. They concluded that there was direct evidence of a deteriorating semen quality as there was a cohort effect with later year of birth that was significantly associated with reduced sperm numbers in adult life.

However, other reports did not find any such time trend (Handelsman *et al*., 1997; Paulsen *et al.,* 1996; Vierula *et al.,* 1996). Fisch and colleagues mention that "*Our data show no decline in sperm counts over a 25-year period in 1283 men who banked sperm before vasectomy at three distinct geographical sites in the United States*" (Fisch *et al.,* 1996).

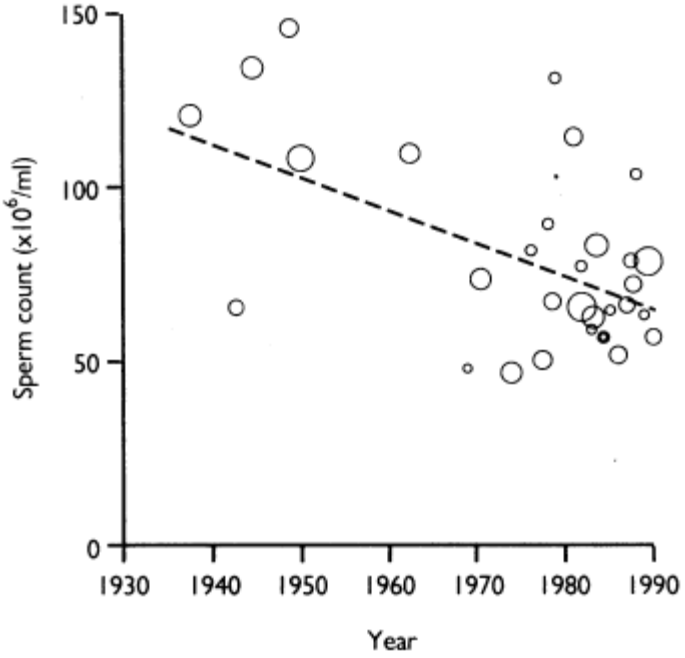

Figure 12. Linear regression of mean sperm concentration including 61 reports between 1938 and 1991 (each weighted according to the number of subjects) Reproduced from Carslen *et al*. (1992).

In order to clarify whether there has been a decline in semen quality, Swan and colleagues published in 2000 a meticulous re-analysis which included 56 of the 61 studies from the Carlsen report (Carlsen *et al.,* 1992) and adjusted for age, period of abstinence, proven fertility, method of analysis, and aim of the individual study (Swan *et al.,* 2000).

This article, again, suggested a decline of sperm concentration worldwide. The results corroborated the hypothesis of sperm concentration decline mentioned by Carlsen and colleagues in 1992, and the observed trends previously reported for 1938–1990 were also seen in data from 1934–1996 (Carlen *et al.*, 1992; Swan *et al.*, 2000).

2.4.2. Geographical differences in semen quality.

In the re-analysis, Swan and colleagues also indicated that the decline of sperm concentration was not a global trend equal in all regions as there were geographical differences (Swan *et al.*, 2000). The sperm concentration decrease was higher in Europe (-2.4%) than in the United States (-0.8%) and other countries (-0.2%) (Fig. 13).

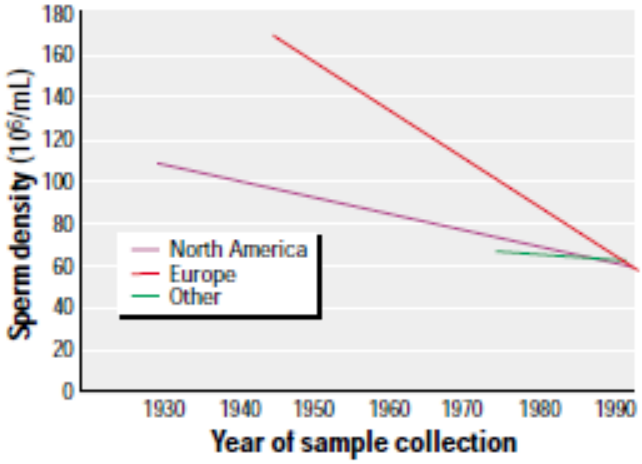

Figure 13. Linear regression of sperm density mean by year and geographic region. Reproduced from Swan *et al.* (2000).

In order to corroborate this finding in fertile males the same author conducted the first study in the United States using standardized methods and strict quality control (Swan *et al.,* 2003). Sperm concentration was significantly lower in Columbia (Missouri), than in New York (New York), Minneapolis (Minnesota) and Los Angeles (California). The total number of motile sperm was also lower in Missouri than in other cities. These data suggested that sperm concentration and motility might be lower in semirural and agricultural areas compared to more urban and less agriculturally exposed areas.

In Northern Europe, a study was also conducted to explore geographical differences in semen quality (Jorgensen *et al*., 2002). An east-west gradient in semen quality was found in a study with 968 young men of the general population. Young men of Estonia and Finland had higher sperm count and percentages of normal morphology than men from Denmark and Norway.

In Japan, a study was undertaken with 324 fertile men from the Kawasaki/Yokohama area in order to explore their semen quality and investigate possible geographical differences with other countries (Iwamoto *et al.,* 2006). However, in this case, as the authors pointed out, *"the study showed that semen quality of fertile men from Japan was at the same low level as Danish men from Copenhagen. Although a possible explanation for regional differences can be differences in lifestyle or other environmental factors, ethnic differences caused by different genetic variation or combinations is a likely contributing factor because Japan differs substantially from the Western countries in ethnicity, but also in lifestyle, even though there is the resemblance in environmental status as an industrialized and affluent society"* (Iwamoto *et al.,* 2006).

2.5. Sperm quality determinants.

As described before, the development of male reproductive organs is an intricate process that begins during fetal development and continues through puberty, resulting in a mature male reproductive tract which relies on hormonal control for conservation of its function.

Poor semen quality, alteration in male reproductive hormones, hypospadias, cryptorchidism, and testicular germ cell cancer adversely impact male reproductive health and can result in infertility. These male reproductive alterations may appear as a consequence of prenatal or adult exposures.

Recognizing environmental exposures that can interfere with male reproductive tract development and adult male functioning is essential for forming strategies to prevent damaging exposure and preserving male reproductive health.

2.5.1. Fetal exposure.

Some studies have referred to an increase in testicular cancer (Skkakebaek *et al*., 2007; Chia *et al.,* 2010; Engholm *et al*., 2010). In 2010, Chia and colleagues published that the frequency of testicular cancer had increased in countries around the world between 1973 and 2002 (Chia *et al.,* 2010).

This increase of testicular cancer incidence has been associated with the reduction of sperm quality (Bray *et al.,* 2006; Jacobsen *et al.,* 2006; Jorgensen *et al*., 2011). In a current publication, it was concluded that the general population of young men from the Turku area showed lower sperm counts in the most recent birth

cohort compared with a cohort only a few years older. In addition, the younger men also had a higher incidence of testis cancer than the older men (Jorgensen *et al.*, 2011).

The most common abnormalities of the male urogenital organs, hypospadias and cryptorchidism, may also have become more frequent in several countries and their increased incidence is parallel with the decline of semen quality. Cryptorchidism increased from 1.8% in 1959–1961 (Buemann *et al.*, 1961) to 9.0% in 1997–2001 (Boisen *et al.*, 2004) in newborn boys from Denmark. Similar temporal trends in the rates of hypospadias have been shown in Denmark, Australia, and the US (Paulozzi *et al.*, 1997; Boisen *et al.*, 2005; Nassar *et al.*, 2007).

In addition, cryptorchidism and hypospadias has been related with increased risk of developing testicular cancer (Giwercman *et al.*, 1987; Dieckmann *et al.*, 2004). Men with a history of cryptorchidism have an almost five-fold risk of developing testicular germ cell tumors. The similarity of risk factors, fetal origin, and frequent preservation of one or more of these conditions in the same patients, strongly suggest a common etiology that is also influencing semen quality. The testicular dysgenesis syndrome (TDS) hypothesis suggests that disturbed testicular development in fetal life may result in one or more postnatal disorders (Skakkebaek *et al.*, 2001).

But the question is what are the determinants by which cryptorchidism, hypospadias and other male reproductive disorders appear in the male? It is obvious that there is not only one factor that would give a clear cut answer, but there might

be complex interactions of genetic and environmental factors that contribute to TDS.

The use of certain substances during pregnancy, thus exposing the fetus, has been associated with male reproductive disorders. The use of nicotine during pregnancy has been associated with an increased prevalence of cryptorchidism (Damgaard *et al.,* 2008). Maternal alcohol consumption has shown a dose-dependent association with cryptorchidism risk (Damgaard *et al.,* 2007). Such life-style factors can be easily assessed in epidemiological studies (Stogaard *et al.,* 2003; Jensen *et al.,* 2004b; Ramlan-Hansen *et al.,* 2007).

Exposures to other environmental chemicals during pregnancy are more difficult to estimate in adults. However, there are studies that have explored the relationship between the exposure to chemical contaminants and disorders in the male reproductive system. In a case control study, concentration of polybrominated diphenyl ethers (antiandrogenic compound) in breast milk was higher in the group of cryptorchid boys compared with controls (Main *et al.,* 2007).

Finally, endocrine disruptors are chemical substances external to the body, which can disturb metabolic processes even at minimal concentrations. Endocrine disrupters with antiandrogenic properties prevent normal masculinization of male fetuses, and androgenic compounds can masculinize female fetuses (Skakkebaek *et al.,* 2001; Damgaard *et al.,* 2006; Kortenkamp *et al.,* 2008.

Shortened anogenital distance (AGD) has been associated with exposure to endocrine-disrupting chemicals, and, AGD is also related to semen quality in humans (Mendiola *et al.,* 2011). These findings suggest, that "*the androgenic*

environment during early fetal life exerts a fundamental influence on both AGD and adult sperm counts in humans".

Diethylstilbestrol (DES) is probably the most known endocrine disruptor for its use in the treatment of pregnant women in the 1970s. DES used during pregnancy also produced undescended testes and impaired spermatogenesis in human adults (Gill *et al.,* 1977; 1979).

2.5.2. Exposure later in life.

Concurrent exposures to contaminants during the life time of an adult individual can also adversely affect the male reproductive system. Toxicants may affect the neuroendocrine system (the hypothalamic-pituitary-testis axis), the testis (Sertoli and Leydig cells) and post-testicular sites (epididymis) (Woodruff *et al.,* 2010).

One of the main group of factors associated with sperm quality impairment are those related to lifestyles. The consumption of both legal and non-legal drugs in adult life, including tobacco (Wang et al., 2001; Saleh et al., 2002; Kunzle *et al.,* 2003; Belcheva *et al*., 2004), alcohol (Martini et al., 2004; Muthusami *et al.,* 2005), cocaine and cannabis (Bracken *et al.,* 1990; Whan *et al.,* 2006; Badawy *et al*., 2009) all seem to have a damaging effect on semen quality.

Obesity (Jensen *et al*., 2004a; Magnusdottir *et al*., 2005; Nguyen *et al*., 2007; Aggerholm *et al*., 2008), psychological stress (Hjollund *et al.,* 2004; Eskiocak et al., 2005; Zorn et al., 2008), and mobile phone use (Fejes *et al*., 2005;

Wdowiak *et al*., 2007; Agarwal *et al*., 2008), have also been related to altered semen parameters in adulthood.

Exposure to environmental contaminants from birth, such as phthalates, non-persistent pesticides, solvents, metals, polychlorinated biphenyls and organochlorine pesticides play an essential role in semen quality impairment (Wagner *et al.*, 1999; Benoff *et al.*, 2000; Jensen *et al.*, 2006; Chery *et al.*, 2008; Mendiola *et al.*, 2008).

Some dietary habits during adulthood have been associated with good or poor semen quality (Eskenazi *et al*., 2005; Chavarro *et al.*, 2008; Mendiola *et al*.; 2009; Mendiola et al., 2010; Chavarro *et al*., 2011). Mendiola and colleagues published in 2010 that *control subjects had a significantly higher intake of carbohydrates, fiber, folate, vitamin C, and lycopene and lower intakes of proteins and total fat.*

However, to the best of our knowledge no other studies have explored the relationship between dietary intake of antioxidant nutrients and semen quality in young healthy men.

2.6. Study population in semen quality studies.

Semen quality investigations have been conducted with selected and unselected groups of subjects. However, most of the research has been conducted with selected men. Men attending infertility clinics have been one of the main selected study population included in semen quality studies (Chavarro *et al*., 2008; Mendiola *et al*., 2008; Boxmeer *et al.,* 2009; Mendiola *et al.,* 2009; Braga *et al.,* 2011). In 2010, Mendiola and colleagues described significant differences in antioxidant intake between fertile and infertile men attending infertility clinic (Mendiola *et al.,* 2010).

Other selected study subjects have been semen donor candidates (Auger *et al.,* 1995; Bujan *et al.,* 1996), volunteers enrolled after advertisement (Irvine *et al.,* 1996; Paulsen *et al.,* 1996; Eskenazi *et al.,* 2005; Fernández *et al.,* 2011; Mendiola *et al.,* 2011), and candidates for vasectomy (Fisch *et al.,* 1996).

Only a few studies have been conducted with unselected populations. In these studies the aim has been to describe semen quality and identify possible determinants. Most of them have been conducted in North European countries (Andersen *et al.,* 2000; Jorgensen *et al.,* 2002; Punab *et al.,* 2002; Paasch *et al.,* 2008). A special effort was made to include populations that might be representative of the general population in those countries, in an attempt, to minimize possible selection bias. For example, a study was conducted in Finland between 1998 and 2006 to examine the causes of the good reproductive health in Finnish men, concluding that this stage of affairs was due to environmental factors (Jorgensen *et al.,* 2010).

HYPOTHESIS AND OBJECTIVES

Hypothesis

1. Semen quality in young university students from the Murcia Region (Spain) in 2011 will present worse semen parameters than those of the study conducted in Almeria (Spain) in 2001.
2. Higher dietary intake from antioxidant nutrients in healthy young university students will be associated with a better semen quality.
3. There is no correlation between the concentrations of lead, cadmium, and mercury in the three body fluids (whole blood, blood plasma, and seminal plasma).

Objectives

1. To describe semen quality in young university students from the Murcia Region (Spain) and to compare their parameters with those of a study conducted in Almeria (Spain) in 2001.
2. To describe the relationship between semen quality and dietary intake from antioxidant nutrients in healthy young university students.
3. To examine the correlations between the concentrations of heavy metals (lead, cadmium, and mercury) in three body fluids (whole blood, blood plasma, and seminal plasma) and their relationship with semen quality.

CHAPTER 1: Sperm concentration in young university students in Southern Spain

Sperm concentration in young university students in Southern Spain.

4.1. Introduction.

In an article published in 1992, Carlsen *et al.* using historical data suggested an overall decline of sperm concentration worldwide (Carlsen *et al.*, 1992). A later review including an additional 47 studies showed a large decline in sperm concentration in Europe (-2.3%), a smaller decline in the US (-0.8%) and no significant trend in other Regions (-0.2%) (Swan *et al.*, 2000).

A previous publication from Almeria Province (Southern Spain) (Fernandez *et al.*, 2012) indicated that Spanish young men had higher sperm count than young men from Northern Europe (Jorgensen *et al.,* 2002; Punab *et al.,* 2002; Paasch *et al.,* 2008). The fieldwork of that study was carried out between 2001 and 2002. However, testicular cancer incidence rates at the same time seem to be increasing among Spanish men (Llanes Gonzalez *et al.*, 2008). According to the testicular dysgenesis syndrome (TDS) concept (Skakkebæk *et al.,* 2001), a link between risk of impaired semen quality and increased risk of testicular cancer has been suggested. In fact, several epidemiological studies have shown similar geographical trends associating decreased semen quality in a population with increasing testis cancer risk (Jørgensen et al., 2002, 2011; Punab *et al.*, 2002; Richthoff *et al*., 2002; Huyghe *et al.,* 2007; Paasch *et al.*, 2008; Chia *et al.,* 2010).

The objective of this study is to explore the hypothesis that semen quality has decreased among Spanish young men.

4.2. Methods.

The Murcia Young Men's Study (MYMS) is a cross-sectional study of healthy young university students (18-23 years old) in the Murcia Region (Spain). MYMS was carried out between October 6th 2010 and November 29th 2011. Written informed consent was obtained from all subjects. This study was approved by The Research Ethics Committee of the University of Murcia.

Flyers stating, "Young healthy male university students wanted for research project" were posted at university campuses to invite students to participate in this study. To be included in MYMS, subjects had to be university students, been born in Spain after December 31, 1987, and able to contact their mother and ask her to complete a questionnaire. Two hundred and forty students contacted us, 17 subjects had any exclusion criteria (had not been born in Spain: 5; had not been born after December 31, 1987: 9; and had not able to contact their mother: 3). Therefore, 223 students (92.9%) met eligibility and were given an appointment to attend the study at the clinic. Lastly, 215 (96.4%) agreed to participate in the study. On the day of attendance, men underwent an andrological examination, provided a semen sample and completed questionnaires on lifestyle, food frequency, smoking exposure, psychological status and quality of life. Participants were rewarded for their participation.

Physical examination

Body weight and height were measured using a digital scale (Tanita SC 330-S, London, UK). Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters. Testes sizes were measured using a Prader orchidometer. Presence of varicocele or other scrotal abnormalities were also evaluated as well as Tanner stage of pubic hair. Testicular location was classified as follows: low in scrotum (normal), high in scrotum, inguinal canal or non-palpable. Testicular consistency was classified as: normal, soft or hard. Presence of varicocele was classified as: no varicocele, only detected during Valsalva procedure, palpable or visible.

Semen analysis

Men were asked to abstain from ejaculation for at least 48 hours before sample collection. Nonetheless, subjects were not excluded if they had not abstained for that period of time (n=30, 14%). Abstinence time was recorded as the time between current and previous ejaculation as reported by the study subject. Men collected semen samples by masturbation at the clinic. Ejaculate volumes were estimated by specimen weight, assuming a semen density of 1.0 g/mL. Sperm concentration was evaluated by haemocytometer (Improved Neubauer; Hauser Scientific Inc., Horsham, PA, USA). For the assessment of sperm concentration, samples were diluted in a solution of 0.6 m $NaHCO_3$ and 0.4% (v⁄v) formaldehyde in distilled water. The haemocytometer chamber was loading with the dilution and allowing spermatozoa to settle in a humid chamber. From the same dilution, two

chambers of the hemocytometer were assessed and at least 200 spermatozoa per replicate were counted. The two replicate counts were compared to see if they are acceptably close. If so, their average were calculated and used in the analyses, if not, new dilutions were prepared. The spermatozoa were classified as either motile or immotile (WHO, 1999) to report the percentage of motile spermatozoa. Briefly, a 10 µL of well-mixed semen was placed on a clean glass slide that had been kept at 37ºC and covered with a 22x22 mm coverslip. The preparation was placed on the heating stage of a microscope at 37º C and immediately examined at x400 magnification. Total sperm count (volume × sperm concentration) and total motile count (volume × sperm concentration × % progressive motile) were also calculated. Smears for morphology were made, air-dried, fixed, Papanicolaou stained and assessed using strict criteria (Menkveld *et al.,* 1999). The same specialized biologist carried out all the semen analyses. To increase consistency and international comparability (inter-laboratory variation) four sets of duplicate semen samples were sent during the study from the University of Copenhagen's Department of Growth and Reproduction to our Andrology Laboratory.

Statistical analyses

Descriptive statistics are presented using untransformed data. Mean and 95%CI published in other studies were used to test similarities with MYMS semen parameters. The potential effect of several covariates on the semen parameters was assessed using linear regression models. Semen volume, sperm concentration and % of normal sperm morphology showed non-normal distributions and were

transformed using the natural log (ln) before analysis. Covariate assessment included: age, BMI, abstinence time (hours), smoking (current smoker vs. not current smoker), time to start semen analysis (minutes) (only for motility) and season (winter vs. spring, summer or winter). All tests were two-tailed and the level of statistical significance was set at 0.05. Statistical analyses were performed with the statistical package IBM SPSS 19.0 (IBM Corporation, Armonk, New York, USA).

4.3. Results.

Table 1 shows a general description of MYMS population and physical examination compared to Almeria study (Fernandez *et al.*, 2012). Median age, BMI and percentage of current smokers were similar in both studies. Median abstinence time was relatively lower in the Almeria study. For MYMS, most participants had normal testis consistency (99%) and testis location (left: 92.1 % - right: 94.4%). Twelve percent of the subjects had surgical scars in the genital area (including lower abdomen) and 15% had varicocele. Table 2 presents the subjects' semen parameters compared to the study conducted by Fernández *et al*. (2012). MYMS participants presented significantly lower sperm concentration (Mean = 52.1, 95%CI 47.1, 57,1 vs. Mean = 72, 95%CI 63.7, 80.3) and total sperm count (Mean = 154, 95%CI 138, 170 vs. Mean = 215, 95%CI 187, 243) compared to the men studied in the Almeria study11. Figure 1 compares the mean (CI 95%) sperm concentration and total sperm count in our study population with the study conducted by Fernández *et al.* (2012). No other semen parameters were significantly different between the two studies. With regard to MYMS covariate assessment, there was only a significant positive association between abstinence time and semen volume (log scale, β=0.01, p=0.03) and a significant negative association between time to start semen analysis and % of motile sperm (β=-0.16; p=0.02). Only about half of our young men (54.9%) were above the cutoff values of normality for all sperm parameters, according to the current WHO guidelines4. In terms of fecundability, 47% of our men had a sperm concentration below 40×10^6/mL (Bonde *et al.*, 1998).

4.4. Discussion.

Our findings suggest that there has been an adverse temporal trend in sperm concentration among young university student in Spain. MYMS participants presented significantly lower sperm concentration and total sperm count compared to Almeria study subjects (Fernández *et al.,* 2012). Both studies were carried out 10 years apart in Spain.

