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CORRELATION BETWEEN SEMINAL PLASMA LEVES OF GROWTH HORMONE, INSULIN-LIKE GROWTH FACTOR-1 AND MALE INFERTILITY

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ABSTRACT

Infertility is a common problem affecting a considerable percentage of the male population worldwide and has been a research topic for many decades. Growth Hormone and Insulin-like Growth Factor 1 have been proposed as factors that may have a pivotal role in male infertility due to their anabolic effect. The aim of this study is to investigate a possible correlation between seminal plasma levels of GH and IGF-1 and sperm parameters that may exert an impact on fertility. The study took place between May 2017 and September 2017 with the participation of 50 patients. A marginally non-significant statistic difference ($p=0.063$) in GH levels has been found between asthenospermic group and normal group. A statistically significant correlation ($p<0.05$) has been found between levels of GH and IGF-1 in the group with asthenospermia and at least one more abnormal parameter. The above correlation has been found also in patients with low sperm concentration, vitality, volume and abnormal morphology. According to our knowledge this is a novel finding and further studies are required in order to clarify the biological significance of this correlation.

ΣΥΝΟΨΗ

Η υπογονιμότητα είναι ένα σύνηθες πρόβλημα που αφορά ένα σημαντικό ποσοστό του παγκόσμιου ανδρικού πληθυσμού και αποτελεί ερευνητικό τομέα εδώ και αρκετές δεκαετίες. Η αυξητική ορμόνη και ο Insulin-like Growth Factor-1 έχουν προταθεί ως παράγοντες που διαδραματίζουν καθοριστικό ρόλο στην ανδρική υπογονιμότητα εξαιτίας της αναβολικής δράσης τους. Ο σκοπός της παρούσας μελέτης είναι η διερεύνηση πιθανής συσχέτισης μεταξύ των επιπέδων GH και IGF-1 του σπερματικού πλάσματος και των σπερματικών παραμέτρεων που πιθανώς επηρεάζουν την ανδρική υπογονιμότητα. Η μελέτη πραγματοποιήθηκε μεταξύ Μαΐου 2017 και Σεπτεμβρίου 2017 με τη συμμετοχή 50 ασθενών. Μια οριακά μη σημαντική στατιστική διαφορά ($\rho=0.063$) στα επίπεδα της GH βρέθηκε ανάμεσα στο φυσιολογικό και το ασθενοσπερμικό γκρουπ. Μια σταστιστικά σημαντική συσχέτιση ($\rho<0.05$) βρέθηκε ανάμεσα στα επίπεδα της GH και του IGF-1 στο γκρουπ με ασθενοσπερμία και τουλάχιστον μια ακόμη μη φυσιολογική σπερματική παράμετρο. Η παραπάνω συσχέτιση βρέθηκε και στους ασθενείς με χαμηλή συγκέντρωση σπερματοζωαρίων, χαμηλή ζωτικότητα σπερματοζωαρίων και χαμηλό όγκο σπέρματος. Σύμφωνα με την γνώση μας πρόκειται για ένα καινοτόμο εύρημα που χρειάζεται περαιτέρω έρευνα προκειμένου να διασαφηνιστεί η βιολογική σημασία της συσχέτισης αυτής.

INTRODUCTION

Spermatogenesis

Spermatogenesis is the multi-step process that begins with the first mitotic division of germ cells in order to become mature spermatozoa. During embryogenesis the primordial germ cells migrate into the gonad and become immature germ cells named spermatogonia. Spermatogonia have 46 chromosomes. After puberty and almost throughout life these cells divide mitotically.