In MYMS, almost half of our young university men had at least one semen parameter below the current WHO criteria for normality (WHO, 2010). It is also remarkable that 47% of our men had a sperm concentration below 40×10^6/mL. This is a worrying finding because the likelihood of pregnancy is significantly decreased if the sperm concentration is below 40×10^6/mL (Bonde *et al.,* 1998). Covariate assessment only showed to have a significant influence on motility and semen volume.

MYMS subjects are comparable to the Almeria study (Fernandez *et al.,* 2012) ones in terms of age and general health conditions. Besides, methodologies are quite similar regarding study target population, motivation, semen analysis, and any other issue that could be a matter of concern for selection bias. Geographical distance should not be an issue since Almeria and Murcia are in the Southern Spain next to each other, and, in principle, both share similar lifestyles, ethnic and environmental characteristics.

MYMS subjects are also comparable to the population participating in a recent study in Rochester (USA) (Mendiola *et al.,* 2012). Actually, MYMS

followed the same protocol and study procedures recently described for the Rochester Young Men's Study (RYMS) (Mendiola *et al.,* 2012). In this case, with comparable population and following identical research procedures, the young university students from Rochester presented $72.6x10^6$/mL (95%CI 60.9, 84.2) of mean sperm concentration while it was $52.1x10^6$/mL (95%CI 47.1, 57.1) in our current study. That could not be explained by a temporal trend, so that this has to be attributed to dissimilar lifestyles, environmental exposures or possible selection bias.

The incidence of testicular cancer has increased in most industrialized countries during the past 5 decades (Richiardi *et al.,* 2004; Bray *et al.,* 2006; Huyghe *et al.,* 2007; Meeks *et al.*, 2012) and is so in Spain (Llanes Gonzalez *et al.,* 2008). Our finding of lower sperm counts in young Spanish men is in agreement with the hypothesis based on the TDS concept, which associates impaired semen quality with an increased risk of testicular cancer and vice versa.

The difference between the Almeria and Murcia studies is in agreement with Carlsen's hypothesis that there has been a worldwide decline in sperm concentration during recent decades (Carlsen *et al.,* 1992). In fact, the yearly rate of sperm decline calculated for the two studies would be -3.0%, even higher than the one estimated by Swan and colleagues for Europe (-2.3%) (Swan *et al.*, 2010).

However, it is unclear why the semen quality may have declined. As Fernández and colleagues (Fernandez *et al.,* 2012) pointed out: "The Southern Spain has experienced an increasing industrialization and modernization in agriculture practices in the recent years and with this, an increased risk of adverse

exposures, and only follow-up studies of new cohorts in the future will be able to determine whether the testicular function of Spanish men becomes affected".

The reasons might be exposure to environmental or occupational pollutants, toxins, differences in lifestyles or dietary habits of the individuals (Tielemans *et al.,* 1999; Homan *et al.,* 2007), as well as prenatal exposures (Skakkebæk *et al.,* 2001). Volatile organic compounds (Wagner et al., 1990), certain halogenated compounds (Whorton & Foliart, 1983), several heavy metals (Benoff *et al.,* 2000; Robins *et al.,* 1997) or xenoestrogens like some polychlorinated biphenyls (Rozati *et al.,* 2002; Spano *et al.,* 2005; Wassermann *et al.,* 1979), organochlorine compounds (pesticides) (Carreño *et al.,* 2007; Juhler *et al.,* 1999; Swan, 2005), and phthalate esters (Duty *et al.,* 2003), have been associated with compromised semen quality and reduced reproductive male function. One of the main hypotheses for the decline of sperm concentration would be related to prenatal exposures to environmental factors such as endocrine disruptors (Skakkebæk *et al.,* 2001). Continued exposures to these factors might explain why researchers still find a secular decline in sperm concentrations (Robins *et al.,* 1997).

In conclusion, our study suggests that there has been an adverse temporal trend in sperm concentration among young Spanish men during the last decade. Southern Spain has gone through a growing innovation and industrialization in many areas in the last decades, and with this, an increased risk of potential adverse exposures, which might affect reproductive parameters in men.

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4.6. Tables.

Table 1. Descriptive characteristics of the participants and the physical examination for MYMS and Almeria Study (Fernández *et al.,* 2010).

	MURCIA STUDY (N=215)		**ALMERIA STUDY (N=273)**	
VARIABLE	MEAN (SD)	MEDIAN (5-95)	MEAN (SD)	MEDIAN (5-95)
Age (years)[a]	19.2 (5.5)	20.4 (18.1-22.8)	21.3 (2.1)	20.9 (18.4-24.9)
BMI (kg/m^2)	24.0 (3.4)	23.7 (19.4-30.0)	23.8 (3.0)	23.7 (19.6-29.4)
Ejaculation abstinence (hour)[b]	79.3 (37.4)	71.0 (39-136)	75 (40)	67 (35-156)
Size, left testis (ml)[c]	20.7 (3.6)	20.0 (14-25)	18.4 (4.7)	20 (12-25)
Size, right testis (ml)[c]	22.0 (3.4)	22.0 (15-27)	18.6 (4.5)	20 (12.25)
Presence of varicocele (%)	15	-	10	-
Current smokers (%)	31.6	-	32.8	-
Have had:				
Good general health (%)[d]	87.9	-	98.4	-
Diabetes or thyroid disease (%)	0.5	-	0.4	-
Prolonged disease (%)[e]	2.8	-	6.7	-
Cryptorchidism (%)[f]	1.9	-	8.7	-
Cryptorchidism treated (%)[g]	1.5	-	3.6	-
Varicocele diagnosed (%)	4.0	-	0.4	-
STD diagnosed (%)[h]	0.9	-	1.6	-
Inguinal hernia diagnosed (%)	3.3	-	1.2	-
Taken medicine (%)[i]	24.3	-	34	-

SD: standard deviation; (5–95): 5th –95th percentile.

[a]Age calculated as difference between day of attendance in study and self-reported day of birth.

[b]Ejaculation abstinence period calculated as difference between time of current ejaculation and self-reported time of previous ejaculation.

[c]Size assessed by palpation.

[d]Question was 'How would you describe your own health?

[e]Question was 'Have you ever had any long-lasting disease?'

[f]Not born with both testicles in scrotum (irrespective of spontaneous descend, treatment or still cryptorchid).

[g]Hormonal, surgical or combination.

[h]Diagnosed with epididymitis, chlamydia or gonorrhoea.

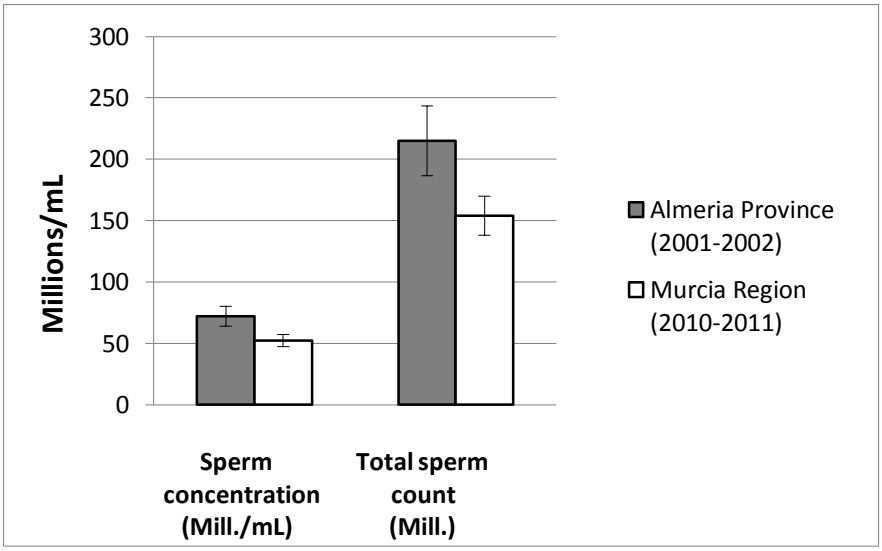
[i]Taken any medication recent 3 months prior to participation in study.

Table 2. Descriptive characteristics of the participants' semen parameters for MYMS and Ameria Study (Fernández et al., 2012).

	MURCIA STUDY (N=215)		**ALMERIA STUDY (N=273)**	
VARIABLE	MEAN (SD)	MEDIAN (5-95)	MEAN (SD)	MEDIAN (5-95)
Semen volume (ml)	3.3 (1.7)	3.0 (1-6.4)	3.1 (1.5)	3.0 (1.0-5.8)
Sperm concentration ($x10^6$/ml)	52.1 (37.1)	44.0 (8.9-129)	72 (70)	51 (5-206)
% Motile sperm (A + B + C)	56.5 (10.9)	57.2 (38.9-74.0)	59 (16)	60 (28-85)
% Normal morphology	10.3 (6.3)	9.0 (2.8-23)	9.4 (5.5)	8.3 (1.9-20.6)
Total sperm count ($x10^6$)	154 (120)	121 (17.8-400)	215 (240)	149 (8-599)

4.7. Figures.

Figure 1. A comparison of mean sperm concentration and total sperm count in young men studies conducted in Spain. Error bars represents confidence intervals at 96%.



CHAPTER 2: Dietary intake of antioxidant nutrients and semen quality in young university students

Dietary intake of antioxidant nutrients and semen quality in young university students.

5.1. Introduction.

Several reports have suggested a decline in semen quality in recent decades (Carlsen *et al.,* 1992; Auger *et al.,* 1995; Swan *et al.,* 2000; Skakkebaek *et al.,* 2006). A special concern has been raised for the low sperm concentration found in young men in some European countries (Jorgensen *et al.,* 2002). Semen quality may be impaired by environmental exposures (Benoff *et al.,* 2000; Rozati *et al.,* 2002; Duty *et al.,* 2003; Spanò *et al.,* 2005; Swan *et al.,* 2005; Carreño *et al.,* 2007), lifestyle (Homan *et al.,* 2007; Braga *et al.,* 2012) or dietary factors. Among the latter, higher intakes of caffeine (Jensen *et al.,* 2010), meat or milk products (Mendiola *et al.,* 2009), saturate fats (Attaman *et al*., 2012), soy foods and soy isoflavones (Chavarro *et al.,* 2008) have been associated with a decreased sperm quality.

However, diet may have a positive contribution as antioxidant intake may have a positive effect on semen quality (Mendiola *et al.,* 2009). It is known that spermatozoa are susceptible to oxidative damage because their plasma membranes are rich in polyunsaturated fatty acids and have low concentrations of scavenging enzymes (de Lamirande *et al.,* 1995). Reactive oxygen species (ROS) levels are higher and levels of seminal plasma antioxidants are significantly lower in

subfertile patients than in the normal fertile control subjects (Kao *et al.,* 2008; Abd-Elmoaty *et al.,* 2010).

Although diet might be an important and modifiable source of antioxidant intake, most information is coming from clinical trials with large doses of antioxidant supplements (Ross *et al.,* 2010). So far, only two observational studies have analyzed dietary intake of specific antioxidant nutrients and semen quality. In 2005 a study on 97 non-smoking healthy men between 20 to 80 years old from a non-clinical setting (Eskenazi *et al.,* 2005) and, in 2010, a study in men attending infertility clinics (Mendiola *et al.,* 2010). Both studies support the hypothesis of a positive association between dietary intake of antioxidant nutrients and semen quality.

In spite of the large interest and concern about semen quality in young men, the relationship between the intake of antioxidant nutrients and semen quality in the young population remains unexplored. The objective of this study is to describe the relationship between dietary intake of antioxidant nutrients and semen quality in healthy young university students.

5.2. Methods.

The Murcia Young Men's Study (MYMS) is a cross-sectional study of healthy young university students (18-23 years old) in the Murcia Region (Spain). MYMS was carried out between October 2010 and November 2011. Written informed consent was obtained from all subjects. The Research Ethics Committee of the University of Murcia approved this study.

Flyers stating, "Young healthy male university students wanted for research project" were posted at university campuses to invite students to participate in this study. To be included in MYMS, subjects had to be university students, been born in Spain after December 31, 1987, and able to contact their mother and ask her to complete a questionnaire. Two hundred and forty students contacted us, 17 subjects had some exclusion criteria (had not been born in Spain: 5; had not been born after December 31, 1987: 9; and had not able to contact their mother: 3). Therefore, 223 students (92.9%) met eligibility and were given an appointment to attend the study at the clinic. Lastly, 215 (89.6%) agreed to participate in the study. In addition, five men reporting an implausible calorie intake greater than 5.000 kilocalories (kcals) were further excluded from the analysis. On the day of attendance, men underwent an andrological examination, provided a semen sample and completed questionnaires on lifestyle, food frequency, smoking exposure, psychological status and quality of life. Participants were rewarded for their participation (€50 gift card).

Physical examination

Body weight and height were measured using a digital scale (Tanita SC 330-S, London, UK). Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters. Testes sizes were measured using a Prader orchidometer. Presence of varicocele or other scrotal abnormalities were also evaluated as well as Tanner stage of pubic hair. Presence of varicocele was classified as: no varicocele, only detected during Valsalva procedure, palpable or visible.

Dietary assessment

We used a semi-quantitative food frequency questionnaire (FFQ) to assess the usual daily intake of foods and nutrients (available at: http://bibliodieta.umh.es/files/2011/07/ CFA101.pdf). The FFQ included 101 food items to capture the major sources of the most relevant nutrients, including specific carotenoids. This questionnaire was a modified version from a previous FFQ based on the Harvard questionnaire (Willett *et al.,* 1995), which we developed and validated using four 1-week dietary records in an adult population in Valencia (Eastern Spain). The validity and reproducibility correlation coefficients (adjusted for energy intake) ranged from 0.38 for reproducibility of carotenoids to 0.44 for validity of vitamin C (Vioque *et al.,* 1995); this is a similar range to other established diet questionnaires (Willet *et al.,* 1998). This FFQ also showed satisfactory biochemical validity when compared to plasma levels (Vioque *et al.,* 2007).

Participants in the study were asked how often, on average, they had consumed each food item over the past year. Serving sizes were specified for each food item in the FFQ. The questionnaire had nine possible responses, ranging from 'never or less than once per month' to 'six or more per day'. Nutrient values were primarily obtained from the food composition tables of the US Department of Agriculture publications as well as other published sources for Spanish foods and portion sizes (Palma *et al.,* 2008; US Department of Agriculture, 2010). In order to obtain average daily nutrient intakes from diet for each individual, we multiplied the frequency of use for each food by the nutrient composition of the portion/serving size specified on the FFQ and added the results across all foods.

Nutrient intakes were adjusted for total energy intake by calculating the residuals from a linear regression with the log e of the nutrient modeled as the dependent variable and the log e of total energy intake as the independent variable (Willett *et al.,* 1998).

Semen analysis

Men were asked to abstain from ejaculation for at least 48 hours before sample collection. Nonetheless, subjects were not excluded if they had not abstained for that period of time (n=30). Abstinence time was recorded as the time between current and previous ejaculation as reported by the study subject. Men collected semen samples by masturbation at the clinic. Ejaculate volumes were estimated by specimen weight, assuming a semen density of 1.0 g/mL. Sperm concentration was evaluated by haemocytometer (Improved Neubauer; Hauser

Scientific Inc., Horsham, PA, USA). For the assessment of sperm concentration, samples were diluted in a solution of 0.6 m $NaHCO_3$ and 0.4% (v⁄v) formaldehyde in distilled water. The haemocytometer chamber was loading with the dilution and allowing spermatozoa to settle in a humid chamber. From the same dilution, two chambers of the haemocytometer were assessed and at least 200 spermatozoa per replicate were counted. The two replicate counts were compared to see if they are acceptably close. If so, their average were calculated and used in the analyses, if not, new dilutions were prepared. The spermatozoa were classified as either motile or immotile (WHO, 2010) to report the percentage of motile spermatozoa (progressive and no progressive). Briefly, a 10 µL of well-mixed semen was placed on a clean glass slide that had been kept at 37ºC and covered with a 22x22 mm coverslip. The preparation was placed on the heating stage of a microscope at 37º C and immediately examined at x400 magnification. Total motile sperm count (volume × sperm concentration × % motile sperm) was also calculated. Smears for morphology were made, air-dried, fixed, Papanicolaou stained and assessed using strict criteria (Menkveld *et al*.., 1990). The same specialized biologist carried out all the semen analyses. To increase consistency and international comparability (inter-laboratory variation) five sets of duplicate semen samples were sent during the study from the University of Copenhagen's Department of Growth and Reproduction to our Andrology Laboratory

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Statistical analyses

Semen volume, sperm concentration, total motile sperm count and percentage of morphologically normal sperm showed non-normal distributions and were transformed using the natural log (ln) before analysis. Nutrient intakes were adjusted for total energy intake using the nutrient residual method (Willet *et al.*, 1998) and further categorized in quartiles. Men with the lowest intake of each micronutrient were considered as the reference group. Linear regression was used to examine the association of each antioxidant with semen quality parameters. Tests for linear trend were performed using the median values of micronutrient intake in each category as a continuous variable and semen parameters as the response variable. The potential effect of body mass index (BMI) (kg/m^2), ejaculation abstinence time (hours), total calorie intake (kcal/day), alcohol intake (g/day), caffeine intake (mg/day), light to extreme exercise (hours/week), presence of varicocele (yes vs. no), smoking (current smoker vs. not current smoker), time to start semen analysis (minutes) and season (winter vs. spring, summer or winter), were assessed using lineal regression models. When inclusion of a potential covariate resulted in a change in the β-coefficient of < 10%, the variable was not retained in final models. We used analysis of covariance (ANCOVA) to calculate adjusted semen parameters for each quartile by relevant covariates. Multivariate ANCOVA models were created with continuous semen parameters as dependent variables, and antioxidant categories and covariates as independent variables. We considered that an association was present when we found statistically significant linear trend across quartiles, or a statistically significant difference in semen

parameters between any of other quartiles. All tests were two-tailed and the level of statistical significance was set at 0.05. Statistical analyses were performed with the statistical package IBM SPSS 19.0 (IBM Corporation, Armonk, New York, USA).

5.3. Results.

Our study population was Caucasian (99%), with a mean age of 19.2 years [Standard Deviation (SD): 5.5], and a BMI of 24.0 (SD: 3.4). Almost thirty two percent were smokers. The mean duration of ejaculation abstinence time was 79.3 hours (SD: 37.4) and the mean time from semen collection to start of semen analysis was 37 minutes (SD: 15.9). Mean sperm concentration was 52.1×10^6/mL (SD: 37.1) and mean motile sperm 56.5% (SD: 10.9). Mean value for morpholically normal spermatozoa was 10.3% (SD: 6.3%). Testicular volume was 20.7 mL (SD: 3.6) for the left testicle and 22.0 mL (SD: 3.4) for the right. Most men (96%) of our men had at least "5" in the Tanner classification of normal pubic hair, and 15% had varicocele in the left testis.

Table 1 presents the covariate mean values by the first and fourth quartile of adjusted dietary intake of antioxidants. For instance, abstinence time showed an increasing effect on total motile sperm count ($p < 0.05$). On the contrary, there was a statistically significant negative association between the time to start semen analysis and percentage of motile sperm ($p < 0.05$). Table 2 shows the semen parameters of the study sample in the first and fourth quartile of adjusted dietary intake of antioxidant nutrients.

Table 3 presents the multivariate adjusted model of dietary intake of antioxidant nutrients and semen parameters. Semen volume was associated with vitamin C intake (p for trend=0.04), being higher for Q2, Q3 and Q4 than for Q1 of intake. Median intake of vitamin C for the first quartile was 63 mg per day. Differences were also found in semen volume and lycopene intakes in the Q2 and

Q4 compared with the lowest quartile of intake. Semen volume was also higher in the Q3 than in Q1 of β-carotene intake. However, the p for trends were not statistically significant for neither lycopene nor β-carotene and semen volume.

Cryptoxanthin (p for trend=0.03) and β-carotene (p for trend =0.04) were associated with total motile sperm count. Lycopene and vitamin C were also associated with higher total motile sperm count (p for trend<0.05), and significant differences were found between the lowest and highest quartiles for both nutrients. Other semen parameters did not show statistically significant differences with dietary intake of antioxidant nutrients.

5.4 Discussion.

Our study suggests a positive association between the dietary intake of several antioxidant nutrients (cryptoxanthin, vitamin C, lycopene and β-carotene) and total motile sperm count in young healthy males. Semen volume increased with higher intakes of vitamin C and β-carotene.

The association between vitamin C and total progressively motile sperm was found by Eskenazi and colleagues in an older population (Eskenazi *et al.,* 2005), although vitamin C was not associated with semen volume. Vitamin C was also associated with being normozoospermic in a case-control study in a clinical setting, though specific semen parameters were not assessed (Mendiola *et al.,* 2010). Vitamin C is a water-soluble antioxidant for ROS found in seminal plasma at higher concentrations than in blood plasma (Agarwal *et al.,* 2011). In an open-label supplementation trial, vitamin C improved sperm count, sperm motility and sperm morphology in oligozoospermic patients (Akmal *et al.,* 2006). The Reference Daily Intake or Recommended Daily Intake (RDI) for vitamin C is 60 mg per day, which is the median value of the first quartile in our study population. Our study raise doubts whether current RDI may underestimate vitamin C requirements needed with regards to semen quality.

For β-carotene, Eskenazi and coworkers, also found that men with higher intake of β-carotene had better sperm concentration and progressive sperm motility than men with low intake (Eskenazi *et al.,* 2005). In that study lycopene and cryptoxantin were not analyzed. Mendiola and colleagues, found that lycopene but not β-carotene was associated with good semen quality (Mendiola *et al.,* 2010). No

previous studies have reported an association between cryptoxanthin and total motile sperm count. However, a study published in 2008 suggested that cryptoxanthin plays a role repairing DNA oxidation damage, in addition to acting as an antioxidant in human cells (Lorenzo *et al.,* 2009).

For other nutrients such as α-carotene, lutein + zeaxanthin, vitamin b6, vitamin b12, vitamin D, vitamin E and folate, we did not find an association with sperm parameters. Similarly, folate intake did not improve semen quality in 97 healthy non-smoking men (Eskenazi *et al.,* 2005) although in a clinical setting, Mendiola and coworkers found higher intake of folate in normozoospermic controls (Mendiola *et al.,* 2010). Conversely, vitamin E was not associated with good sperm quality in the case-control study (Mendiola *et al.,* 2010) but it was with progressive sperm motility and total progressively motile sperm in healthy individuals (Eskenazi *et al.,* 2005). Supplementation with selenium and vitamin E in infertile men improved sperm quality and had protective effects especially on motility (Moslemi *et al.,* 2011).

Some possible limitations of our study design should be discussed. Only one sample of semen was taken for each subject. However, there are indications that one semen sample may be sufficient to characterize semen quality of the individuals in epidemiological studies (Carlsen *et al.,* 2005; Stokes-Riner *et al.,* 2007). Bias due to measurement errors may also occur since there is no perfect method to assess diet. However, the FFQ used in this study was previously validated in an adult population of the same area in Spain and it has been used in other populations (Guxens *et al.,* 2011). If any, any bias in assessing diet should be

not differential which should reinforce our results. And finally, there might be selection bias as the subjects were university student volunteers. However, during the recruitment, the study was not advertised as a fertility study and participation was ensured because subjects were rewarded for participating. The proportion of individuals with andrological anomalies was within the expected range in this population.

In conclusion, our study suggests that some sperm parameters are sensitive to dietary intake of antioxidant nutrients, and that current recommendations of vitamin C intake may be insufficient to reach the optimum benefit in terms of semen quality.

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5.6 Tables.

Table 1. Covariate values by the first and fourth quartile of adjusted dietary intake of antioxidant nutrients.

Range values for Q1 and Q4	**BMI**		**Total caloric intake**		**Smokers (%)**	**Alcohol intake (g/day)**		**Caffeine intake (mg/day)**		**Abstinence time (hours)**		**Time to perform analysis (minutes)**	
α-carotene													
Q1 (3.8-118 µg/day)	24.5	(3.7)	2489	(994)	33%	9.6	(11.8)	99.4	(90.4)	75.0	(28.9)	40.1	(12.2)
Q4 (404-1651 µg/day)	23.6	(3.2)	2774	(941)	26%	7.6	(6.3)	126	(141)	82.5	(49.6)	39.4	(9.5)
β-carotene													
Q1 (101-1492 µg/day)	24.2	(3.3)	2464	(787)	28%	8.7	(10.8)	95.8	(99.5)	76.4	(22.0)	39.2	(11.2)
Q4 (4228-10996 µg/day)	24.5	(3.8)	2647	(931)	29%	7.4	(6.8)	137	(158)	79.7	(42.2)	38.0	(9.0)
Lutein+Zeaxanthin													
Q1 (226-784 µg/day)	24.2	(3.3)	2379	(762)	37%	9.8	(10.9)	110	(111)	72.5	(20.4)	37.5	(11.4)
Q4 (2481-10229 µg/day)	24.7	(4.1)	2557	(1062)	34%	7.8	(6.6)	130	(163)	78.3	(36.6)	40.4	(12.4)
Lycopene													
Q1 (1330-2446 µg/day)	24.1	(3.4)	2477	(766)	34%	8.3	(7.8)	115	(113)	78.2	(30.5)	39.6	(10.4)
Q4 (5869-14583 µg/day)	24.2	(3.4)	2383	(732)	32%	9.4	(9.3)	124	(167)	75.2	(28.9)	34.3	(9.2)
Vitamin B6													
Q1 (1-1.9 mg/day)	23.5	(3.4)	2582	(778)	26%	11.6	(12.7)	108	(123)	76.2	(29.6)	38.6	(12.2)
Q4 (2.9-5.2 mg/day)	24.4	(3.8)	2507	(973)	12%	6.7	(6.8)	115	(131)	88.3	(51.3)	38.3	(9.2)
Vitamin B12													
Q1 (4.0-8.7 µg/day)	24.2	(3.4)	2506	(974)	41%	11.8	(11.8)	114	(148)	74.4	(27.7)	38.1	(11.0)
Q4 (15.7-55.9 µg/day)	24.7	(3.9)	2560	(1007)	32%	8.7	(8.7)	111	(89.6)	77.2	(41.7)	38.5	(10.5)

Vitamin C													
Q1 (7.8-76.6 mg/day)	23.8	(3.2)	2573	(1004)	35%	10.9	(12.7)	104	(111)	74.3	(24.3)	37.9	(11.1)
Q4 (143-518 mg/day)	24.2	(4.1)	2512	(809)	37%	8.5	(7.1)	139	(161)	75.9	(28.8)	38.3	(10.6)
Vitamin D													
Q1 (0.43-2.4 µg/day)	24.3	(3.7)	2586	(884)	39%	11.3	(8.7)	108	(124)	80.6	(28.2)	37.3	(10.6)
Q4 (4.9-14.1 µg/day)	23.6	(2.9)	2615	(1045)	25%	7.5	(6.4)	143	(154)	81.4	(44.7)	38.6	(11.2)
Vitamin E													
Q1 (4.4-8.4 mg/day)	24.0	(3.7)	2640	(1081)	34%	10.6	(11.5)	120	(119)	78.1	(19.4)	37.5	(10.4)
Q4 (11.7-21.3 mg/day)	24.2	(3.8)	2697	(930)	22%	6.4	(6.2)	110	(106)	85.3	(50.8)	38.2	(10.9)
Folate													
Q1 (97.8-241 µg/day)	23.9	(2.9)	2633	(1114)	37%	11.4	(11.6)	107	(121)	72.9	(27.9)	38.5	(11.0)
Q4 (336-605 µg/day)	24.6	(3.8)	2573	(835)	26%	9.2	(7.8)	140	(165)	87.5	(49.1)	38.7	(9.8)
Cryptoxanthin													
Q1 (2.7-157 µg/day)	23.7	(3.2)	2655	(1023)	30%	9.8	(12.3)	102	(111)	73.6	(25.2)	38.8	(10.9)
Q4 (405-856 µg/day)	23.9	(3.5)	2380	(838)	34%	9.3	(7.7)	135	(159)	74.5	(30.2)	38.4	(10.4)

Note: Continuous variables are shown as mean and standard deviation unless otherwise indicated.

Table 2. Semen parameters of the study sample in the first and fourth quartile of adjusted dietary intake of antioxidant nutrients.

Range values for Q1 and Q4	**Volume**		**Motile Sperm**		**Morphologically Normal sperm**		**Sperm Concentration**		**Total Motile Sperm Count**	
	(ml)	**SD**	**%**	**SD**	**%**	**SD**	**($x10^6$/ml)**	**SD**	**($x10^6$)**	**SD**
α-carotene										
Q1 (3.8-118 µg/day)	3.4	(2.2)	55.2	(11.1)	10.5	(5.3)	54.1	(38.7)	88.2	(70)
Q4 (404-1651µg/day)	3.4	(1.5)	55.4	(9.7)	9.9	(6.9)	48.2	(33.7)	87.0	(69.7)
β-carotene										
Q1 (101-1492 µg/day)	3.3	(2.2)	55.7	(11.7)	9.1	(4.8)	59.4	(42.7)	89.2	(69.1)
Q4 (4228-10996 µg/day)	3.2	(1.6)	56.6	(9.9)	10.1	(5.5)	56.8	(34.3)	104	(83.1)
Lutein+Zeaxanthin										
Q1 (226-784 µg/day)	3.5	(2.2)	56.3	(12.5)	8.3	(4.8)	52.7	(41.5)	85.4	(68.2)
Q4 (2481-10229 µg/day)	3.4	(1.5)	56.8	(9.8)	10.0	(5.3)	57.1	(33.8)	104	(70.3)
Lycopene										
Q1 (1330-2446 µg/day)	3.0	(1.8)	56.6	(11.9)	9.7	(5.6)	55.6	(43.4)	86.6	(81.8)
Q4 (5869-14583 µg/day)	3.3	(1.7)	59.0	(9.9)	11.6	(6.2)	56.3	(33.7)	110	(86.0)
Vitamin B6										
Q1 (1-1.9 mg/day)	3.3	(1.8)	56.6	(10.0)	10.3	(6.5)	56.8	(43.4)	92.4	(81.9)
Q4 (2.9-5.2 mg/day)	3.2	(1.6)	54.7	(8.8)	10.4	(6.4)	53.5	(35.8)	89.9	(71.4)
Vitamin B12										
Q1 (4.0-8.7 µg/day)	3.5	(1.8)	57.3	(10.3)	9.1	(5.1)	50.0	(34.9)	89.9	(73.9)
Q4 (15.7-55.9 µg/day)	2.9	(1.3)	55.3	(12.7)	10.9	(7.3)	54.5	(37.9)	88.6	(76.7)
Vitamin C										
Q1 (7.8-76.6 mg/day)	2.9	(2.1)	56.9	(10.9)	9.6	(5.7)	54.1	(41.2)	77.0	(74.0)
Q4 (143-518 mg/day)	3.3	(1.5)	55.9	(9.7)	10.4	(5.9)	54.5	(33.3)	97.9	(68.1)

Vitamin D										
Q1 (0.43-2.4 µg/day)	3.0	(1.7)	56.6	(11.6)	10.1	(6.7)	53.5	(37.7)	85.0	(71.2)
Q4 (4.9-14.1 µg/day)	3.4	(1.6)	55.7	(9.3)	9.9	(6.0)	49.2	(36.8)	84.7	(69.6)
Vitamin E										
Q1 (4.4-8.4 mg/day)	3.2	(1.6)	55.7	(11.8)	9.8	(5.6)	49.6	(37.3)	77.8	(68.6)
Q4 (11.7-21.3 mg/day)	3.2	(1.4)	54.9	(10.5)	9.7	(5.7)	51.7	(36.0)	86.3	(65.3)
Folate										
Q1 (97.8-241 µg/day)	3.3	(2.2)	56.8	(11.6)	8.9	(5.8)	55.2	(43.7)	85.2	(72.4)
Q4 (336-605 µg/day)	3.3	(1.6)	54.7	(10.4)	10.4	(5.8)	53.8	(34.9)	94.8	(73.2)
Cryptoxanthin										
Q1 (2.7-157 µg/day)	3.1	(2.3)	56.1	(10.4)	9.8	(5.6)	53.3	(39.6)	81.5	(82.2)
Q4 (405-856 µg/day)	3.3	(1.4)	58.2	(9.6)	11.2	(5.7)	52.6	(33.3)	95.8	(61.2)

Note: Continuous variables are shown as mean and standard deviation unless otherwise indicated.

Table 3. Multivariate adjusted model of dietary intake of antioxidant nutrients and semen parameters.

	Volume		Motile Sperm		Morphologically Normal Sperm		Sperm Concentration		Total Motile Sperm Count	
Median for each quartile	**(ml)**	**CI 95%**	**%**	**CI 95%**	**%**	**CI 95%**	**($x10^6$/ml)**	**CI 95%**	**($x10^6$)**	**CI 95%**
α-carotene										
Q1 (73.9 µg/day)	2.8	2.4, 3.3	56.3	53.4, 59.2	9.5	8.0, 11.4	41.3	31.7, 53.8	62.4	48.1, 81.0
Q2 (178 µg/day)	3.4	2.1, 2.9	57.5	54.7, 60.2	7.7	6.5, 9.2	35.3	27.3, 45.7	46.2	35.9, 59.6
Q3 (280 µg/day)	3.0	2.5, 3.5	58.1	55.4, 60.8	9.7	8.2, 1.5	41.1	31.9, 53.0	81.0	63.1, 104.
Q4 (795µg/day)	3.0	2.5, 3.6	55.2	52.4, 55.0	7.8	6.6, 9.3	35.5	24.9, 42.4	61.2	46.9, 79.9
p - trend	0.26		0.33		0.33		0.26		0.73	
β-carotene										
Q1 (1158 µg/day)	2.6	2.2, 3.0	56.3	53.5, 59.1	8.6	7.2, 10.3	44.9	34.8, 58.0	59.6	46.2, 76.7
Q2 (1927 µg/day)	2.5	2.1, 3.0	56.8	53.9, 59.6	8.1	6.7, 9.6	29.8	23.0, 38.8	47.6	36.5, 61.9
Q3 (3192 µg/day)	3.3	2.8, 3.9[a]	58.0	55.3, 60.7	9.6	8.1, 1.3	36.8	28.6, 47.4	66.5	51.7, 85.5
Q4 (5286 µg/day)	2.9	2.4, 3.4	56.2	53.3, 59.0	8.4	7.0, 0.0	39.2	30.2, 50.8	76.1	58.4, 99.1
p - trend	0.22		0.97		0.97		0.98		0.04[a]	
Lutein+Zeaxanthin										
Q1 (618 µg/day)	2.9	2.4, 3.4	56.7	53.9, 59.5	7.8	6.5, 9.3	38.9	30.1, 50.1	59.2	45.9, 76.3
Q2 (115 µg/day)	2.9	2.4. 3.4	57.4	54.6, 60.2	9.3	7.8, 11.1	34.7	26.7, 45.1	61.9	47.6, 80.6
Q3 (1858 µg/day)	2.5	2.1, 3.0	56.4	53.6, 59.2	9.0	7.6, 10.7	33.7	26.1, 43.6	50.7	39.2, 65.5
Q4 (3157 µg/day)	3.0	2.6, 3.6	56.9	54.0, 59.7	8.6	7.2, 10.3	43.3	33.2, 56.5	79.5	60.8, 104
p - trend	0.70		0.94		0.94		0.45		0.15	
Lycopene										
Q1 (1780 µg/day)	2.4	2.0, 2.8	57.0	54.2, 59.8	8.7	7.3, 10.3	39.6	30.6, 51.2	48.6	37.6, 62.6
Q2 (3199 µg/day)	3.2	2.8, 3.8[a]	56.7	53.9, 59.5	8.2	6.9, 9.7	33.9	26.1, 43.9	69.5	53.7, 90.0

Q3 (4647 µg/day)	2.7	2.3, 3.1	55.2	52.4, 58.1	8.1	6.8, 9.7	33.4	25.8, 43.5	53.6	41.3, 69.6
Q4 (7053 µg/day)	3.0	2.6, 3.6[a]	58.3	55.5, 61.2	9.7	8.2, 11.6	43.5	33.5, 56.4	79.9	61.7, 103[a]
p - trend	0.15		0.57		0.57		0.52		0.03[a]	
Vitamin B6										
Q1 (1.8 mg/day)	2.8	2.4, 3.4	56.6	53.9, 59.4	8.9	7.5, 10.6	44.2	34.1, 57.2	65.9	50.9, 85.3
Q2 (2.2 mg/day)	2.8	2.4, 3.3	56.7	53.9, 59.4	8.7	7.3, 10.4	37.9	29.2, 49.4	63.1	48.4, 82.2
Q3 (2.5 mg/day)	2.9	2.5, 3.5	59.0	56.3, 61.8	8.5	7.1, 10.1	33.1	25.6, 42.9	62.8	48.3, 81.5
Q4 (3.1 mg/day)	2.7	2.2, 3.2	54.8	52.0, 57.7	8.5	7.1, 10.1	35.1	27.0, 45.6	54.8	41.8, 72.0
p - trend	0.72		0.48		0.48		0.20		0.34	
Vitamin B12										
Q1 (6.5 µg/day)	2.9	2.5, 3.4	57.4	54.6, 60.1	8.3	7.0, 9.9	37.1	28.8, 47.8	63.9	49.3, 82.8
Q2 (9.9 µg/day)	2.7	2.3, 3.2	58.1	55.3, 60.9	8.2	6.9, 9.8	47.1	36.4, 61.0	75.6	58.3, 98.0
Q3 (13.0 µg/day)	3.1	2.6, 3.7	56.1	53.4, 58.9	9.1	7.7, 10.8	30.4	23.6, 39.3	55.5	42.7, 72.0
Q4 (21.8 µg/day)	2.5	2.1, 3.0	55.7	52.8, 58.6	9.0	7.5, 10.7	36.9	28.4, 47.8	53.4	41.0, 69.5
p - trend	0.28		0.30		0.30		0.61		0.16	
Vitamin C										
Q1 (62.6 mg/day)	2.3	1.9, 2.7	56.9	54.0, 59.8	8.4	7.0, 10.0	41.9	32.3, 54.5	49.2	37.8, 63.8
Q2 (93.4 mg/day)	3.0	2.6, 3.6[a]	56.9	54.1, 59.7	8.9	7.5, 10.6	40.0	31.0, 51.7	66.7	51.6, 86.1
Q3 (127 mg/day)	3.0	2.5, 3.5[a]	57.5	54.7, 60.3	8.7	7.3, 10.4	27.2	21.2, 35.1	56.2	43.3, 73.0
Q4 (175 mg/day)	3.0	2.6, 3.6[a]	56.1	53.4, 58.9	8.7	7.3, 10.3	42.9	33.4, 55.3	77.4	59.9, 100[a]
p - trend	0.04[a]		0.74		0.74		0.85		0.04[a]	
Vitamin D										
Q1 (1.8 µg/day)	2.6	2.2, 3.0	56.3	53.5, 59.1	8.8	7.4, 10.5	40.8	31.4, 52.9	59.4	45.6, 77.2
Q2 (2.8 µg/day)	3.0	2.5, 3.5	57.5	54.6, 60.3	7.9	6.6, 9.4	33.5	25.9, 43.3	62.1	47.8, 80.6
Q3 (4.2 µg/day)	2.8	2.4, 3.4	57.9	55.1, 60.7	9.5	8.0, 11.3	38.2	29.4, 49.7	72.6	55.6, 94.7
Q4 (5.9 µg/day)	2.8	2.4, 3.4	55.6	52.8, 58.5	8.5	7.2, 10.2	37.6	29.0, 48.8	54.1	41.7, 70.2

p - trend	0.59		0.70		0.70		0.93		0.72	
Vitamin E										
Q1 (7.3 mg/day)	2.7	2.2, 3.2	56.4	53.6, 59.2	8.6	7.2, 10.2	36.4	27.9, 47.4	49.9	38.5, 64.9
Q2 (9.2 mg/day)	3.0	2.6, 3.6	58.9	56.1, 61.6	8.3	7.0, 9.8	37.8	29.3, 48.8	66.8	51.8, 86.1
Q3 (10.6 mg/day)	2.8	2.3, 3.3	57.6	54.8, 60.3	10.0	8.4, 11.9	41.7	32.0, 54.3	71.7	55.2, 93.1
Q4 (13.0 mg/day)	2.7	2.3, 3.2	54.3	51.4, 57.1	7.9	6.7, 9.4	34.1	26.2, 44.3	59.9	45.8, 78.3
p - trend	0.93		0.20		0.20		0.77		0.36	
Folate										
Q1 (210 µg/day)	2.7	2.3, 3.2	57.3	54.6, 60.1	7.8	6.5, 9.3	40.1	30.8, 52.3	60.2	46.1, 78.6
Q2 (264 µg/day)	2.6	2.2, 3.1	59.1	56.4, 61.7	8.9	7.5, 10.6	41.1	31.8, 53.0	65.0	50.2, 84.0
Q3 (302 µg/day)	3.0	2.6, 3.6	56.4	53.8, 59.1	9.2	7.8, 10.9	33.4	25.9, 43.3	60.6	46.6, 78.6
Q4 (382 µg/day)	2.8	2.4, 3.4	55.0	52.2, 57.7	8.6	7.2, 10.2	35.5	27.4, 46.1	60.0	46.2, 79.5
p - trend	0.54		0.12		0.12		0.40		0.95	
Cryptoxanthin										
Q1 (105 µg/day)	2.5	2.1, 2.9	56.1	53.2, 58.9	8.5	7.1, 10.1	41.8	32.0, 54.4	53.8	41.3, 70.0
Q2 (209 µg/day)	2.7	2.3, 3.2	57.0	54.2, 59.9	8.9	7.5, 10.6	34.2	26.3, 44.4	54.3	41.8, 70.5
Q3 (318 µg/day)	3.0	2.6, 3.6	56.0	53.3, 58.8	7.8	6.6, 9.3	34.5	26.8, 44.6	63.8	49.4, 82.4
Q4 (505 µg/day)	3.1	2.6, 3.6	58.1	55.4, 60.9	9.5	8.0, 11.2	39.9	30.8, 51.7	77.0	59.3, 100
p - trend	0.06		0.34		0.36		0.98		0.03[a]	

[a]Statistically significant.

Semen parameters are presented by the mean unless otherwise indicated.

Note: Tests for linear trend were performed using the median value for each quartile. Multivariate model adjusted for season, BMI, presence of varicocele, total calorie intake, light to extreme exercise, alcohol and caffeine intake, smoking, time to start analysis and abstinence time.

CHAPTER 3: Correlations between different heavy metals in diverse body fluids (studies of human semen quality)

Correlations between different heavy metals in diverse body fluids (studies of human semen quality).

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6.1. Introduction.

Over time there has been a significant decline of human fertility (Skakkebaek *et al.,* 2006). Like other European countries, Spain is since 1981 well below the 2.1 children needed to maintain replacement level (INE). Birth rate have declined mainly due to changes in lifestyle and social mores and increased contraception (Mills *et al.*, 2011).

These demographic transformations, as much as they are socially valued and desirable, have important clinical consequences. The fertility decline has resulted in a major delay in the average age of conception. The first pregnancy is postponed to ages at which women fecundity is decreased (Dunson *et al.*, 2004). That may be one important reason why the medical reproductive units have taken on such a relevant role in developed countries. Between 2002 and 2004 more than 6% of Danish children were born through assisted reproduction techniques (Skakkebaek *et al*., 2006). Consequently social and medical considerations about infertility have become an important concern in recent years.