Some of the spermatogonia enter into their first meiotic division and become primary spermatocytes. At the prophase of this meiotic division the chromosomes undergo cross-over and as a result the primary spermatocytes become tetraploid cells (46 sets of chromosomes). Completing this meiotic division, the daughter cells become secondary spermatocytes, which have 23 sets of chromosomes (22 autosomal and a duplicated X or Y chromosomes). Undergoing another meiosis the secondary spermatocytes become spermatids which are haploid cells, having one of each autosomal chromosomes and a X or Y chromosome. Spermatids form the inner layer of the epithelium and are found in rather discrete aggregates as the cells derived from a single spermatogonium tend to remain together and differentiate synchronously [Boron, 2012]

For the production of mature spermatozoa by spermatids another process is required, the spermiogenesis. Spermatids are located adjacent to the lumen of the seminiferous tubules during the early stages of spermiogenesis and are surrounded by processes of Sertoli cell cytoplasm. The Sertoli cells are large, polyhedral cells extending from the basement membrane toward the lumen of the seminiferous tubule. Tight junctions connect the adjacent Sertoli cells to forming a blood-testis barrier providing a protective environment for developing germ cells. In addition, gap junctions between the Sertoli cells and developing spermatozoa may represent a mechanism for transferring material between these two types of cells. Release of the spermatozoa from the Sertoli cell has been called spermiation [Guyton & Hall, 2011].

During spermiation spermatozoa are immotile and are passively moved from the Sertoli cells through the seminiferous tubules to the rete testes and to epididymis. Spermatozoa are transferred to epididymis with assistance by ciliary action of the luminal epithelium and contractility of the smooth muscle elements of the efferent duct wall. [Vanders, 2001]

Sperm are stored in the epididymis, where they undergo a process of maturation before they are capable of progressive motility and fertilization. Spermatozoa released at ejaculation are fully motile and capable of fertilization providing they undergo

changes during their journey to the salpinx, whereas spermatozoa obtained directly from the testis are functionally immature insofar as they cannot penetrate an ovum. However, these immature spermatozoa can fertilize if they are injected into an ovum employing the technique of Intracytoplasmic Sperm Injection ICSI [Vanderzwalmen et al., 1995]. During maturation in the epididymis, spermatozoa undergo changes in motility, metabolism, and morphology. Spermatozoa derived from the head of the epididymis are often unable to fertilize ova, whereas larger proportions of spermatozoa captured from the body are fertile. Spermatozoa obtained from the tail of the epididymis, or from the vas deferens, are almost always capable of fertilization. [Boron, 2012]

The epididymis empties into the vas deferens, which is responsible for the movement of sperm along the tract. The vas deferens contains well-developed muscle layers that facilitate sperm movement. The vas deferens passes through the inguinal canal, traverses the ureter, and continues medially to the posterior and inferior aspect of the urinary bladder, where it is joined by the duct arising from the seminal vesicle; together, they form the ejaculatory duct.

Almost 74 days are needed for spermatogonia to become mature spermatozoa and each stage of the process has a specific duration. The rate of spermatogenesis is constant and cannot be accelerated by hormones such as gonadotropins or androgens. Germ cells must comply with a specific timeline in their differentiation. If the environment is unfavorable and makes it impossible for them to continue their differentiation at the normal rate they degenerate. The production rate of spermatozoa is the highest for a man in his 20's and drops progressively throughout life. In men older than 50 years old a significant decrease in the percentage of morphologically normal and motile spermatozoa is found. [Chrysikopoulos, 2001]

Biology of semen

Only 10% of the volume of semen is sperm cells. According to World Health Organisation the normal concentration of sperm cells is greater than 15 million/mL, and the typical ejaculate volume is greater than 1.5 mL. The typical ejaculate content varies between 150 and 600 million spermatozoa.

Aside from the sperm cells, the remainder of the semen (i.e., 90%) is seminal plasma, the extracellular fluid of semen. Very little seminal plasma accompanies the spermatozoa as they move through the testes and epididymis. The seminal plasma originates primarily from the accessory glands (the seminal vesicles, prostate gland, and the bulbourethral glands). The seminal vesicles contribute around 70% of the volume of semen. Aside from the sperm, the remaining 20% represents epididymal fluids, as well as secretions of the prostate gland and bulbourethral glands.

Seminal plasma contains a plethora of sugars and ions. Fructose and citric acid are contributed to the seminal plasma by the accessory glands, and their concentrations

vary with the volume of semen ejaculated. Ascorbic acid and traces of B vitamins are also found in human seminal plasma.

Seminal plasma also contains low molecular weight polypeptides and proteins. Sperm functionality is relative to these proteins, which have a major role in sperm interactions with the various environments along the female genital tract towards the oocyte vestments. Specific peptides and proteins act as signals for the female immune system to modulate sperm rejection or tolerance, perhaps even influencing the relative intrinsic fertility of the male and/or couple by attaining a status of maternal tolerance towards embryo and placental development [Rodriguez-Martinez et al., 2011].

Male Infertility

Infertility is defined as the inability of a couple to achieve pregnancy after at least one year of unprotected intercourse (WHO). Infertility is a common problem and according to recent researche about 15% of couples are infertile worldwide. Around 50% of total infertility is attributed to male factor infertility alone or combined with female infertility. Another study supports that infertility affects almost 7% of the total male population [Lotti & Maggi, 2015]. These numbers may be even higher because male infertility is not well reported due to social reasons especially in the developing countries or the patriarchal societies.

According to European Urology Association almost 70% of male infertility is a result of urogenital abnormalities, malignancies, urogenital track infections, increased scrotal temperature, endocrine disturbances, genetic abnormalities and immunological factors. In the other 30% no associated factor can be found although the semen analysis is abnormal. This is called idiopathic infertility. A part of idiopathic infertility is suspected to be caused by epigenetic disorders or life style habits.

Causes

Urogenital abnormalities may be congenital or acquired and refer to cryptorchidism, hypospadias (dislocation of the urethra), congenital absence of vas deference (may be a symptom of cystic fibrosis), obstructions, testicular torsion and orchitis (a result of either viral or bacterial infection).

Cryptorchidism is the absence of one or both testes from the scrotum. It is one of the most common congenital defects of the male reproductive system. It affects approximately 2-4% of new-born boys and may have an impact at the health of the male adult. Testosterone and Insuline-like factor 3 (INSL3) are major regulators of the testicular descent. The cause of the abnormal descent may be genetic, including mutations on the INSL3 gene, chromosomal alterations or polymorphisms [Foresta et al., 2008]. Environmental reasons may be another cause of cryptorchidism including maternal lifestyle habits like increased alcohol consumption [Daamgard et al., 2007]

and smoking [Jensen et al., 2007]. Other factors like pre-mature birth and low body weight of the infant is found to increase the risk of cryptorchidism.

Increased scrotal temperature may be a result of **varicocele**. Varicocele is the enlargement of the veins in the scrotum. It affects almost 15% of all adult males and is amongst the most common causes of male infertility. Varicocele has an adverse effect on spermatogenesis not only due to increased temperature but also causes increased intratesticular pressure, hypoxia, reflux of toxic metabolites from the adrenal glands and hormonal abnormalities [Kantartzi et al., 2007]. Scrotal hyperthermia may also be caused by febrile illness or lifestyle habits (such as laptop use [Sheynkin et al., 2005]).

Urogenital track infections are accounted for almost 15% of male infertility. The infections may affect different locations of the urogenital track, which may be the testes, the epididymis and the accessory male glands [Pellati et al., 2008]. The spermatozoa may be affected during the different stages of their development resulting in reduced vitality and reduced sperm count.

Endocrine disturbances may be the cause of infertility in a small percentage of men. Usually they are a result of a dysfunction in hypothalamic-pituitary-testicular axis and on rare occasions they are a result of thyroid or adrenal disorders. The causes of the endocrine disturbances may be genetic or acquired. Acquired causes include tumors, inflammation or injury of the hypothalamus or the pituitary.

Genetic disorders may act indirectly (as described above) or directly towards male infertility. Genetic syndromes that include an abnormal chromosomal number are a cause of infertility. Reciprocal translocations, ring chromosomal abnormalities, Robertsonian translocations, inversions and reciprocal translocations are associated with male infertility. Mutations affecting the Y chromosome are possible causes of male infertility such as the AZF deletions [Shamsi et al., 2011]

Immune Infertility is an autoimmune disease caused by anti-sperm antibodies. Abnormalities of the blood-testis barrier may cause the production of anti-sperm antibodies. The anti-sperm antibodies may inhibit sperm functions that are important for fertilization mostly affecting zona and oolemma binding [Bohring & Krause, 2003].