In parallel, it has been hypothesized that there is a worldwide decline in male semen quality (Carlsen *et al.,* 1992; Irvine *et al.,* 1996; Spanò *et al.,* 2005;

Hause *et al.,* 2006), but it is clearly not uniform (Swan *et al.,* 2003). The decline in semen quality has been linked to environmental and work-related toxic exposures (Indulski *et al.,* 1997; Rubes *et al.,* 2005). For example, heavy metals may compromise male reproduction, as demonstrated by epidemiological and animal studies (Fatima *et al.,* 2010; Telisman *et al.,* 2000; Hernández-Ochoa *et al.,* 2005; Mendiola *et al.,* 2011; Hovatta *et al.,* 1998; Meeker *et al.,* 2008; Akinloye *et al.,* 2006; Benoff *et al.,* 2009; Chia *et al.,* 1994; Choy *et al.,* 2002; Rignell-Hydbom *et al.,* 2007).

Our research interests are related to the measurement of the exposure to lead (Pb), cadmium (Cd) and mercury (Hg), and its relationship with human semen quality. The main results published on that issue are summarized in Table 1. Those studies were done using the World Health Organization (WHO) guidelines for semen analysis published in 1987 (WHO, 1987), 1992 (WHO, 1992) and in 1999 (WHO, 1999). However, in 2010 the WHO published new criteria for the assessment of semen samples (WHO, 2010) and all the sperm parameter cut-offs were lowered. The changes in the three main semen parameters through time (1987- 2010) are summarized in table 2.

Lead

There is considerable agreement that high or even moderate concentrations of lead cause fertility problems in humans. Fatima et al. showed that >40µg/dL of lead in blood produced a decline of sperm concentration (<20x10^6 cells/mL). In addition, they observed lower motility (<50%) and morphology (<14%), with >35µg/dL in whole blood (Fatima *et al.,* 2010). Telisman and colleagues showed

significantly lower sperm density and motility with high blood lead concentrations (36.7 µg/dL) (Telisman *et al.,* 2000). High concentrations of lead seem to be clearly associated with sperm damage.

However, there are conflicting results about the effect on semen quality at low lead exposures. Hernandez-Ochoa and colleagues found that low lead concentrations in seminal fluid (0.2 µg/dL) were associated with impaired semen quality; 44% of motility, 32% of normal morphology and $11x10^6$ cell/mL of sperm concentration (Hernández-Ochoa *et al.,* 2005). In contrast, Mendiola et al. found a relationship between levels of lead ten times higher in the spermatic fluid (2.93 µg/dL) and low motility, but no effect on morphology (>14%) or sperm concentration (>$20x10^6$ cells/mL) (Mendiola *et al.*, 2011). Similarly, Hovatta et al. reported that lead concentrations in seminal plasma of 2.5 µg/dL did not affect sperm concentration ($96x10^6$ cells/mL) (Howatta *et al.,* 1998). Moreover, Mendiola et al. found that lead concentrations of 9.75 µg/dL measured in whole blood and 2.78 µg/dL in blood plasma had no effect on morphology (>14%), motility (>50%), or sperm concentration (>$20x10^6$ cells/mL) (Mendiola *et al.,* 2011). Meeker et al. also reported no effect on sperm concentration ($42.7x10^6$ cells/mL) or motility (55%) with 1.5 µg/dL of lead concentration in whole blood (Meeker *et al.,* 2008).

Cadmium

At high concentrations, cadmium could affect semen quality. According to Akinloye et al., men with high concentrations of cadmium in seminal plasma (65 µg/dL) had $5.16x10^6$ cells/ml of sperm count, and 36% of motile sperms (Akinloye *et al.,* 2006).

As seen with lead, there is no agreement on the effect of low concentrations of cadmium on semen quality. Telisman et al. found that even low concentrations of cadmium <1 µg/dL in whole blood were associated with head pathologic sperms (Telisman *et al.,* 2000). Benoff and colleagues concluded that sperm concentration, motility and morphology are affected even with low seminal plasma concentrations of cadmium (0.028 µg/dL) (Benoff *et al.,* 2009). Mendiola and colleagues also found that low concentrations of cadmium in seminal plasma (0.085 µg/dl) were moderately associated with low sperm motility (<50%), but had no effect on morphology (>14%) or sperm concentration (>20x10^6 cells/mL) (Mendiola *et al.,* 2011). Equally, Hovatta et al. showed no correlation between higher cadmium concentrations in seminal fluid (0.15 µg/dL) and sperm concentration (96x10^6 cells/mL) [16]. Chia and colleagues did not find an impairment of morphology (>50%) and motility (>50%) with low concentrations of cadmium in whole blood (0.095 µg/dl) (Chia *et al.,* 1994). Similarly, Mendiola et al. showed that cadmium measured in whole blood (0.10 µg/dL) and blood plasma (0.08 µg/dL) did not impaired morphology (>14%), motility (>50%), or sperm concentration (>20x10^6 cells/ml) (Mendiola *et al.,* 2011). Moreover, Meeker et al. reported no effect of low cadmium concentrations in whole blood (0.04 µg/dL) on sperm density (42.7x10^6 cells/mL) and motility (55%) (Meeker *et al.,* 2008).

Mercury

There is clear evidence that very high concentrations of mercury in the body will harm sperm. Choy et al. showed that high concentrations of total mercury (inorganic and organic) measured in whole blood (40.6 mmol/ L) resulted in <50%

of progressive motility, <14% of normal morphology and <$20x10^6$ cells/mL of sperm concentration (Choy *et al.*, 2002).

However, Mendiola et al. did not find an alteration of motility (>50%), morphology (>14%) or sperm concentration (>$20x10^6$ cells/mL) at low concentrations of total mercury in seminal plasma (1.18 µg/dl). Besides, low concentrations measured in whole blood (1.99 µg/dL) and blood plasma (0.6 µg/dL), were no related to decreased morphology (>14%), motility (>50%) or sperm concentration (>$20x10^6$ cells/mL) (Mendiola *et al.*, 2011). Rignell-Hydbom et al. found no association with sperm motility (54%) or concentration ($48x10^6$ sperm cells/mL) at low concentrations of organic mercury in whole blood (0.225 µg/dL) (Rignell-Hydbom *et al.*, 2007). In addition, Meeker et al. reported that low mercury concentrations in whole blood (0.11 µg/dL) did not affect motility (55%) and sperm concentration ($42.7x10^6$ cells/mL) (Meeker *et al.*, 2008).

Justification of the study

There are at least two problems in assessing whether low concentrations of heavy metals have an impact on human semen quality. First of all, there are just a few studies published on that issue so far. A second problem relates to the variables measured. That is the biological samples in which the concentrations of heavy metals are measured, and the parameters used to measured semen quality (motility, morphology and sperm concentration).

To measure the effect produced by low doses of a heavy metal in the reproductive organs, it is necessary to clarify where to perform those measurements. Concentrations of heavy metals may be measured in the whole

blood, in blood plasma and in seminal plasma. However, it is not clear whether measurements in one or another fluid are equivalent, nor to what extend there are correlations between the three measurements of these heavy metals in the different body fluids.

The objectives of this study are: 1) to examine whether there are correlations between the concentrations of heavy metals (lead, cadmium and mercury) in the three body fluids (whole blood, blood plasma and seminal plasma), and 2) to explore whether any one of the three measures relates better than the others with the semen quality parameters.

6.2. Methods.

Study population, design and semen analysis

The study population, hormone and semen analyses have been previously described elsewhere (Mendiola *et al.,* 2008; Mendiola *et al.,* 2009). Sixty-one men were participating in a study to explore the role of environmental toxins and lifestyles on male infertility. Briefly, the men of couples attending three infertility centers in Southeastern Spain between 2005 and 2007 were classified on the basis of semen quality, following WHO criteria (WHO, 1999). Subjects provided two semen samples and were requested to observe a 3- to 5-day abstinence period. The importance of the abstinence period was stressed on the interviews with the participants (Mendiola *et al.,* 2008). The average of the two samples was used in our statistical analysis. Semen parameters evaluated included: ejaculate volume, sperm concentration, percentage of motile sperm, and percentage of normal forms following Kruger's strict criteria (WHO, 1999). All patients were interviewed face-to-face by the same interviewer and completed a comprehensive occupational and lifestyle questionnaire (Mendiola *et al.,* 2008). This study was approved by the Institutional Review Board. Patients were included in the study after giving informed written consent.

Measurements of metals

A total of 181 biological samples were analyzed for Pb, Cd and Hg, including 61 samples of seminal plasma, 61 of blood plasma and 59 of whole blood, as two samples were lost during the study. Biological samples were

dispensed into aliquots and frozen and stored at -40 ºC until analysis. Anodic Stripping Voltammetry (ASV) was used for measuring Pb and Cd concentrations. ASV was carried out using a voltamperometer with VA 663 stand and VA 608 controller (Metrohm 626, Herisau, Switzerland). The voltamperometric cell was equipped with a drop of mercury as the working electrode, an Ag/AgCl/KCl 3M reference electrode and a platinum auxiliary electrode.

Determination of total Hg was carried out by thermal decomposition, amalgamation and atomic absorption spectrophotometry, using a Mercury analyzer with quartz sample boats (DMA-80 Direct Mercury Analyzer, Milestone, Shelton CT, USA).

The highest grade purity reagents were employed in this procedure including nitric acid 65% and perchloric acid 70% (Suprapur®, Merck, Darmstadt, Germany). The ultrapure water was purified with Millipore Simplicity 185 (Millipore GmbH, Molsheim, France) obtaining conductivity values of 0.054 µS/cm.

In order to prepare the working standard solutions, commercially available standard solutions for Pb 1 g/L and Cd 1 g/L (Tritisol®, Merck, Darmstadt, Germany) and Hg 1 g/L (Certipur®, Merck, Darmstadt, Germany) were used. The limits of detection (LOD) for the body's fluid metal levels were as follows: lead, 21 µg/L; cadmium, 0.11 µg/L and mercury, 0.1 µg/L. To guarantee the accuracy and precision of the applied technique regarding heavy metals, whole blood reference materials (SeronormTM Trace Elements Whole Blood, SERO AS, Billingstad, Norway) were employed.

Sample preparation

Pb and Cd determinations were performed using 0.2 mL of the biological sample deposited inside of 25 mL borosilicate glass. Acid digestion was carried out by adding 2 mL of nitric acid and 2 mL of perchloric acid and evaporating it to dryness. Once the sample was dry and cooled down, 100 µl of perchloric acid and 15 mL of double-distilled water were added, transferring the final volume into a voltamperometric cell.

Biological samples were measured by ASV according to the following method (WHO, 1992). Briefly, differential pulse (DP) with hanging mercury drop electrode (HMDE) was used, the voltage sweep was from -0.70 to +0.15 volts and the peak voltage were located at -0.58 and -0.40 volts for Cd and Pb respectively. Deaeration, preconcentration and resting time (without stirring) were 180, 120 and 40 seconds respectively. Sensitivity was 0.05 nAmp/mm and 0.2 nAmp/mm for Cd and Pb respectively. Standard addition method was applied to perform the current analyses, adding known values of a standard solution (2, 4 and 6 ng for Cd and 20, 40 and 60 ng for Pb) to obtain a calibration curve, then the values of the measurements were interpolated into that curve. Mercury determination was carried out following EPA method 7473 (EPA, 2007) and 0.2 mL of the biological sample was transferred directly into the quartz sample boats. To obtain a calibration curve, standard solutions of 5, 10, 20, 30, 100, 200 and 500 ng of Hg were employed.

Statistical analysis

The statistical analysis encompassed descriptive and inferential analyses. Basic, dispersion as well as frequency parameters were calculated for descriptive analyses. Statistical analyses were performed to explore possible patterns in the concentrations of heavy metals measured in blood serum, whole blood and seminal plasma. Spearman's rank correlations and scatter plots were employed for comparison of variables. In the inferential analysis, the mean comparison tests and discriminant analysis were performed. All tests were two-tailed and the level of statistical significance was set at 0.05. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

6.3. Results.

Table 3 shows lead (Pb), cadmium (Cd) and mercury (Hg) concentrations in µg/dL (mean, standard error, median and interquartile range), in whole blood, blood plasma and seminal plasma.

Figure 1 to 8 show the scatter plots of the concentrations of the three metals in the three body fluids. As may be observed, men with low concentration of one heavy metal in a fluid can show low or high concentrations of the same metal in another fluid. There is a wide dispersion of data, and there are no associations between the measurements made of the metals in one fluid and the concentrations measured in the two other fluids.

Table 4 presents the results of the Spearman's correlation between the concentrations of lead, cadmium and mercury in whole blood, blood plasma and seminal plasma. Although the correlation coefficients were above 0.5 for some determinations, no significant correlations were found between the concentrations of the same metal y the three biological fluids. The correlation between the concentration of lead in blood plasma and whole blood was 0.57 (p=0.67), between cadmium in seminal plasma and whole blood was -0.50 (p=0.72), and between mercury in seminal plasma and whole blood -0.34 (p=0.80).

Figure 9 to 18 show the relationship between the concentrations of lead, cadmium and mercury measured in each fluid. As may be observed, there is a linear relationship, since men with low concentration of a given metal in a biological fluid also had low concentration of the other two metals in the same fluid. And,

reversely, men with high concentration of a given metal in a biological fluid also had high concentration of the other metals in the same body fluid.

Spearman's correlation coefficients and scatter plots revealed a high correlation between the concentrations of the three metals in the same biological fluids. Table 5 shows the correlation of the three heavy metals (Pb, Cd and Hg) in the same biological fluid (whole blood, blood plasma or seminal plasma). High and statistically significant correlations were observed between the three heavy metals for the same biological fluid. In seminal plasma, the correlations between cadmium and lead was 0.74 (p-value <0.005), and between mercury and lead 0.76 (p value <0.005).

To explore whether these correlations were determined by associations with other factors, exploratory scatter plots were generated between the concentrations of the three metals in the three biological fluids and possible confounding variables. Possible confounders were such as "occupation", "tobacco smoke", "exposure to toxics at work" or "using metals at work". No patterns were observed. Hypothesis tests were used to detect significant differences in the mean concentrations of metals and the possible confounding factors used in the scatter plots. Not significant differences were found (data not shown).

As a final alternative, metal concentrations were categorized in two, three and four groups using the mean values, tertiles and quartiles respectively. Discriminant analysis was then used to detect whether any of the factors was related to the categories of the metal concentrations. To this end, different

discriminant analysis evaluating the overall Wilks' lambda and the owners of each factor were produced, but none of them were satisfactory.

6.4. Discussion.

Using the Spearman's correlation coefficient and scatter plots revealed a high correlation between the measured concentrations of the 3 heavy metals in the same biological fluids. However, no similar relationship was observed when comparing the concentrations in different body fluids of the same metal.

It would be reasonable to expect that subjects with high and low levels of exposure to any metal would show similar positions (low or high concentrations) in the measurements made in any body fluid. However, we found no correlation between the concentrations of any of the metal in the three biological samples analyzed (whole blood, blood plasma and seminal plasma).

Other authors, similarly, found no correlation between the concentrations of the same metal in different fluids (Hernández-Ochoa *et al.*, 2005; Benoff *et al.*, 2009). Benoff and colleagues found no correlation between cadmium concentrations in seminal plasma and blood plasma. Hernandez-Ochoa et al. also reported no correlation in blood lead concentrations between whole blood-plasma blood, whole blood-seminal plasma, or blood plasma-sperm in 68 Mexican men.

There are some possible hypotheses for these phenomena. The three heavy metals are bound and transported by erythrocytes (ATSDR, 1999; ATSDR, 2007; ATSDR, 2008). Given that metals are transported by red cells, unmeasured differences in the concentration of red cells in our study population may result in different concentration of the metals in the blood. However, this hypothesis cannot be tested, mainly due to information on red cell concentration was not collected.

Surprisingly, the concentrations of Pb, Cd and Hg were correlated in the same biological samples. Howatta *et al.* also found that the concentrations of cadmium and lead in seminal plasma were correlated (Hovatta *et al.,* 1998). We do not have a firm hypothesis of why that may happen.

Correlations of the three heavy metals in the same body biological fluid may be due to an interaction between the different metals in the same compartment, so that the concentration of one metal determines the concentration of the others. We are not aware of lead, cadmium or mercury modulates each other. However, it has been published that selenium produces the redistribution of Hg from plasma to erythrocytes at higher ratio (ORct *et al.,* 2009), and the modification of hepatic zinc by cadmium (Braga *et al.,* 2011). Therefore, it could be that a given heavy metal might modulate proteins and/or enzymes in the cells and influence the concentration of other heavy metals (Coddou *et al.,* 2005; Borges *et al.,* 2007; Korashy *et al.,* 2008).

As to how to measure the effect produced by heavy metal concentrations on semen quality, it would be better to measure those metals in seminal plasma than in blood plasma or whole blood. Heavy metal concentrations in blood samples do not necessarily reflect the seminal plasma ones, since heavy metal concentrations reaching the seminal plasma could be quite different.

Heavy metals have a strong capacity to induce oxidative stress in body cells by disintegration of the lipid membrane, and spermatozoa are quite sensible to oxidative stress (Ercal *et al.,* 2001; Grotto *et al.,* 2010). Thus, in principle, it would be more accurate to measure heavy metal concentrations in seminal plasma -than in

other fluids- in order to determine sperm damage. Numerous antioxidants such as vitamin C, vitamin E, glutathione, coenzyme Q10, some fruits, etc, may diminish the oxidative stress caused by heavy metals (Sheweita *et al.,* 2005; Mendiola *et al.,* 2009;Tito *et al.,* 2011).

Furthermore, as it can be seen in table 1, high concentrations of heavy metals can alter sperm morphology, motility and concentration individually. However, an alteration of the three semen parameters can be observed with very low heavy metal concentrations only in seminal plasma, showing us that this body fluid might reflect better the sperm damage.

Finally, our findings might be attributed to chance or bias. The sample of individuals included in the study was small and the lack of statistically significant correlations may be a consequence of that. Our findings are, however, consisting with those (Hernández-Ochoa *et al.,* 2005; Hovatta *et al.,* 1998; Benoff *et al.,* 2009) of that have explored the same correlations leading us to believe that they cannot be attributed to random or systematic error.

Our study suggests that there is no correlation between the concentrations of any of the metals in the three biological samples analyzed (whole blood, blood plasma and seminal plasma), and there is a correlation between the concentrations of Pb, Cd and Hg in the same biological samples. According to our results and previous publications, seminal plasma might be the best body fluid for assessing impairment of human semen parameters.

6.5. References.

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6.6. Tables.

Table 1: Review of the measurement in the exposure to lead (Pb), cadmium (Cd) and mercury (Hg), and its relation with semen quality.

		Semen Quality		
		Morphology	**Motility**	**Sperm Concentration**
Lead	**Whole Blood**	Fatima 2010: C=>35 µg/dl Mr= <14% 1999 criteria Mendiola 2011: C= 9.75 µg/dl Mr=>14% 1999 criteria*	Telisman 2000: C=36.7 µg/dl Mt=p <0.02 1987 criteria Fatima 2010: C=>35 µg/dl Mt= <50% 1999 criteria Mendiola 2011: C= 9.75 µg/dl Mt=>50% 1999 criteria* Meeker 2008: C=1.5 µg/dl Mt=55% 1999 criteria*	Fatima 2010: C=>40 µg/dl SpC= <20x10^6 cells/ml 1999 criteria Telisman 2000: C=36.7 µg/dl SpC= p<0.05 1987 criteria Mendiola 2011: C= 9.75 µg/dl SpC=>20x10^6 cells/ml 1999 criteria* Meeker 2008: C= 1.5 µg/dl SpC= 42.7x10^6 cells/ml 1999 criteria*
	Blood Plasma	Mendiola 2011: C= 2.88 µg/dl Mr=>14% 1999 criteria*	Mendiola 2011: C= 2.88 µg/dl Mt=>50% 1999 criteria	Mendiola 2011: C= 2.88 µg/dl SpC=>20x10^6 cells/ml 1999 criteria
	Seminal Plasma	Mendiola 2011: C= 2.93 µg/dl Mr=>14% 1999 criteria* Hdez-Ochoa 2005: C=0.2 µg/dl Mr= 32% 1999 criteria	Mendiola 2011: C=2.93 µg/dl Mt=<50% 1999 criteria* Hdez-Ochoa 2005: C=0.2 µg/dl Mt= 44% 1999 criteria	Mendiola 2011: C= 2.93 µg/dl SpC=>20x10^6 cells/ml 1999 criteria* Hovatta 1998: C=2.5 µg/dl SpC=96x10^6 cells/ml 1992 criteria Hdez-Ochoa 2005: C=0.2 µg/dl SpC=11x10^6 cells/ml 1999 criteria
Cadmium	**Whole Blood**	Telisman 2000: C=<1 µg/dl Mr= p<0.005 1987 criteria Mendiola 2011: C= 0.10 µg/dl Mr=>14% 1999 criteria* Chia 1994: C=0.095 µg/dl Mr= >50% 1987 criteria	Mendiola 2011: C= 0.10 µg/dl Mt=>50% 1999 criteria* Chia 1994: C=0.095 µg/dl Mt= >50% 1987 criteria Meeker 2008: C=0.04 µg/dl Mt=55% 1999 criteria	Mendiola 2011: C= 0.10 µg/dl SpC=>20x10^6 cells/ml 1999 criteria* Meeker 2008: C=0.04 µg/dl SpC= 42.7x10^6 cells/ml 1999 criteria

	Blood Plasma	Mendiola 2011: C= 0.08 µg/dl Mr=>14% 1999 criteria*	Mendiola 2011: C= 0.08 µg/dl Mot=>50% 1999 criteria*	Mendiola 2011: C= 0.08 µg/dl SpC=>20x10^6 cells/ml 1999 criteria*
	Seminal Plasma	Mendiola 2011: C= 0.085 µg/dl Mr=>14% 1999 criteria* Bennof 2009 : C=0.028 µg/dl Mr=p<0.05 1992 criteria	Akinloye 2006: C=65 µg/dl Mt=35.75 % 1999 criteria Mendiola 2011: C=0.085 µg/dl Mt=<50% 1999 criteria* Bennof 2009: C=0.028 µg/dl Mt= p<0.05 1992 criteria	Akinloye 2006: C=65 µg/dl SpC=5.16x10^6 cells/ml 1999 criteria Hovatta 1998: C=0.15 µg/dl SpC=96x10^6 cells/ml 1992 criteria Mendiola 2011: C= 0.085 µg/dl SpC=>20x10^6 cells/ml 1999 criteria* Bennof 2009: C=0.028 µg/dl SpC=p<0.05 1992 criteria
Mercury	**Whole Blood**	Choy 2002: C=40.6 mmol/L Mr=<14% 1999 criteria Mendiola 2011: C= 1.99 µg/dl Mr=>14% 1999 criteria*	Choy 2002: C=40.6 mmol/L Mt=<50% 1999 criteria Mendiola 2011: C= 1.99 µg/dl Mt=>50% 1999 criteria* Rignell-Hydbom 2007: C=0.225 µg/dl Mt=54% 1999 criteria Meeker 2008: C=0.11 µg/dl Mt=55% 1999 criteria	Choy 2002: C=40.6 mmol/L SpC=<20x10^6 cells/ml 1999 criteria Mendiola 2011: C= 1.99 µg/dl SpC=>20x10^6 cells/ml 1999 criteria* Rignell-Hydbom 2007: C=0.225 µg/dl SpC=48x10^6 cells/ml 1999 criteria Meeker 2008: C=0.11 µg/dl SpM= 42.7x10^6 cells/ml 1999 criteria
	Blood Plasma	Mendiola 2011: C= 0.6 µg/dl Mr=>14% 1999 criteria*	Mendiola 2011: C= 0.6 µg/dl Mt=>50% 1999 criteria*	Mendiola 2011: C= 0.6 µg/dl SpC=>20x10^6 cells/ml 1999 criteria*
	Seminal Plasma	Mendiola 2011: C= 1.18 µg/dl Mr=>14% 1999 criteria*	Mendiola 2011: C=1.18 µg/dl Mt= >50% 1999 criteria*	Mendiola 2011: C= 1.18 µg/dl SpC=>20x10^6 cells/ml 1999 criteria*

Note: This table shows author, publication year, concentration of metal in whole blood, blood plasma and seminal plasma, their effect on semen quality parameters, and the WHO criteria used to clasificate the semen quality.
C= Concentration of the metal
Mr= Morphology
Mt= Motility
SpC= Sperm concentration
*Mendiola et al. use Kruger's strict criteria (14% of normal forms) as a cutoff for sperm morphology [25].