According to recent research another cause of infertility may be epigenetic disorders. Epigenetic changes occur in many stages during spermatogenesis. These changes include DNA methylation and phosphorylation, hyperacytelation, histone degradation, ubiquitylation and sumoylation [Dada et al., 2012]. miRNAs also play an important role in the fertility as there are more than 300 differentially regulated miRNAs when comparing fertile and azoospermic men. [Gunes et al., 2016].

Lifestyle habits or work conditions may also have an impact in male fertility. Gasoline, lead and zync fumes have been found to have a negative result in male

fertility. Smoking, obesity and drugs may also be a cause of infertility [Sharma et al., 2013].

SEMEN ANALYSIS DIAGNOSIS

The most reliable diagnostic approach for male infertility is the semen analysis. The semen analysis includes the collection of semen after at least 3 days of abstinence (optimal abstinence time 2-5 days find out what WHO suggests. There are few basic criteria for the evaluation of the sample. These criteria have been updated regularly by W.H.O and according to the last update (5^{th} Edition WHO Laboratory Manual for the Examination and Processing of Human Semen, 2010) for a male to be categorized as fertile the following must stand:

Parameter	Levels
Semen Volume (mL)	>1,5
Sperm Concentration (/mL)	>15.000.000
Total Sperm Count	>39.000.000
Total Motility	>40%
Progressive Motility	>32%
Vitality	>58%
Morphology (normal)	>4%

There are more criteria that have an important role in fertility, they present with much less prevalence and are optional during the analysis.

PARAMETER	LEVELS
pH	>7.2
Peroxidase-positive leukocytes	<1.000.000
MAR test	<50%
Immunobead test	<50%
Seminal Zinc (μmol)	>=2,4
Seminal Fructose (μmol)	>=13
Seminal Neutral Glucosidase (μmol)	<=20

The threshold of 4% for normal morphology spermatozoa is according to Kruger strict criteria. For a spermatozoon to be characterized as normal the following conditions must be met:

- Head: Oval shape and smooth configuration, length 5 to 6 μm, diameter 2.5 to 3.5 μm
- Acrosome: 40 to 70% of head area
- Tail: 45 μm
- No neck, midpiece or tail defects
- Borderline forms are counted as abnormal

[Kruger et al., 1986 & 1988]

Approach and management of male infertility within the scope of Assisted Reproduction Technologies (ART)

Male infertility is a dysfunction with complex etiology so there are few treatment options. Drug administration, surgical corrections and Assisted Reproductive Techniques are some of them. Since a large portion of infertility is caused by idiopathic reasons, or other treatments may not have the expected results, ART are commonly performed.

Intrauterine Insemination (IUI) is an assisted reproductive technique (ART) in which sperm is transferred to a woman's uterine cavity in order to achieve fertilization. This procedure has a long history of usage, mostly used in animals during the early years. The protocol has changed over the course of time and after the discovery of modern laboratory techniques in order to achieve higher success rates. After collection of the semen, the sample is processed and washed in the laboratory, separating the sperm from the other proteins and cells of the seminal fluid and only the highly motile spermatozoa are transferred to the uterine cavity using a catheter. IUI is commonly used as the first line of treatment managing an infertile couple with aetiology that does not include male factor. However it may also be employed when mildly low sperm count or motility is detected. IUI has a success rate of approximately 10% per cycle (Soria et al., 2012), which is much less than other ARTs but is less invasive and less costly.

The first successful IVF took place in 1978 and created many new opportunities for couples suffering with infertility [Steptoe and Edwards, 1978] During the first years of IVF practice success rates were relatively low. IVF procedure includes gonadotropin medication treatment for women in order to cause controlled ovarian stimulation resulting to production of multiple follicles of the ovaries. After the follicles have reached a certain degree of development, other medications are administered (usually chorionic gonadotropin) which trigger oocyte release. The oocytes are retrieved using ultrasound guided transvaginal paracentesis and aspiration of follicular fluid.

After collection of both the oocytes and the semen, selection procedures occur for both samples. The oocyte selection is based mainly on the assessment of the cumulus oocyte complex morphology. Further research has lead to the proposal and enrichment of assessment criteria of selection such as on intrinistic (mitochondrial status, glucose-6-phosphate dehydrogenase 1 activity) and extrinistic factors (apoptosis of follicular cells or the levels of transforming of growth factor beta superfamily in follicular fluid or serum) [Wang et al., 2007] or morphological parameters (zona pellucida imaging) obtained by light or polarized light microscopy. During sperm selection motile spermatozoa separation are separated from the semen, mucus and other immotile or non-viable spermatozoa, using a technique called sperm

washing. This is performed by density gradient centrifugation or a direct swim up technique. [Volpes et al., 2016]

The spermatozoa and the oocyte are incubated together in a culture media at a rate of about 75000:1 (day 0). The incubation time preferred for better outcome is 1-4 hours [Zhang et al., 2013]. If fertilization occurs two nuclei will be visible at the fertilized oocyte following 16-18 hours post incubation(day 1). The fertilized oocyte then is transferred to the appropriate growth medium. Embryos grow in culture until cleavage stage (days 2-4) or until blastocyst stage (days 5-6). During the cleavage (except day 4) or blastocyst stage embryos are available either for embryo transfer or cryopreservation. The embryo qualified for embryo transfer is evaluated using mainly a morphological scoring system. Morphological parameters are acquired through microscopy at certain times or time-lapse microscopy. Embryos could also be evaluated through Preimplantation Genetic Diagnosis (PGD). Embryos may be biopsied for PGD during 3 stages (polar body {preconception Diagnosis}, cleavage stage and blastocyst stage). [Geraedts & De Wert, 2009]

In case of severe male infertility Intracytoplasmic Sperm Injection (ICSI) is preferred instead of the standard IVF. First human birth using ICSI was reported in 1992 [Palermo et al., 1992] . A complete absence of spermatozoa occurs in 10-15% of infertile men. This condition is named azoospermia and is categorized into obstructive and non-obstructive. In case of obstructive azoospermia sperm retrieval occurs with Percutaneous or Microscopic Epididymal Sperm Aspirations (PESA/MESA). The non-obstructed condition requires a more inasive procedure called Testicular Sperm Extraction (TESE) and micro-TESE. [Esteves, 2013]

The procedure requires the deposition of a single spermatozoon into the cytoplasm of an oocyte at MII stage of metaphasis. The oocyte is held with the aid of a holding pipette while the injection pipette penetrates the zona pellucida and the single spermatozoon is released into the cytoplasm [Palermo et al., 1992]. In order to avoid damage to the meiotic spindle (that may lead to genetic abnormalities) the polar body is rotated at the 11o clock position during sperm injection. ICSI is usually performed at 4 hours after oocyte retrieval [Simopoulou et al., 2016]. The fertilized oocyte is then transferred to another medium similar to the classic IVF process.

Growth Hormone

Growth hormone is secreted by somatotrophs in the anterior pituitary, is a part of the somatotropin prolactin family of hormones and plays a major role in growth regulation. GH is coded by a single gene (GH1), related with 4 other genes, and is located on chromosome 17 where all 5 genes are interspersed in the same transcriptional orientation. Those genes share a remarkable degree of sequence identity and form a gene cluster, the growth hormone locus, which is thought to have evolved from continuous duplications. Alternative splicing of the mRNA coded by

those genes results to different isomorphs of the 5 growth hormones. The GH1 is expressed in the pituitary but not in the placental tissue in contrast to the 4 other genes of the growth hormone locus.

Like other hormones GH is synthesized as a larger prehormone. The prehormone is processed by the endoplasmic reticulum and the Golgi system, resulting to the removal of several small peptides. The predominant form of GH is a 22-kDA polypeptide with two sulfhydryl bonds.

Growth hormone secretion is regulated by growth hormone–releasing hormone and somatostatin and ghrelin. GH is secreted in pulses, throughout the day mostly during the first hours of sleep. The coordination of GH secretion by the somatotrophs during a secretory pulse presumably occurs in response to both positive and negative hypothalamic control signals.