Table 2: Changes in the three main semen parameters through time (1987- 2010). A semen parameter was considered normal when the values were equal or above the presented figures (WHO, 1987; WHO, 1992; WHO, 1997; WHO 2010).

	1987	**1992**	**1999**	**2010**
Sperm concentration ($x10^6$ cells/ml)	20-200[1]	≥20	≥20	≥15
Motility (%)	≥60	≥50	≥50	≥40
Morphology (%)	≥50	≥30	≥14	≥4

[1]range

Table 3: Heavy metal concentrations in seminal, blood plasma and whole blood.

	LEAD (µg/dL)		**CADMIUM** (µg/dL)		**MERCURY** (µg/dL)	
	MEAN (SE)	MEDIAN (IQR)	MEAN (SE)	MEDIAN (IQR)	MEAN (SE)	MEDIAN (IQR)
Blood plasma (n=61)	2.88 (0.22)	2.90 (2.72-3.05)	0.08 (0.007)	0.08 (0.07-0.08)	0.6 (0.22)	0.58 (0.42-0.72)
Whole blood (n=61)	9.75 (2.28)	10.10 (7.50-11.90)	0.10 (0.02)	0.10 (0.09-0.12)	1.99 (0.69)	1.96 (1.47-2.46)
Seminal plasma (n=61)	2.93 (0.32)	2.90 (2.72-3.15)	0.08 (0.01)	0.08 (0.07-0.09)	1.18 (0.35)	1.13 (0.92-1.49)

SE: Standard Error
IQR: Interquartile Range

Table 4: Spermean's correlation coefficients between metal concentrations in seminal and blood plasma, and whole blood.

		Blood Plasma		**Whole Blood**	
		R	P-value	R	P-value
Lead	**Blood Plasma**			0.57	0.67
	Seminal Plasma	0.13	0.32	-0.08	0.55
Cadmium	**Blood Plasma**			0.14	0.30
	Seminal Plasma	0.12	0.36	-0.50	0.72
Mercury	**Blood Plasma**			0.17	0.19
	Seminal Plasma	-0.13	0.34	-0.34	0.80

Table 5: Spermean's correlation coefficients between seminal plasma, blood plasma, and whole blood, with metal concentrations.

		Cadmium		**Mercury**	
		R	P-value	R	P-value
Seminal Plasma	**Lead**	0.740	0.001	0.760	0.001
	Cadmium			0.870	0.001
Blood Plasma	**Lead**	0.550	0.001	0.750	0.001
	Cadmium			0.700	0.001
Whole Blood	**Lead**	0.850	0.001	0.950	0.001
	Cadmium			0.792	0.001

6.7. Figures.

Figure 1: Relation between lead concentrations in seminal plasma and blood plasma.

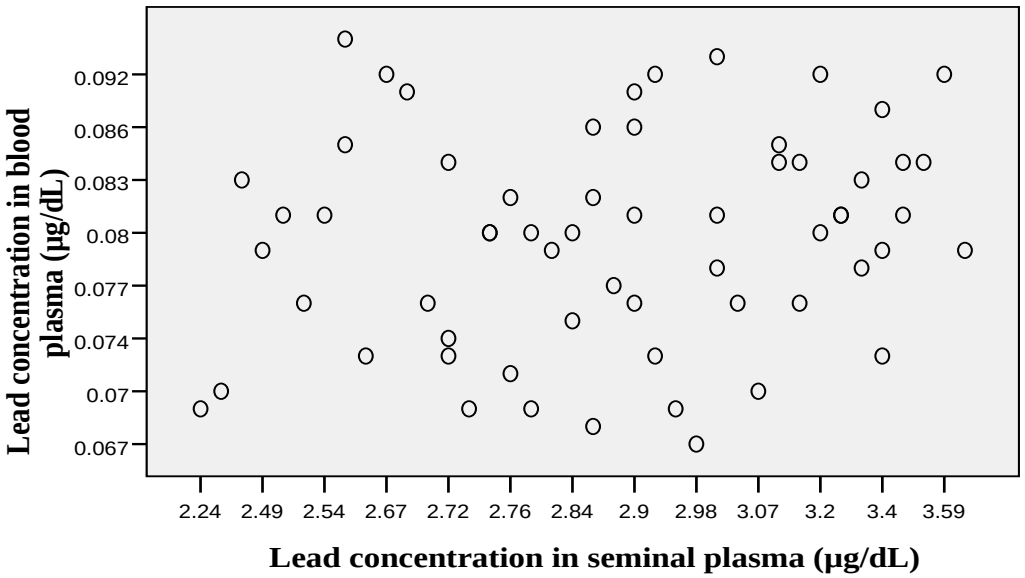


Figure 2: Relation between lead concentrations in seminal plasma and whole blood.

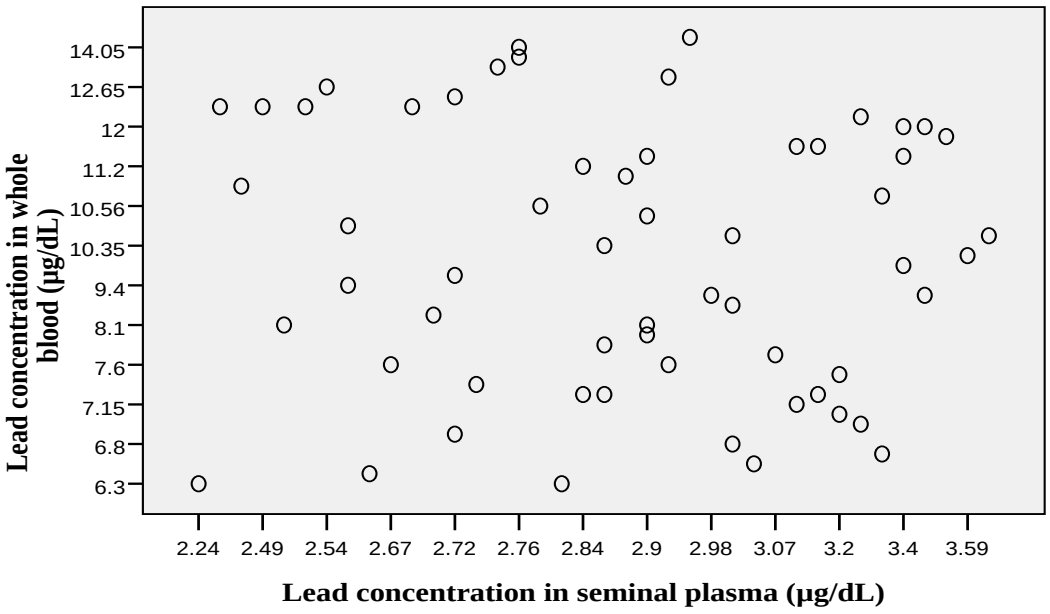


Figure 3: Relation between lead concentrations in blood plasma and whole blood.

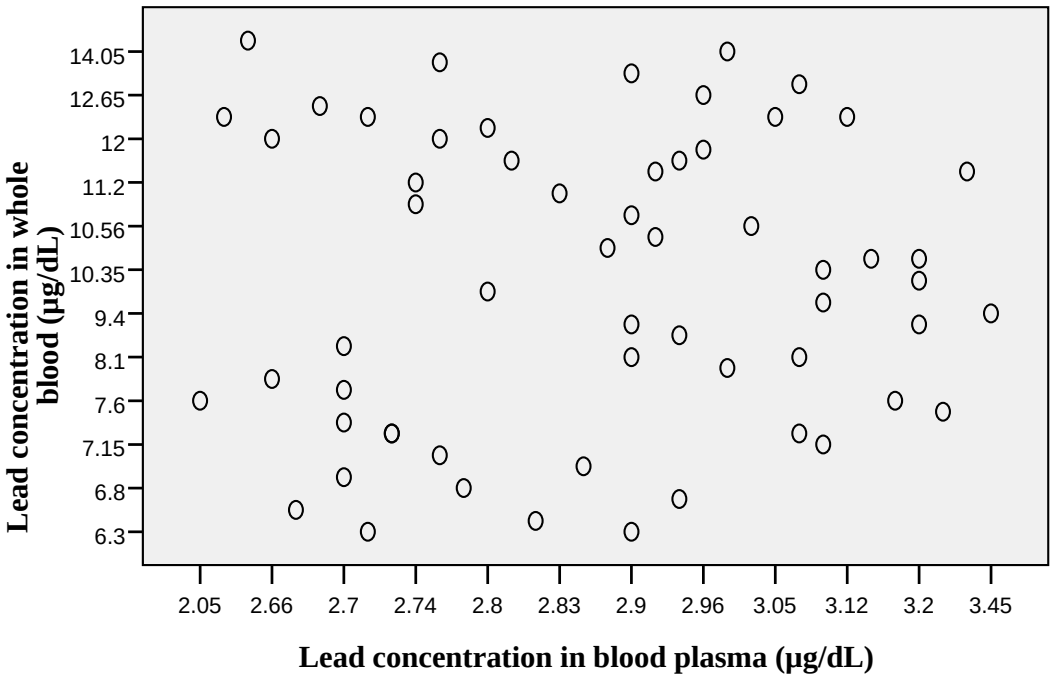


Figure 4: Relation between cadmium concentrations in seminal plasma and blood plasma.

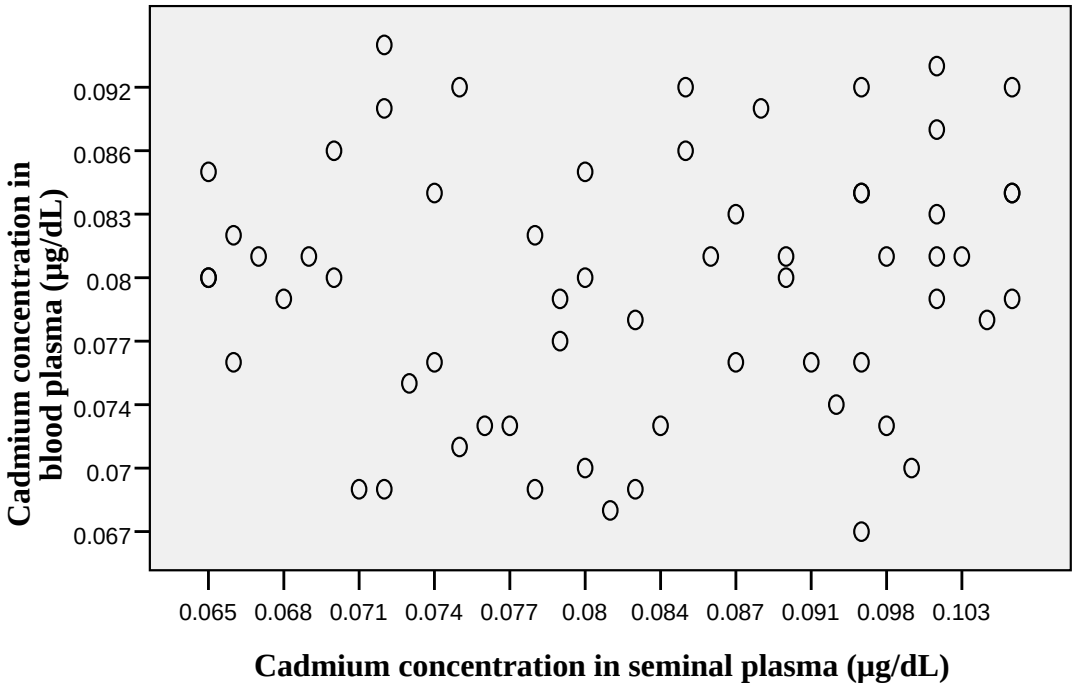


Figure 5: Relation between cadmium concentrations in seminal plasma and whole blood.

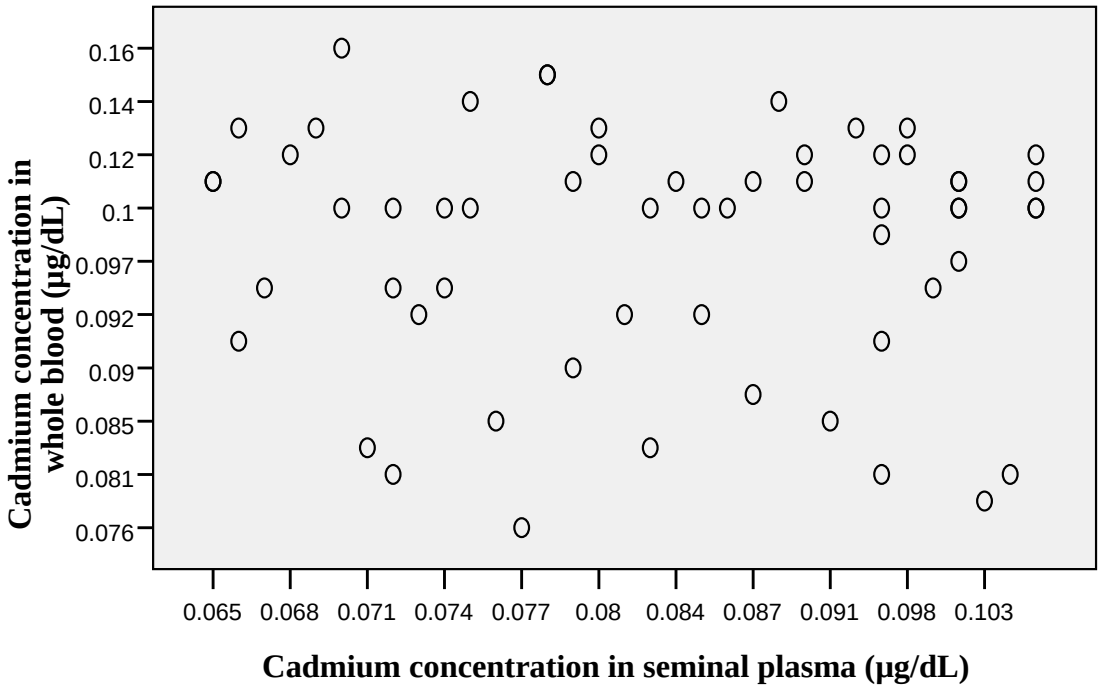


Figure 6: Relation between cadmium concentrations in blood plasma and whole blood.

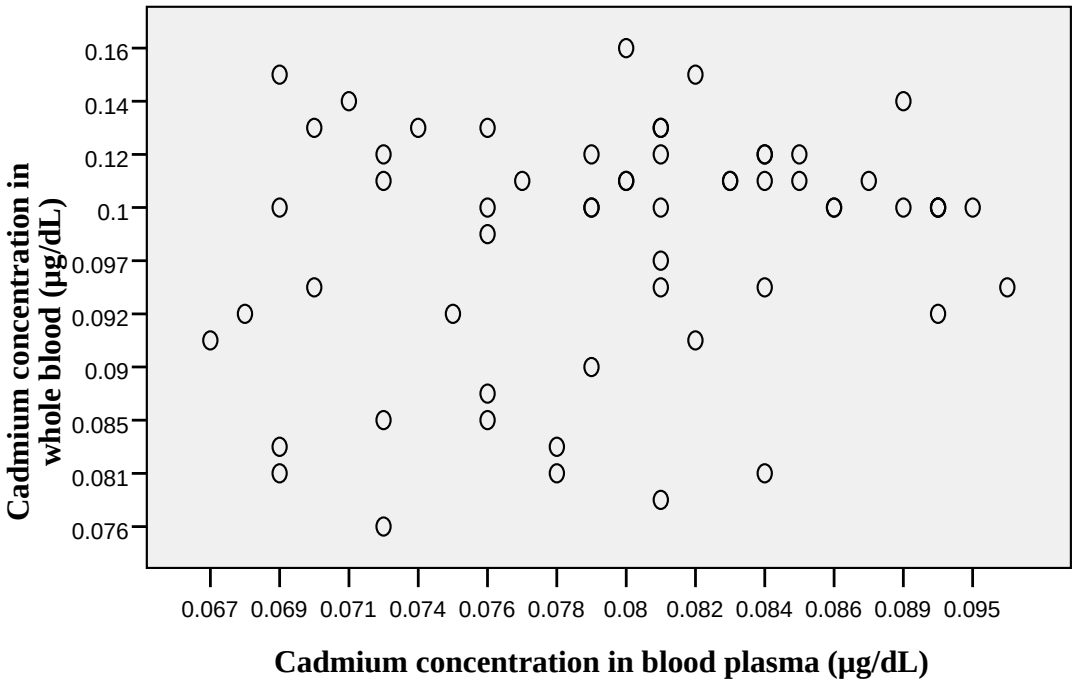


Figure 7: Relation between mercury concentrations in blood plasma and whole blood.

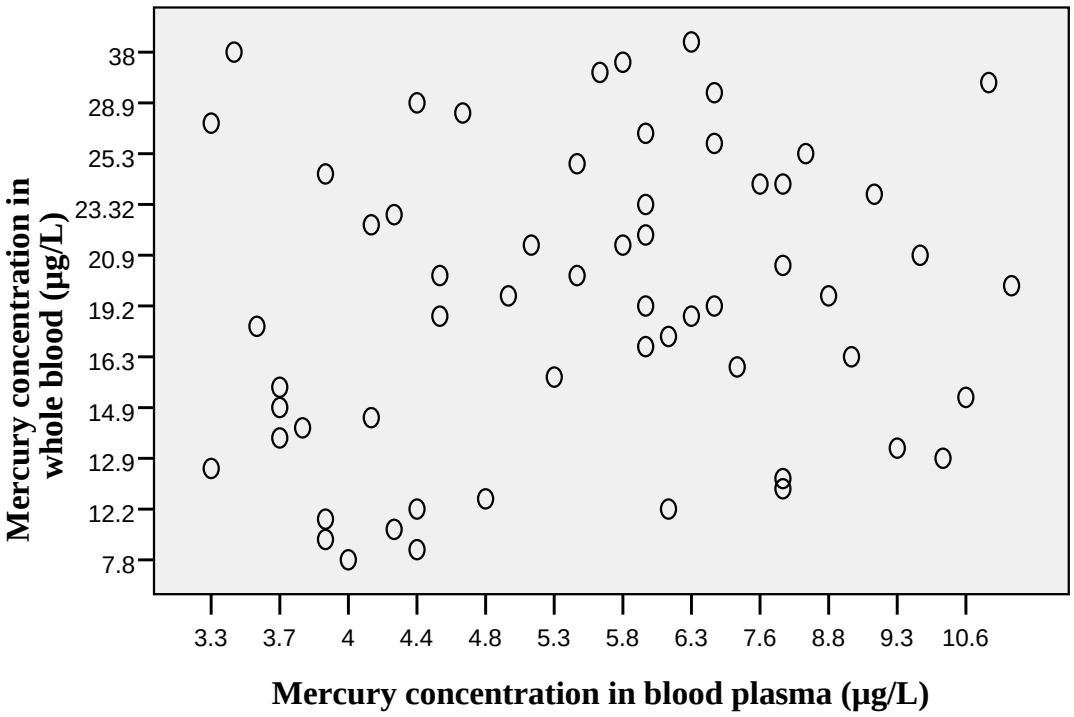


Figure 8: Relation between mercury concentrations in seminal plasma and whole blood.