Growth Hormone Releasing Hormone Small diameter neurons in the arcuate nucleus of the hypothalamus secrete GH-releasing hormone (GHRH), a 43 amino acid peptide that reaches the somatotrophs in the anterior pituitary. This neuropeptide promotes GH secretion by the somatotrophs. GHRH is made principally in the hypothalamus, but it can also be found in neuroectodermal tissue outside the Central Nervous System.

The hypothalamus also synthesizes Somatostatin, a 14 amino acid neuropeptide. Somatostatin is produced in the periventricular region of the hypothalamus and is secreted into the hypophyseal portal blood. It is a potent inhibitor of GH secretion. Somatostatin is also produced elsewhere in the brain and in selected tissues outside the Central Nervous System. The primary regulation of GH secretion is stimulatory, because sectioning the pituitary stalk, thereby interrupting the portal blood flow from the hypothalamus to the pituitary, leads to a decline in GH secretion. It also appears that the pulses of GH secretion are entrained by the pulsatile secretion of GHRH.

Ghrelin is a 28 amino acids hormone. It is released by distinct endocrine cells within the mucosal layer of the stomach in response to fasting. Endocrine cells of the gastrointestinal tract also produce ghrelin, although the highest ghrelin concentrations are found in the fundus of the stomach. The arcuate nucleus of the hypothalamus also makes small amounts of ghrelin. Infusion of ghrelin either into the bloodstream or into the cerebral ventricles markedly increases growth hormone secretion.

GH has anabolic, lipolytic and antinatriuretic action. GH deficiency in children results to a reduced growth rate according to growth charts. Children with reduced GH levels may suffer from pituitary dwarfism. GH deficiency in adults may result to reduced lean body mass, reduced extracellular water and higher fat mass respectively [Carroll et al., 1998]. It may also result to psychological problems (depression, anxiety, lack of well-being), muscular system dysfunctions (fatigue, lack of strength, fibromyalgia syndrome and neuromuscular dysfunction), cardiovascular abnormalities (impaired cardiac function, increased low-density lipoprotein, prothrombotic state, accelerated

atherogenesis), decreased insulin sensitivity and decreased sweating and thermoregulation [Gupta, 2011]. Growth hormone has been associated with fertility as GH is required for timing the onset of puberty and the induction of sexual maturation both in males and females. Moreover GH modulates gonadotropin secretion and regulates the growth and actions of secondary sexual tissues, including the activation of uterus in females and the prostate and seminal vesicles in males [Hull & Harvey, 2014].

Excessive GH secretion during childhood is the cause of gigantism development. An excess of GH after puberty results in the clinical syndrome of acromegaly. This condition is characterized by the growth of bone and many other somatic tissues, including skin, muscle, heart, liver, and the gastrointestinal tract. The lengthening of long bones is not part of the syndrome because the epiphyseal growth plates close at the end of puberty. Thus, acromegaly causes progressive thickening of bones and soft tissues of the head, hands, feet, and other parts of the body. If untreated, these somatic changes cause significant morbidity and shorten life as a result of joint deformity, hypertension, pulmonary insufficiency, and heart failure.

IGF-1

Insulin-like Growth Factor 1 (IGF-1) is similar to insulin in function and structure and is a member of a family of proteins involved in mediating growth and development. The encoded protein is processed from a precursor, bound by a specific receptor, and secreted. The gene coding this protein is the IGF1 gene and is located at the q arm of chromosome 12 at position 23.2. The human IGF-1 gene consists of six exons, including two leader exons, and has two promoters. IGF-1 precursor protein includes different isoforms (IGF-1Ea, IGF-1Eb, IGF-1Ec or MGF) which undergo post-translational modification [Philippou et al., 2007]. IGF-1 is a small peptide containing 70 amino acids with a molecular weight of 7649 Da.

The production of IGF-1 is induced by GH in liver and its paracrine production is also regulated by GH. IGF-1 acts as a suppressor of GH secretion. An increase in the circulating concentration of IGF- 1 suppresses GH secretion through both direct and indirect mechanisms. IGF-1 exerts a direct action on the pituitary to suppress GH secretion by the somatotrophs. IGF-1 also appears to suppress GHRH release in the hypothalamus and to increase somatostatin secretion.