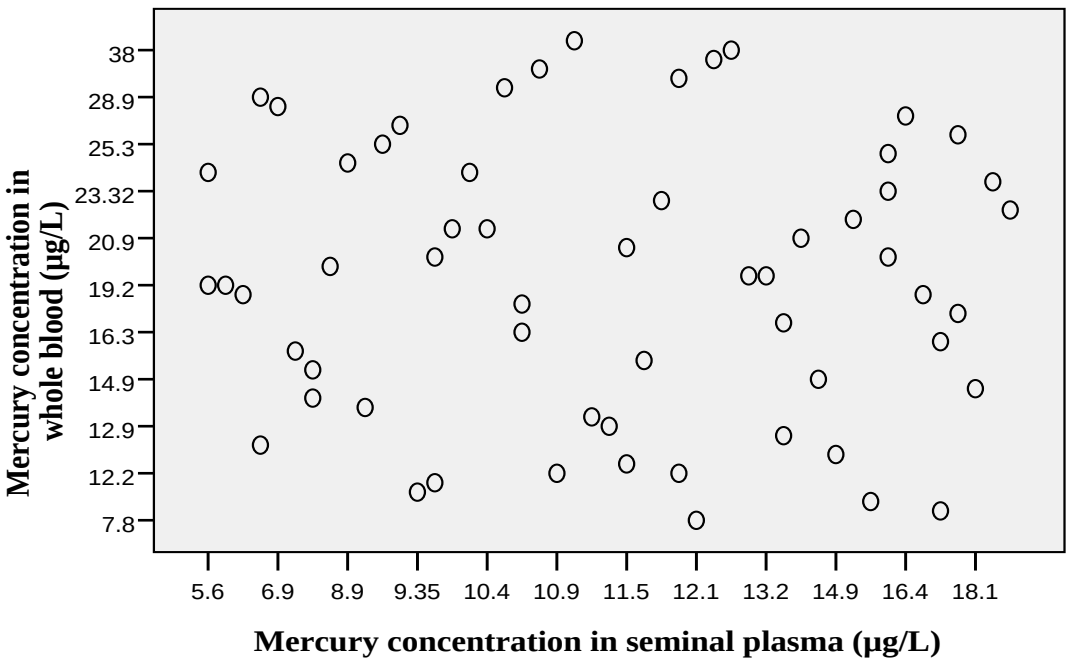


Figure 9: Relation between mercury concentrations in blood plasma and whole blood.

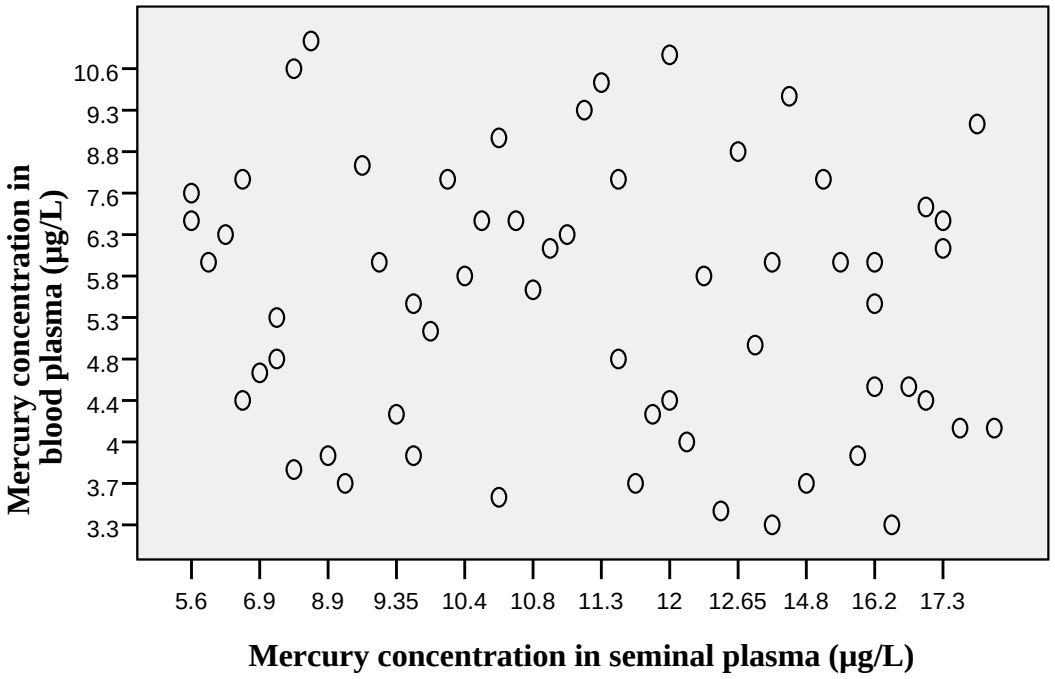


Figure 10: Relation between lead and cadmium concentrations in whole blood.

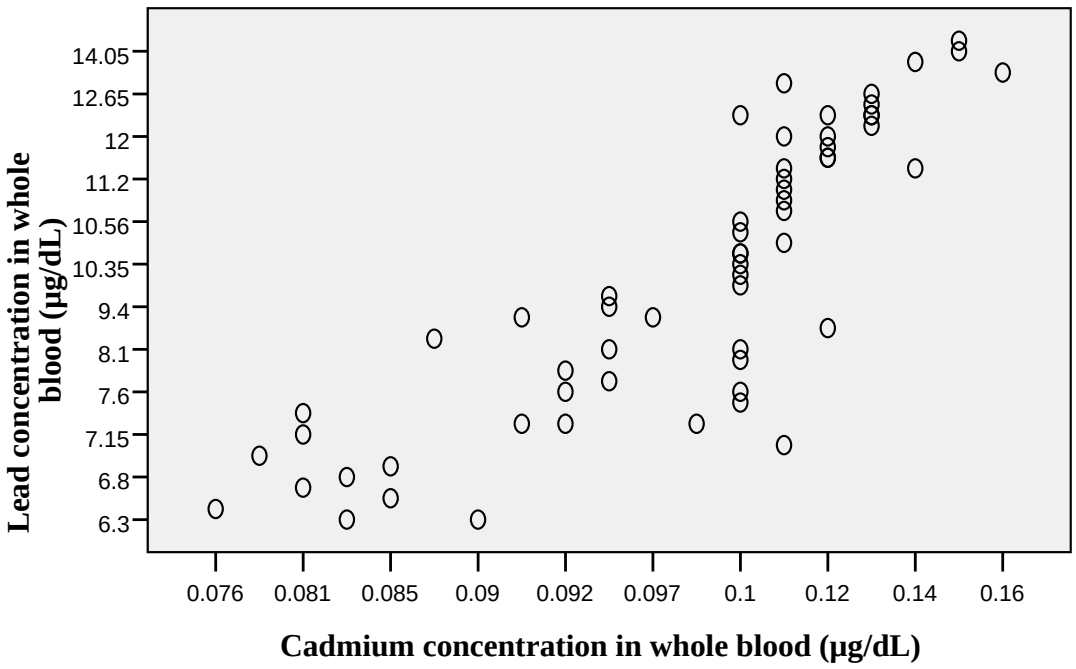


Figure 11: Relation between lead and mercury concentrations in whole blood.

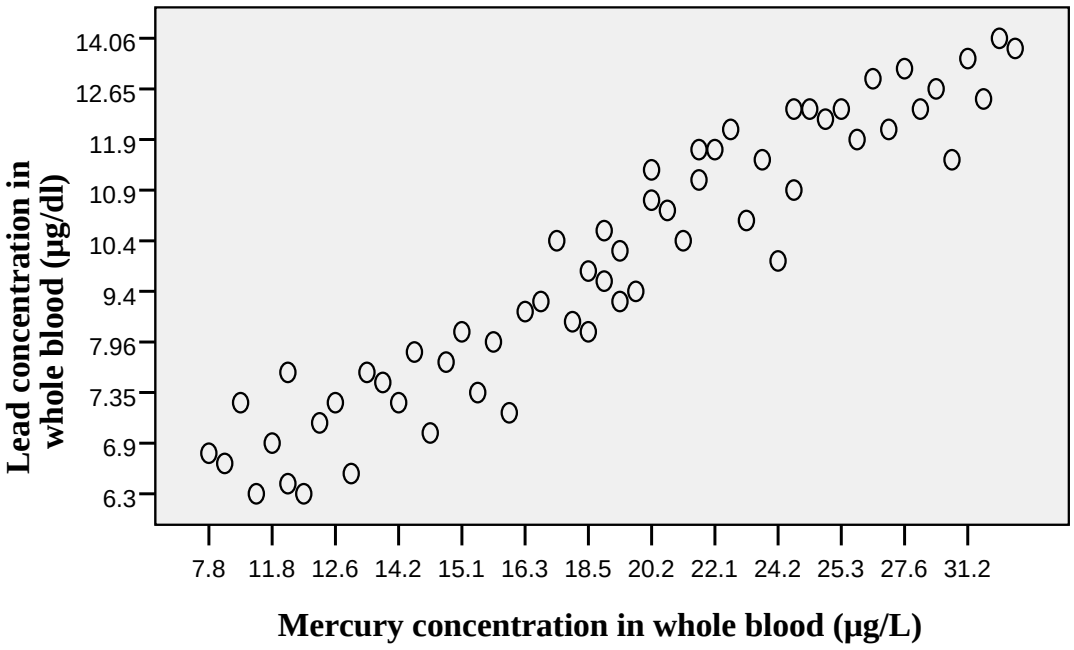


Figure 12: Relation between cadmium and mercury concentrations in whole blood.

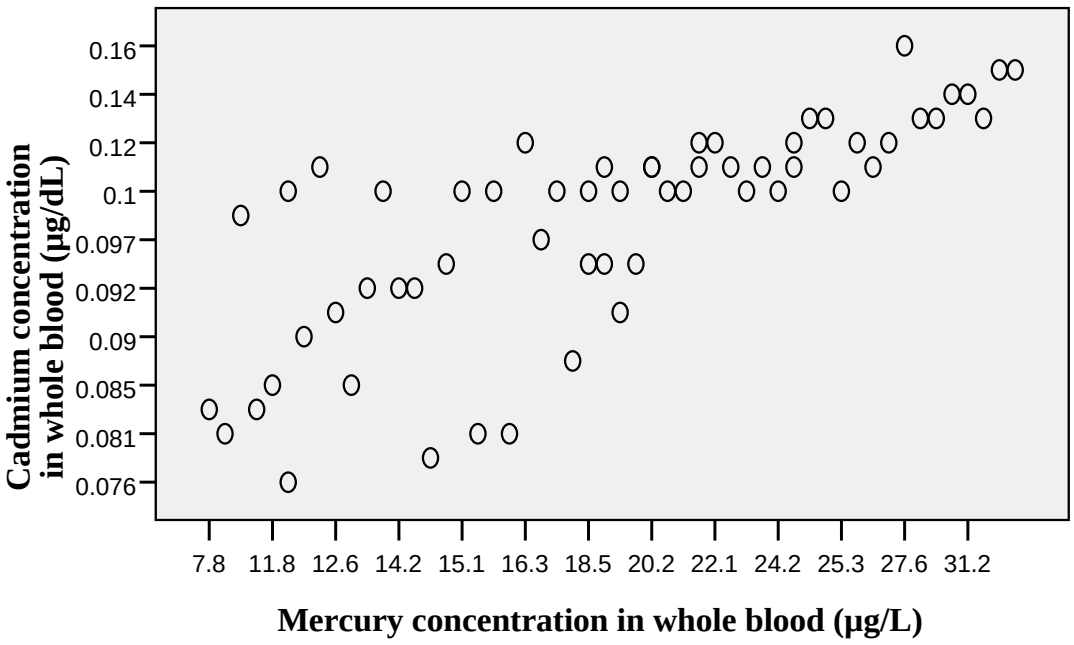


Figure 13: Relation between lead and cadmium concentrations in blood plasma.

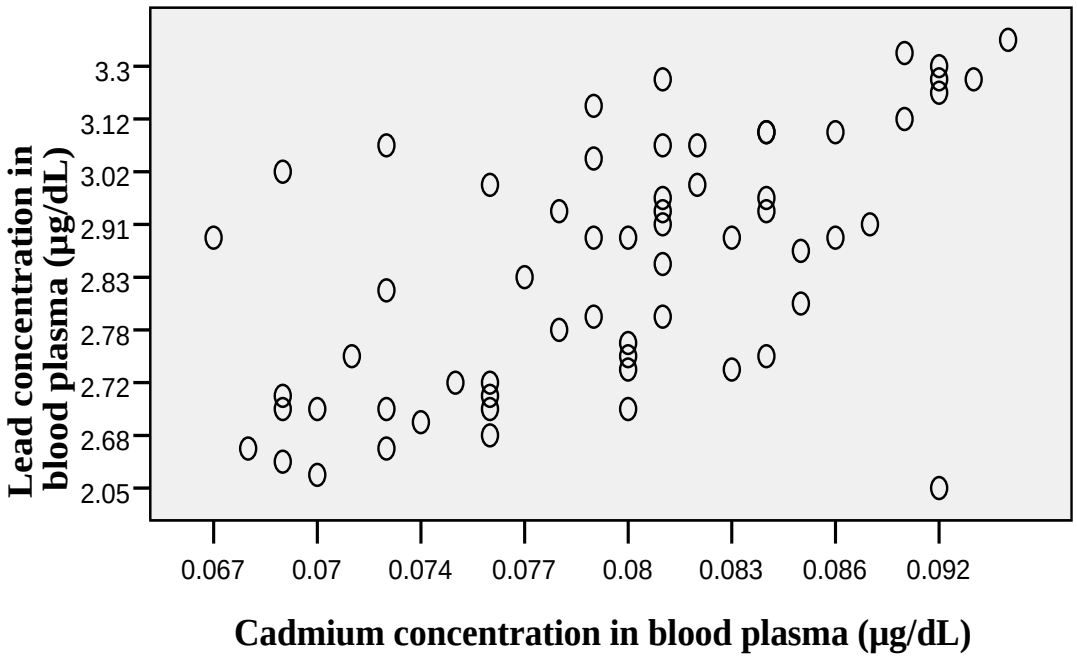


Figure 14: Relation between lead and mercury concentrations in blood plasma.

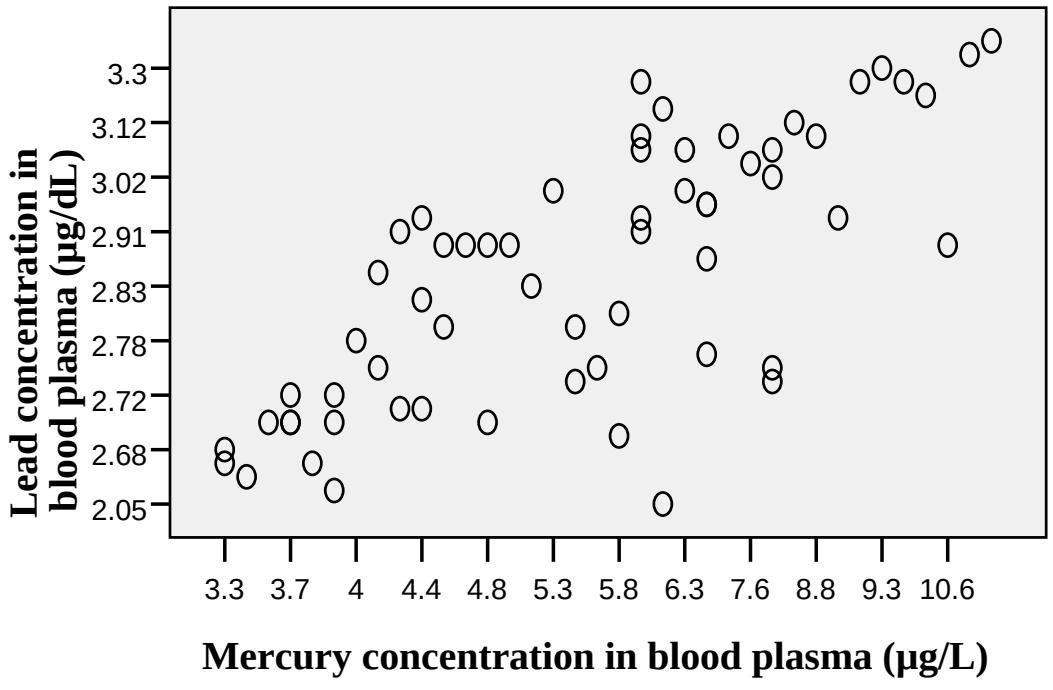


Figure 15: Relation between cadmium and mercury concentrations in blood plasma.

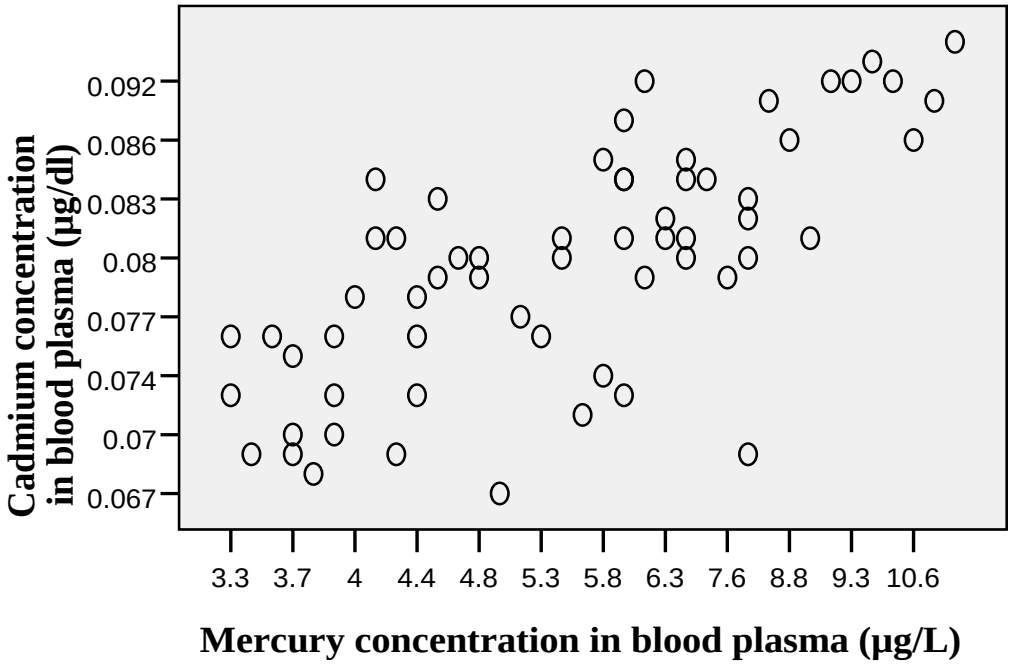


Figure 16: Relation between lead and cadmium concentrations in seminal plasma.

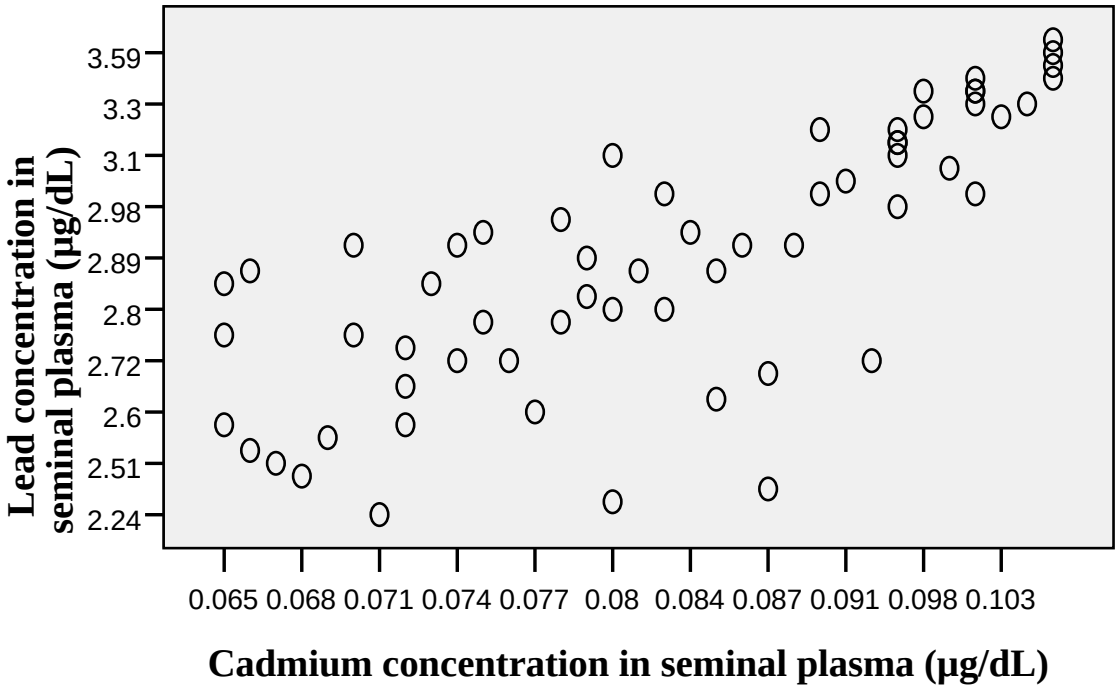


Figure 17: Relation between lead and mercury concentrations in seminal plasma.

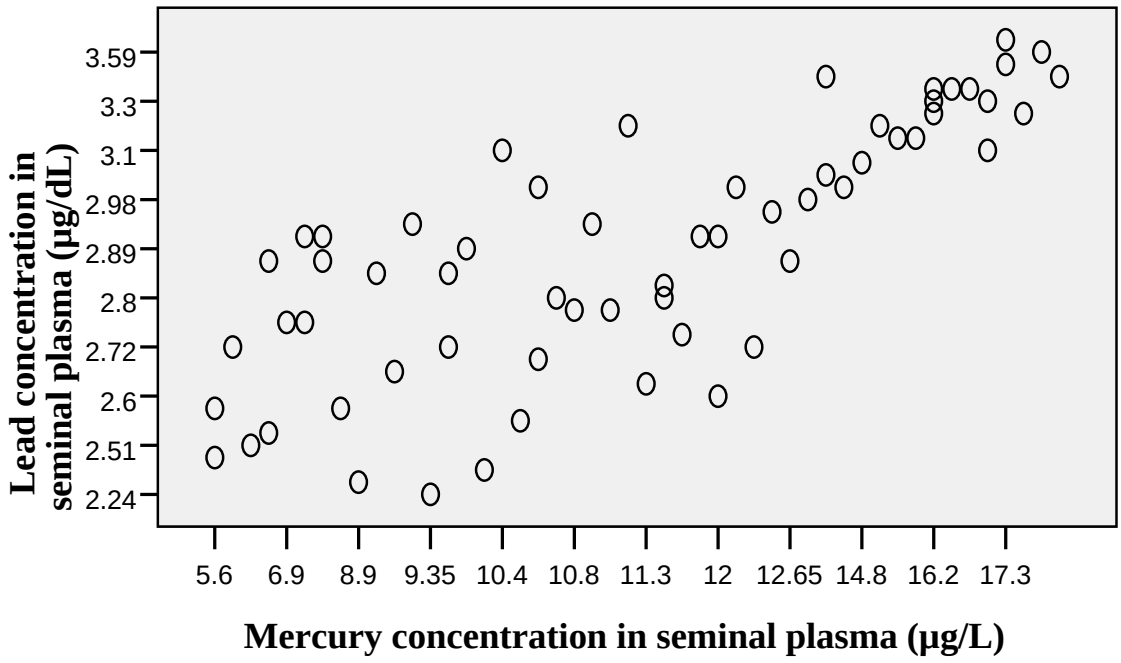
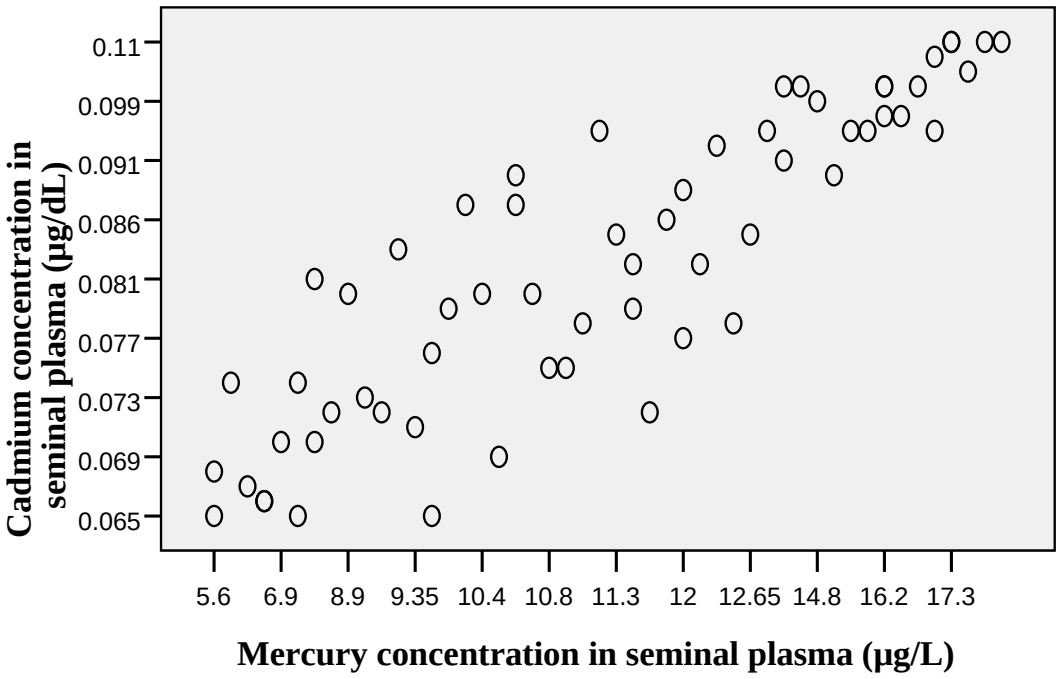


Figure 18: Relation between cadmium and mercury concentrations seminal plasma.



CONCLUSIONS

Conclusions

1. Almost half of our young university men had at least one semen parameter below the current WHO criteria for normality.

2. There has been an adverse temporal trend in sperm concentration among young Spanish men during the last decade. Young university students presented significantly lower sperm concentration and total sperm count than those found in a study conducted in Almeria in 2001.

3. The yearly rate of sperm decline calculated for the two studies would be -3.0%, even higher than the one estimated by Swan *et al.* for Europe (-2.3%).

4. A positive association has been found between the dietary intake of several antioxidant nutrients (cryptoxanthin, vitamin C, lycopene and β-carotene) and total motile sperm count.

5. Semen volume increased with higher intakes of vitamin C and β-carotene in young healthy students from Murcia.

6. Our study raise doubts whether current RDI may underestimate vitamin C requirements needed with regards to semen quality. The Recommended Daily Intake (RDI) for vitamin C is 60 mg per day, which is the median value of the first quartile in our study population.

7. There was no correlation between the concentrations of any of the metals in the three biological samples analyzed (whole blood, blood plasma and seminal plasma).

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Review Article

Correlations between Different Heavy Metals in Diverse Body Fluids: Studies of Human Semen Quality

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It has been hypothesized that exposure to heavy metals may impair male reproduction. To measure the effect produced by low doses of heavy metals on semen parameters, it is necessary to clarify in which body fluids those measurements must be performed. Sixty-one men attending infertility clinics participated in our study. Concentrations of lead, cadmium, and mercury were measured in whole blood, blood plasma, and seminal plasma using spectroanalytical and electrochemical methods. Semen analyses were performed according to World Health Organization criteria. For statistical analysis, Spearman's rank correlations, mean comparison tests, and discriminant analysis were calculated. Significant correlations between the measured concentrations of the three heavy metals in the same biological fluids were observed. However, no similar relationship was seen when comparing the concentrations in different body fluids of the same metal. According to our results and previous publications, seminal plasma might be the best body fluid for assessing impairment of human semen parameters.

1. Introduction

Over time there has been a significant decline of human fertility [1]. Like other European countries, Spain is since 1981 well below the 2.1 children needed to maintain replacement level [2]. Birth rate, have declined mainly due to changes in lifestyle and social mores and increased contraception [3].

These demographic transformations, as much as they are socially valued and desirable, have important clinical consequences. The fertility decline has resulted in a major delay in the average age of conception. The first pregnancy is postponed to ages at which women fecundity is decreased [4]. That may be one important reason why the medical reproductive units have taken on such a relevant role in developed countries. Between 2002 and 2004, more than 6% of Danish children were born through assisted reproduction techniques [1]. Consequently, social and medical considerations about infertility have become an important concern in recent years.

In parallel, it has been hypothesized that there is a worldwide decline in male semen quality [5–8], but it is clearly not uniform [9]. The decline in semen quality has been linked to environmental and work-related toxic exposures [10, 11]. For example, heavy metals may compromise male reproduction, as demonstrated by epidemiological and animal studies [12–22].

Our research interests are related to the measurement of the exposure to lead (Pb), cadmium (Cd), and mercury (Hg), and its relationship with human semen quality. The main results published on that issue are summarized in Table 1. Those studies were done using the World Health Organization (WHO) guidelines for semen analysis published in 1987 [23], 1992 [24], and 1999 [25]. However, in 2010, the WHO published new criteria for the assessment of semen samples [26], and all the sperm parameter cutoffs were lowered. The changes in the three main semen parameters through time (1987–2010) are summarized in Table 2.

1.1. Lead. There is considerable agreement that high or even moderate concentrations of lead cause fertility problems in humans. Fatima et al. showed that >40 μg/dL of lead in blood produced a decline of sperm count (<20 $\times$ 10^6 cells/mL). In addition, they observed lower motility (<50%) and morphology (<14%), with >35 μg/dL in whole blood [12]. Telisman and colleagues showed significantly lower sperm density and motility with high blood lead concentrations (36.7 μg/dL) [13]. High concentrations of lead seem to be clearly associated with sperm damage.

However, there are conflicting results about the effect on semen quality at low lead exposures. Hernandez-Ochoa and colleagues found that low lead concentrations in seminal fluid (0.2 μg/dL) were associated with impaired semen quality, 44% of motility, 32% of normal morphology, and 11 $\times$ 10^6 cell/mL of sperm concentration [14]. In contrast, Mendiola et al. found a relationship between levels of lead ten times higher in the spermatic fluid (2.93 μg/dL) and low motility, but no effect on morphology (>14%) or sperm concentration (>20 $\times$ 10^6 cells/mL) [15]. Similarly, Hovatta et al. reported that lead concentrations in seminal plasma of 2.5 μg/dL did not affect sperm concentration (96 $\times$ 10^6 cells/mL) [16]. Moreover, Mendiola et al. found that lead concentrations of 9.75 μg/dL measured in whole blood and 2.78 μg/dL in blood plasma had no effect on morphology (>14%), motility (>50%), or sperm concentration (>20 $\times$ 10^6 cells/mL) [15]. Meeker et al. also reported no effect on sperm concentration (42.7 $\times$ 10^6 cells/mL) or motility (55%) with 1.5 μg/dL of lead concentration in whole blood [17].

1.2. Cadmium. At high concentrations, cadmium could affect semen quality. According to Akinloye et al., men with high concentrations of cadmium in seminal plasma (65 μg/dL) had 5.16 $\times$ 10^6 cells/mL of sperm count and 36% of motile sperms [18].

As seen with lead, there is no agreement on the effect of low concentrations of cadmium on semen quality. Telišman et al. found that even low concentrations of cadmium <1 μg/dL in whole blood were associated with head pathologic sperms [13]. Benoff and colleagues concluded that sperm concentration, motility, and morphology are affected even with low seminal plasma concentrations of cadmium (0.028 μg/dL) [19]. Mendiola and colleagues also found that low concentrations of cadmium in seminal plasma (0.085 μg/dL) were moderately associated with low sperm motility (<50%) but had no effect on morphology (>14%) or sperm concentration (>20 $\times$ 10^6 cells/mL) [15]. Equally, Hovatta et al. showed no correlation between higher cadmium concentrations in seminal fluid (0.15 μg/dL) and sperm concentration (96 $\times$ 10^6 cells/mL) [16]. Chia and colleagues did not find an impairment of morphology (>50%) and motility (>50%) with low concentrations of cadmium in whole blood (0.095 μg/dL) [20]. Similarly, Mendiola et al. showed that cadmium measured in whole blood (0.10 μg/dL) and blood plasma (0.08 μg/dL) did not impaired morphology (>14%), motility (>50%), or sperm concentration (>20 $\times$ 10^6 cells/mL) [15]. Moreover, Meeker et al. reported no effect of low cadmium concentrations in whole blood (0.04 μg/dL) on sperm density (42.7 $\times$ 10^6 cells/mL) and motility (55%) [17].

1.3. Mercury. There is clear evidence that very high concentrations of mercury in the body will harm sperm. Choy et al. showed that high concentrations of total mercury (inorganic and organic) measured in whole blood (40.6 mmol/L) resulted in <50% of progressive motility, <14% of normal morphology, and <20 $\times$ 10^6 cells/mL of sperm concentration [21].

However, Mendiola et al. did not find an alteration of motility (>50%), morphology (>14%), or sperm concentration (>20 $\times$ 10^6 cells/mL) at low concentrations of total mercury in seminal plasma (1.18 μg/dL). Besides, low concentrations measured in whole blood (1.99 μg/dL) and blood plasma (0.6 μg/dL) were not related to decreased morphology (>14%), motility (>50%), or sperm concentration (>20 $\times$ 10^6 cells/mL) [15]. Rignell-Hydbom et al. found no association with sperm motility (54%) or concentration (48 $\times$ 10^6 sperm cells/mL) at low concentrations of organic mercury in whole blood (0.225 μg/dL) [22]. In addition, Meeker et al. reported that low mercury concentrations in whole blood (0.11 μg/dL) did not affect motility (55%) and sperm concentration (42.7 $\times$ 10^6 cells/mL) [17].

1.4. Justification of the Study. There are at least two problems in assessing whether low concentrations of heavy metals have an impact on human semen quality. First of all, there are just a few studies published on that issue so far. A second problem relates to the variables measured; that is the biological samples in which the concentrations of heavy metals are measured, and the parameters used to measure semen quality (motility, morphology, and sperm concentration).

To measure the effect produced by low doses of a heavy metal in the reproductive organs, it is necessary to clarify where to perform those measurements. Concentrations of heavy metals may be measured in the whole blood, in blood plasma, and in seminal plasma. However, it is not clear whether measurements in one or another fluid are equivalent, nor to what extent there are correlations between the three measurements of these heavy metals in the different body fluids.

The objectives of this study are (1) to examine whether there are correlations between the concentrations of heavy metals (lead, cadmium, and mercury) in the three body fluids (whole blood, blood plasma, and seminal plasma) and (2) to explore whether any one of the three measures relates better than the others with the semen quality parameters.

Table 1: Review of the measurement in the exposure to lead (Pb), cadmium (Cd), and mercury (Hg) and its relation with semen quality.

		Semen quality		
		Morphology	Motility	Sperm concentration
Lead	Whole blood	Fatima 2010 [12]: (i) C ≥ 35 μg/dL (ii) Mr ≤ 14% (iii) 1999 criteria Mendiola 2011 [15]: (i) C = 9.75 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria*	Telišman 2000 [13]: (i) C = 36.7 μg/dL (ii) Mt = $P < 0.02$ (iii) 1987 criteria Fatima 2010 [12]: (i) C ≥ 35 μg/dL (ii) Mt ≤ 50% (iii) 1999 criteria Mendiola 2011 [15]: (i) C = 9.75 μg/dL (ii) Mt ≥ 50% (iii) 1999 criteria* Meeker 2008 [17]: (i) C = 1.5 μg/dL (iii) Mt = 55% (iii) 1999 criteria*	Fatima 2010 [12]: (i) C ≥ 40 μg/dL (ii) SpC ≤ 20×10^6 cells/mL (iii) 1999 criteria Telišman 2000 [13]: (i) C = 36.7 μg/dL (ii) SpC = $P < 0.05$ (iii) 1987 criteria Mendiola 2011 [15]: (i) C = 9.75 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria* Meeker 2008 [17]: (i) C = 1.5 μg/dL (ii) SpC = 42.7×10^6 cells/mL (iii) 1999 criteria*
	Blood blasma	Mendiola 2011 [15]: (i) C = 2.88 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 2.88 μg/dL (ii) Mt ≥ 50% (iii) 1999 criteria	Mendiola 2011 [15]: (i) C = 2.88 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria
	Seminal blasma	Mendiola 2011 [15]: (i) C = 2.93 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria* Hernández-Ochoa 2005 [14]: (i) C = 0.2 μg/dL (ii) Mr = 32% (iii) 1999 criteria	Mendiola 2011 [15]: (i) C = 2.93 μg/dL (ii) Mt ≤ 50% (iii) 1999 criteria* Hernández-Ochoa 2005 [14]: (i) C = 0.2 μg/dL (ii) Mt = 44% (iii) 1999 criteria	Mendiola 2011 [15]: (i) C = 2.93 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria* Hovatta 1998 [16]: (i) C = 2.5 μg/dL (ii) SpC = 96×10^6 cells/mL (iii) 1992 criteria Hernández-Ochoa 2005 [14]: (i) C = 0.2 μg/dL (ii) SpC = 11×10^6 cells/mL (iii) 1999 criteria
Cadmium	Whole blood	Telišman 2000 [13]: (i) C ≤ 1 μg/dL (ii) Mr = $P < 0.005$ (iii) 1987 criteria Mendiola 2011 [15]: (i) C = 0.10 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria* Chia 1994 [20]: (i) C = 0.095 μg/dL (ii) Mr ≥ 50% (iii) 1987 criteria	Mendiola 2011 [15]: (i) C = 0.10 μg/dL (ii) Mt ≥ 50% (iii) 1999 criteria* Chia 1994 [20]: (i) C = 0.095 μg/dL (ii) Mt ≥ 50% (iii) 1987 criteria Meeker 2008 [17]: (i) C = 0.04 μg/dL (ii) Mt = 55% (iii) 1999 criteria	Mendiola 2011 [15]: (i) C = 0.10 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria* Meeker 2008 [17]: (i) C = 0.04 μg/dL (ii) SpC = 42.7×10^6 cells/mL (iii) 1999 criteria
	Blood plasma	Mendiola 2011 [15]: (i) C = 0.08 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 0.08 μg/dL (ii) Mot ≥ 50% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 0.08 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria*

Table 1: Continued.

		Semen quality		
		Morphology	Motility	Sperm concentration
	Seminal plasma	Mendiola 2011 [15]: (i) C = 0.085 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria*	Akinloye 2006 [18]: (i) C = 65 μg/dL (ii) Mt = 35.75% (iii) 1999 criteria	Akinloye 2006 [18]: (i) C = 65 μg/dL (ii) SpC = 5.16×10^6 cells/mL (iii) 1999 criteria
		Bennof 2009 [19]: (i) C = 0.028 μg/dL (ii) Mr = $P < 0.05$ (iii) 1992 criteria	Mendiola 2011 [15]: (i) C = 0.085 μg/dL (ii) Mt ≤ 50% (iii) 1999 criteria*	Hovatta 1998 [16]: (i) C = 0.15 μg/dL (ii) SpC = 96×10^6 cells/mL (iii) 1992 criteria
			Bennof 2009 [19]: (i) C = 0.028 μg/dL (ii) Mt = $P < 0.05$ (iii) 1992 criteria	Mendiola 2011 [15]: (i) C = 0.085 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria*
				Bennof 2009 [19]: (i) C = 0.028 μg/dL (ii) SpC = $P < 0.05$ (iii) 1992 criteria
Mercury	Whole blood	Choy 2002 [21]: (i) C = 40.6 mmol/L (ii) Mr ≤ 14% (iii) 1999 criteria	Choy 2002 [21]: (i) C = 40.6 mmol/L (ii) Mr ≤ 50% (iii) 1999 criteria	Choy 2002 [21]: (i) C = 40.6 mmol/L (ii) SpC ≤ 20×10^6 cells/mL (iii) 1999 criteria
		Mendiola 2011 [15]: (i) C = 1.99 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 1.99 μg/dL (ii) Mt ≥ 50% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 1.99 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria*
			Rignell-Hydbom 2007 [22]: (i) C = 0.225 μg/dL (ii) Mt = 54% (iii) 1999 criteria	Rignell-Hydbom 2007 [22]: (i) C = 0.225 μg/dL (ii) SpC = 48×10^6 cells/mL (iii) 1999 criteria
			Meeker 2008 [17]: (i) C = 0.11 μg/dL (ii) Mt = 55% (iii) 1999 criteria	Meeker 2008 [17]: (i) C = 0.11 μg/dL (ii) SpM = 42.7×10^6 cells/mL (iii) 1999 criteria
	Blood plasma	Mendiola 2011 [15]: (i) C = 0.6 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 0.6 μg/dL (ii) Mt ≥ 50% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 0.6 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria*
	Seminal plasma	Mendiola 2011 [15]: (i) C = 1.18 μg/dL (ii) Mr ≥ 14% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 1.18 μg/dL (ii) Mt ≥ 50% (iii) 1999 criteria*	Mendiola 2011 [15]: (i) C = 1.18 μg/dL (ii) SpC ≥ 20×10^6 cells/mL (iii) 1999 criteria*

Note: This table shows author, publication year, concentration of metal in whole blood, blood plasma, and seminal plasma, their effect on semen quality parameters, and the WHO criteria used to classify the semen quality.
C: concentration of the metal, Mr: morphology, Mt: motility, SpC: sperm concentration.
*Mendiola et al. use Kruger's strict criteria (14% of normal forms) as a cutoff for sperm morphology [25].

2. Materials and Methods

2.1. Study Population, Design, and Semen Analysis. The study population, hormone, and semen analyses have been previously described elsewhere [27, 28]. Sixty-one men were participating in a study to explore the role of environmental toxins and lifestyles on male infertility. Briefly, the men of couples attending three infertility centers in southeastern Spain between 2005 and 2007 were classified on the basis of semen quality, following WHO criteria [25]. Subjects provided two semen samples and were requested to observe a 3- to 5-day abstinence period. The importance of the abstinence

Table 2: Changes in the three main semen parameters through time (1987–2010). A semen parameter was considered normal when the values were equal or above the presented figures [23–26].

	1987	1992	1999	2010
Sperm concentration ($\times 10^6$ cells/mL)	20–200[1]	≥20	≥20	≥15
Motility (%)	≥60	≥50	≥50	≥40
Morphology (%)	≥50	≥30	≥14	≥4

[1] range.

period was stressed on the interviews with the participants [27]. The average of the two samples was used in our statistical analysis. Semen parameters evaluated included ejaculate volume, sperm concentration, percentage of motile sperm, and percentage of normal forms following Kruger's strict criteria [25]. All patients were interviewed face-to-face by the same interviewer and completed a comprehensive occupational and lifestyle questionnaire [27]. This study was approved by the Institutional Review Board. Patients were included in the study after giving informed written consent.

2.2. Measurements of Metals. A total of 181 biological samples were analyzed for Pb, Cd, and Hg, including 61 samples of seminal plasma, 61 of blood plasma, and 59 of whole blood, as two samples were lost during the study. Biological samples were dispensed into aliquots and frozen and stored at −40°C until analysis. Anodic stripping voltammetry (ASV) was used for measuring Pb and Cd concentrations. ASV was carried out using a voltamperometer with VA 663 stand and VA 608 controller (Metrohm 626, Herisau, Switzerland). The voltamperometric cell was equipped with a drop of mercury as the working electrode, an Ag/AgCl/KCl 3 M reference electrode, and a platinum auxiliary electrode.

Determination of total Hg was carried out by thermal decomposition, amalgamation, and atomic absorption spectrophotometry, using a mercury analyzer with quartz sample boats (DMA-80 Direct Mercury Analyzer, Milestone, Shelton CT, USA).

The highest grade purity reagents were employed in this procedure including nitric acid 65% and perchloric acid 70% (Suprapur, Merck, Darmstadt, Germany). The ultrapure water was purified with Millipore Simplicity 185 (Millipore GmbH, Molsheim, France) obtaining conductivity values of 0.054 μS/cm.

In order to prepare the working standard solutions, commercially available standard solutions for Pb 1 g/L and Cd 1 g/L (Tritisol, Merck, Darmstadt, Germany) and Hg 1 g/L (Certipur, Merck, Darmstadt, Germany) were used. The limits of detection (LOD) for the body's fluid metal levels were as follows: lead, 21 μg/L; cadmium, 0.11 μg/L, and mercury, 0.1 μg/L. To guarantee the accuracy and precision of the applied technique regarding heavy metals, whole blood reference materials (Seronorm Trace Elements Whole Blood, SERO AS, Billingstad, Norway) were employed.

2.3. Sample Preparation. Pb and Cd determinations were performed using 0.2 mL of the biological sample deposited inside of 25 mL borosilicate glass. Acid digestion was carried out by adding 2 mL of nitric acid and 2 mL of perchloric acid and evaporating it to dryness. Once the sample was dry and cooled down, 100 μL of perchloric acid and 15 mL of double-distilled water were added, transferring the final volume into a voltamperometric cell.

Biological samples were measured by ASV according to the following method [24]. Briefly, differential pulse (DP) with hanging mercury drop electrode (HMDE) was used, the voltage sweep was from −0.70 to +0.15 volts, and the peak voltage was located at −0.58 and −0.40 volts for Cd and Pb respectively. Deaeration, preconcentration, and resting time (without stirring) were 180, 120, and 40 seconds, respectively. Sensitivity was 0.05 nAmp/mm and 0.2 nAmp/mm for Cd and Pb, respectively. Standard addition method was applied to perform the current analyses, adding known values of a standard solution (2, 4, and 6 ng for Cd and 20, 40, and 60 ng for Pb) to obtain a calibration curve, then the values of the measurements were interpolated into that curve. Mercury determination was carried out following EPA method 7473 [29], and 0.2 mL of the biological sample was transferred directly into the quartz sample boats. To obtain a calibration curve, standard solutions of 5, 10, 20, 30, 100, 200, and 500 ng of Hg were employed.

2.4. Statistical Analysis. The statistical analysis encompassed descriptive and inferential analyses. Basic, dispersion as well as frequency parameters were calculated for descriptive analyses. Statistical analyses were performed to explore possible patterns in the concentrations of heavy metals measured in blood serum, whole blood, and seminal plasma. Spearman's rank correlations and scatter plots were employed for comparison of variables. In the inferential analysis, the mean comparison tests and discriminant analysis were performed. All tests were two-tailed, and the level of statistical significance was set at 0.05. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Results. Table 3 shows lead (Pb), cadmium (Cd), and mercury (Hg) concentrations in μg/dL (mean, standard error, median, and interquartile range), in whole blood, blood plasma, and seminal plasma.

Figures 1(a)–1(i) show the scatter plots of the concentrations of the three metals in the three body fluids. As may be observed, men with low concentration of one heavy metal in a fluid can show low or high concentrations of the same metal in another fluid. There is a wide dispersion of data, and

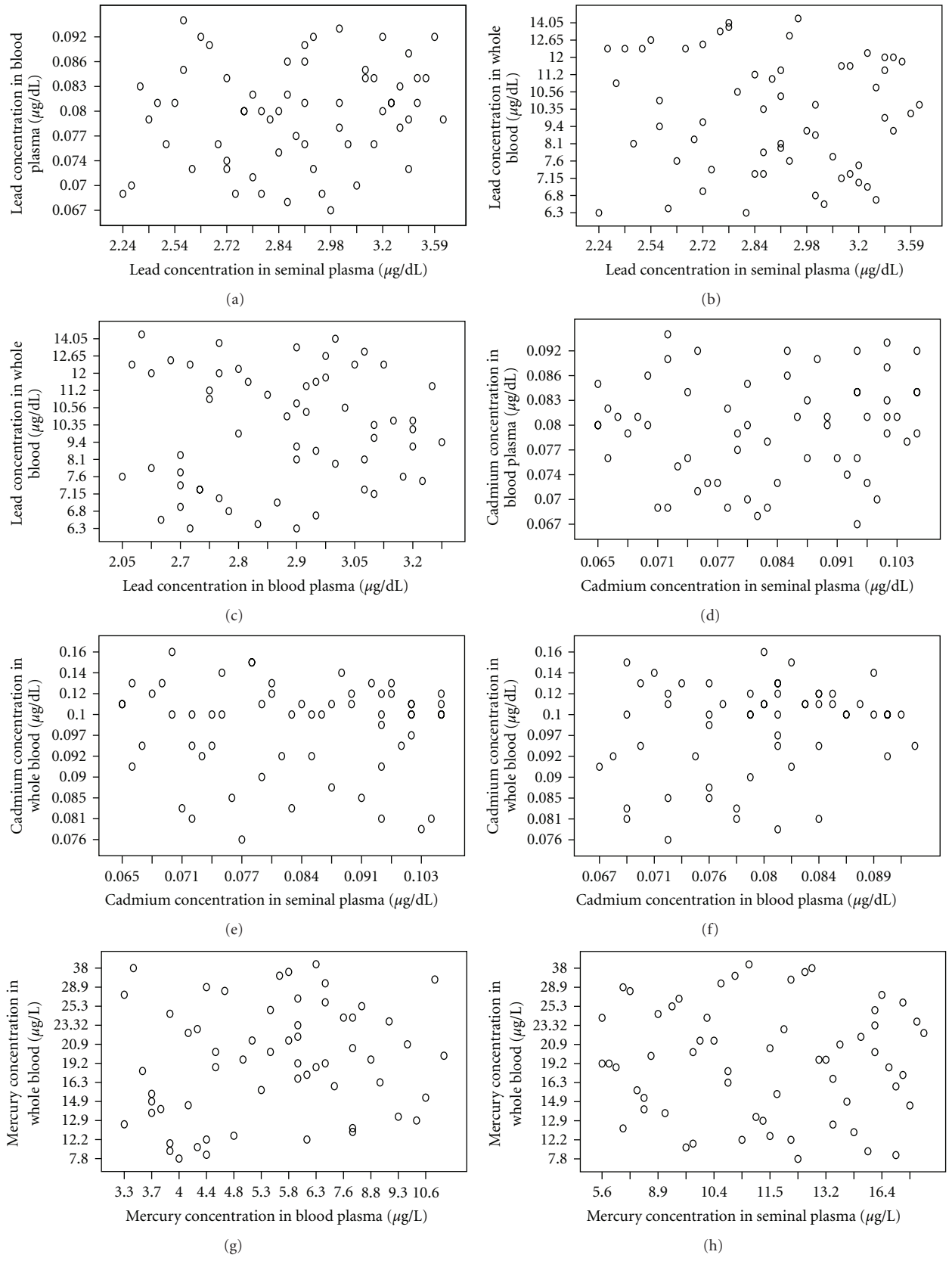
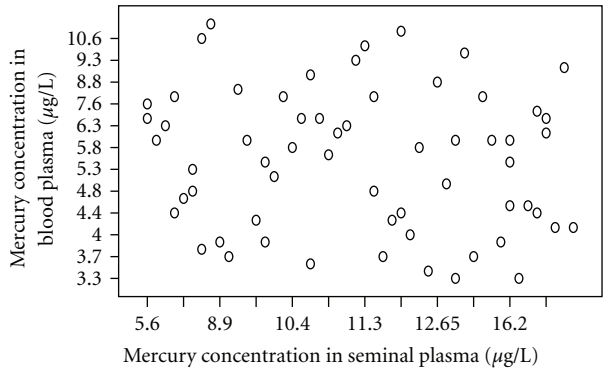


Figure 1: Continued.



(i)

Figure 1: (a) Relation between lead concentrations in seminal plasma and blood plasma. (b) Relation between lead concentrations in seminal plasma and whole blood. (c) Relation between lead concentrations in blood plasma and whole blood. (d) Relation between cadmium concentrations in seminal plasma and blood plasma. (e) Relation between cadmium concentrations in seminal plasma and whole blood. (f) Relation between cadmium concentrations in blood plasma and whole blood. (g) Relation between mercury concentrations in blood plasma and whole blood. (h) Relation between mercury concentrations in seminal plasma and whole blood. (i) Relation between mercury concentrations in blood plasma and whole blood.

Table 3: Heavy metal concentrations in seminal, blood plasma, and whole blood.

	Lead (μg/dL)		Cadmium (μg/dL)		Mercury (μg/dL)	
	Mean (SE)	Median (IQR)	Mean (SE)	Median (IQR)	Mean (SE)	Median (IQR)
Blood plasma ($n = 61$)	2.88 (0.22)	2.90 (2.72–3.05)	0.08 (0.007)	0.08 (0.07–0.08)	0.6 (0.22)	0.58 (0.42–0.72)
Whole blood ($n = 61$)	9.75 (2.28)	10.10 (7.50–11.90)	0.10 (0.02)	0.10 (0.09–0.12)	1.99 (0.69)	1.96 (1.47–2.46)
Seminal plasma ($n = 61$)	2.93 (0.32)	2.90 (2.72–3.15)	0.08 (0.01)	0.08 (0.07–0.09)	1.18 (0.35)	1.13 (0.92–1.49)

SE: standard Error, IQR: interquartile range.

there are no associations between the measurements made of the metals in one fluid and the concentrations measured in the two other fluids.

Table 4 presents the results of the Spearman's correlation between the concentrations of lead, cadmium, and mercury in whole blood, blood plasma, and seminal plasma. Although the correlation coefficients were above 0.5 for some determinations, no significant correlations were found between the concentrations of the same metal and the three biological fluids. The correlation between the concentration of lead in blood plasma and whole blood was 0.57 ($P = 0.67$), between cadmium in seminal plasma and whole blood was -0.50 ($P = 0.72$), and between mercury in seminal plasma and whole blood -0.34 ($P = 0.80$).

Figures 2(a)–2(i) show the relationship between the concentrations of lead, cadmium, and mercury measured in each fluid. As may be observed, there is a linear relationship, since men with low concentration of a given metal in a biological fluid also had low concentration of the other two metals in the same fluid. And, reversely, men with high concentration of a given metal in a biological fluid also had high concentration of the other metals in the same body fluid.

Spearman's correlation coefficients and scatter plots revealed a high correlation between the concentrations of the three metals in the same biological fluids. Table 5 shows the correlation of the three heavy metals (Pb, Cd, and Hg) in the same biological fluid (whole blood, blood plasma, or seminal plasma). High and statistically significant correlations were observed between the three heavy metals for the same biological fluid. In seminal plasma, the correlation between cadmium and lead was 0.74 (P value < 0.005) and between mercury and lead 0.76 (P value < 0.005).

To explore whether these correlations were determined by associations with other factors, exploratory scatter plots were generated between the concentrations of the three metals in the three biological fluids and possible confounding variables. Possible confounders were such as "occupation," "tobacco smoke," "exposure to toxics at work" or "using metals at work." No patterns were observed. Hypothesis tests were used to detect significant differences in the mean concentrations of metals and the possible confounding factors used in the scatter plots. Not significant differences were found (data not shown).

As a final alternative, metal concentrations were categorized in two, three, and four groups using the mean values, tertiles, and quartiles, respectively. Discriminant analysis was then used to detect whether any of the factors was related to the categories of the metal concentrations. To this end, different discriminant analysis evaluating the overall Wilks' lambda and the owners of each factor were produced, but none of them were satisfactory.

3.2. Discussion. Using the Spearman's correlation coefficient and scatter plots revealed a high correlation between

Table 4: Spearman's correlation coefficients between metal concentrations in seminal and blood plasma, and whole blood.

		Blood plasma		Whole blood	
		R	*P* value	*R*	*P* value
Lead	Blood plasma			0.57	0.67
	Seminal plasma	0.13	0.32	−0.08	0.55
Cadmium	Blood plasma			0.14	0.30
	Seminal plasma	0.12	0.36	−0.50	0.72
Mercury	Blood plasma			0.17	0.19
	Seminal plasma	−0.13	0.34	−0.34	0.80

Table 5: Spermean's correlation coefficients between seminal plasma, blood plasma, and whole blood, with metal concentrations.

		Cadmium		Mercury	
		R	*P* value	*R*	*P* value
Seminal plasma	Lead	0.740	0.001	0.760	0.001
	Cadmium			0.870	0.001
Blood plasma	Lead	0.550	0.001	0.750	0.001
	Cadmium			0.700	0.001
Whole blood	Lead	0.850	0.001	0.950	0.001
	Cadmium			0.792	0.001

the measured concentrations of the 3 heavy metals in the same biological fluids. However, no similar relationship was observed when comparing the concentrations in different body fluids of the same metal.

It would be reasonable to expect that subjects with high and low levels of exposure to any metal would show similar positions (low or high concentrations) in the measurements made in any body fluid. However, we found no correlation between the concentrations of any of the metal in the three biological samples analyzed (whole blood, blood plasma, and seminal plasma).

Other authors, similarly, found no correlation between the concentrations of the same metal in different fluids [14, 19]. Benoff and colleagues found no correlation between cadmium concentrations in seminal plasma and blood plasma. Hernandez-Ochoa et al. also reported no correlation in blood lead concentrations between whole blood-plasma blood, whole blood-seminal plasma, or blood plasma-sperm in 68 Mexican men.

There are some possible hypotheses for these phenomena. The three heavy metals are bound and transported by erythrocytes [30–32]. Given that metals are transported by red cells, unmeasured differences in the concentration of red cells in our study population may result in different concentration of the metals in the blood. However, this hypothesis cannot be tested, mainly due to information on red cell concentration was not collected.

Surprisingly, the concentrations of Pb, Cd, and Hg were correlated in the same biological samples. Howatta et al. also found that the concentrations of cadmium and lead in seminal plasma were correlated [16]. We do not have a firm hypothesis of why that may happen.

Correlations of the three heavy metals in the same body biological fluid may be due to an interaction between the different metals in the same compartment, so that the concentration of one metal determines the concentration of the others. We are not aware of lead, cadmium, or mercury modulate each other. However, it has been published that selenium produces the redistribution of Hg from plasma to erythrocytes at higher ratio [33] and the modification of hepatic zinc by cadmium [34]. Therefore, it could be that a given heavy metal might modulate proteins and/or enzymes in the cells and influence the concentration of other heavy metals. [35–37].

As to how to measure the effect produced by heavy metal concentrations on semen quality, it would be better to measure those metals in seminal plasma than in blood plasma or whole blood. Heavy metal concentrations in blood samples do not necessarily reflect the seminal plasma ones, since heavy metal concentrations reaching the seminal plasma could be quite different.

Heavy metals have a strong capacity to induce oxidative stress in body cells by disintegration of the lipid membrane, and spermatozoa are quite sensible to oxidative stress [38, 39]. Thus, in principle, it would be more accurate to measure heavy metal concentrations in seminal plasma—than in other fluids—in order to determine sperm damage. Numerous antioxidants such as vitamin C, vitamin E, glutathione, coenzyme Q10, and some fruits may diminish the oxidative stress caused by heavy metals [28, 40, 41].

Furthermore, as it can be seen in Table 1, high concentrations of heavy metals can alter sperm morphology, motility, and concentration individually. However, an alteration of the three semen parameters can be observed with very low heavy metal concentrations only in seminal plasma, showing us that this body fluid might reflect better the sperm damage.

Finally, our findings might be attributed to chance or bias. The sample of individuals included in the study was

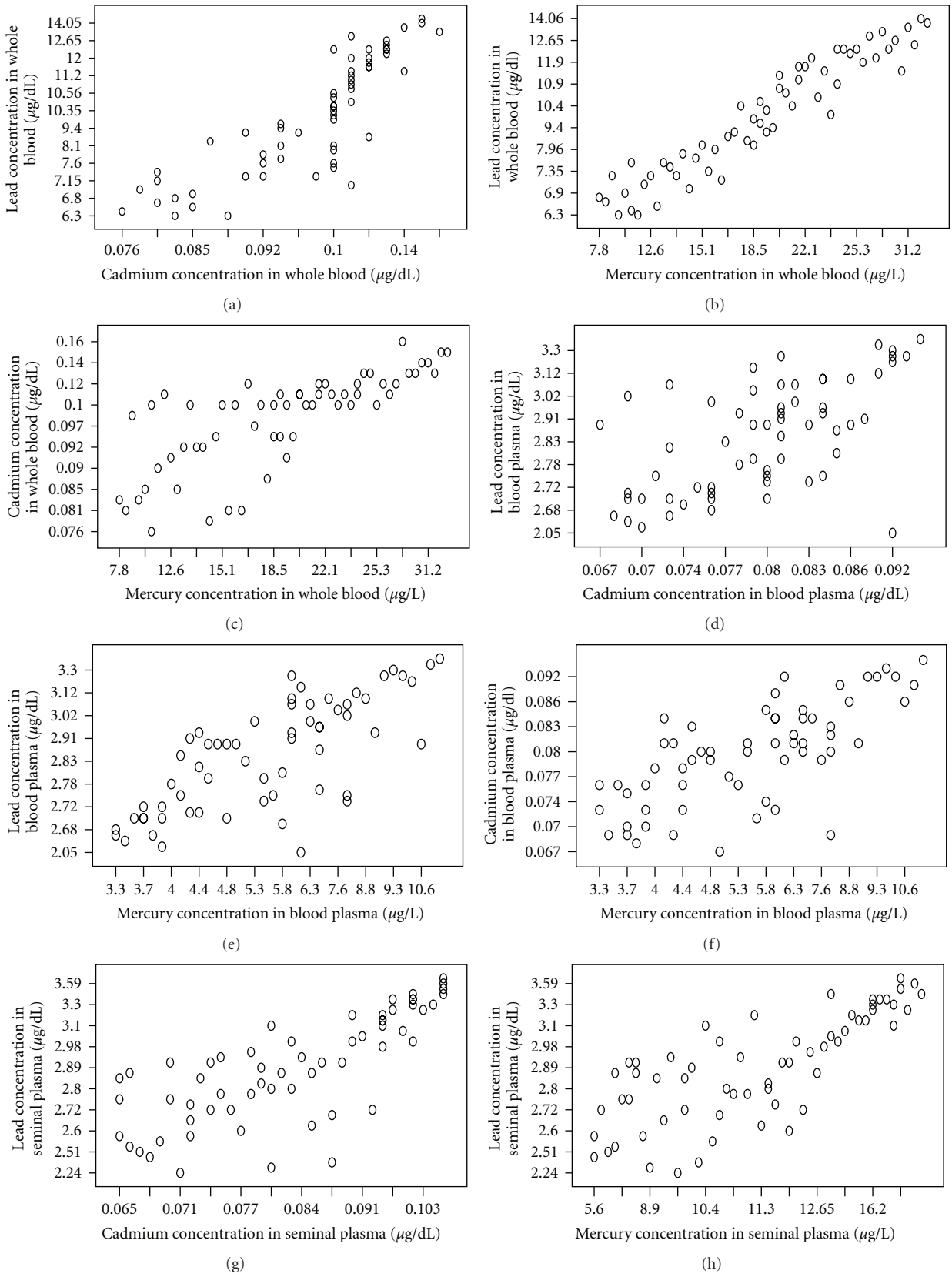
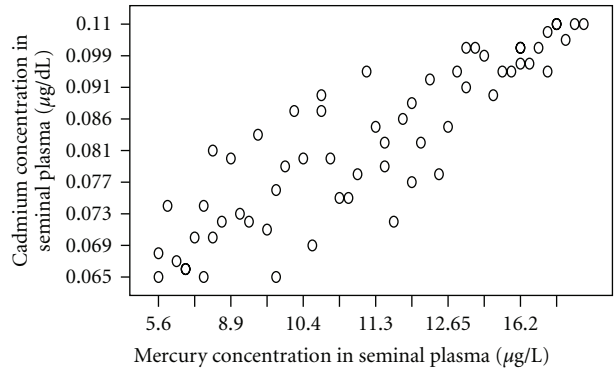


Figure 2: Continued.



(i)

Figure 2: (a) Relation between lead and cadmium concentrations in whole blood. (b) Relation between lead and mercury concentrations in whole blood. (c) Relation between cadmium and mercury concentrations in whole blood. (d) Relation between lead and cadmium concentrations in blood plasma. (e) Relation between lead and mercury concentrations in blood plasma. (f) Relation between cadmium and mercury concentrations in blood plasma. (g) Relation between lead and cadmium concentrations in seminal plasma. (h) Relation between lead and mercury concentrations in seminal plasma. (i) Relation between cadmium and mercury concentrations seminal plasma.

small and the lack of statistically significant correlations may be a consequence of that. Our findings are, however, consisting with those [14, 16, 19] of that have explored the same correlations leading us to believe that they cannot be attributed to random or systematic error.

4. Conclusions

Our study suggests that there is no correlation between the concentrations of any of the metals in the three biological samples analyzed (whole blood, blood plasma, and seminal plasma) and there is a correlation between the concentrations of Pb, Cd, and Hg in the same biological samples. According to our results and previous publications, seminal plasma might be the best body fluid for assessing impairment of human semen parameters.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

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