IGF-1 is a hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults. IGF-1 is produced throughout life. The highest rates of IGF-1 production occur during the pubertal growth spurt.

Its primary action is mediated by binding to its specific receptor, the insulin-like growth factor 1 receptor (IGF1R), which is present on many cell types in many tissues. Binding to the IGF1R, a receptor tyrosine kinase, initiates intracellular signaling. IGF-1 binds to at least two cell surface receptors: the IGF-1 receptor (IGF1R), and the insulin receptor. The IGF1R binds

IGF-1 at significantly higher affinity than the insulin receptor. Like the insulin receptor, the IGF1R receptor is a tyrosine kinase receptor.

IGF-1 binds with Insulin-like Growth Factor Binding Proteins (IGFBPs) with high affinity. IGFBPs block IGF-1 ability to activate IGF1R. IGF affinity for IGFBPs can be decreased with limited proteolysis. IGFBPs may bind to specific IGFBP receptors (IGFBPR) and then internalize into the cell or transmit inhibitory signal from cell surface [Baxter, 2014].

IGF-1 deficiency has been linked with growth retardation. Laron syndrome is an example of IGF-1 deficiency in children and results to dwarfism, retarded skeletal maturation and organ growth, osteopenia and muscle underdevelopment. IGF-1 deficiency has been associated with an number of other conditions, like chronic liver disease, cardiovascular diseases including reduced heart contractibility, angiotensin II sensitivity and altered expression of genes coding proteins required for proper cardiac structure and function [Gonzalez-Guerra et al., 2017]. Metabolic syndrome, neurodegenerative diseases, musculoskeletal disorders, renal diseases and catabolic syndrome are also linked with IGF-1 deficiency [Puche et al., 2012].

Furthermore IGF-1 deficiency is associated with intrauterine growth restriction implying the major role of IGF-1 in normal fetal and placental growth and differentiation. Intrauterine growth restriction (IUGR) is the second most frequent cause of perinatal morbidity and mortality, defined as the inability to achieve the expected weight for gestational age. [Martin-Estal et al., 2016].

In testicular tissues the production of IGF-1 is independent of GH [Richards et al., 2002] and is localized in spermatogenic and Leydig cells [Yoon et al., 2011]. The stimulation of IGF-1 in reproductive tissues and cells is yet a controversial issue. IGF-1 in human males is thought to be stimulated by gonadotropins [Grizard, 1994] and more specifically FSH and testosterone [Itoh et al., 1994]. Similar findings exist in fish [Sambroni et al., 2013]. In granulosa cells of mice females IGF-1 seems to regulate FSH [Zhou et al., 1997]. Gonadotropin axis hormone stimulates the somatotropic hormone axis to produce pubertal growth spurt [Rogol, 2004]. IGF-1 and FSH act synergistically in regulating FSHR [Minegishi et al., 2000] AKT and steroidogenic genes [Zhou et al., 2013] in rat granulosa cells. IGF-1 regulates FOXO1 which downregulates production of gonadotropins [Skarra et al., 2015].

Association between Male Infertility, IGF-1 and GH

There is wide controversy about the roles of Growth Hormone and Insulin-like Growth Factor 1 in male fertility. Although both these hormones regulate human growth and secondary sexual tissues, it is yet uncertain if they have a direct effect on fertility.

GH has been identified as a potential treatment for infertility. Research has shown that GH improves semen volume, count and motility [Kalra et al., 2008]. GH improved motility, but not sperm count, in asthenozoospermic and oligozoospermic men, with reported pregnancies in the asthenozoospermic group but not in the oligozoospermic

group [Ovesen et al., 1996]. Recombinant-hGH was found to increase sperm concentration and motility in half of the male subjects with idiopathic oligozoospermia [Radicioni et al., 1994].

On the other hand GH treatment did not increase sperm count on men with severe idiopathic oligozoospermia [Lee et al., 1995]. Moreover male Growth Hormone Receptor knock-out dwarf chickens had no difference in fertility in comparison with normal ones [Zheng et al., 2007]. Male rats with GHR-ko abolished only part of their fertility (14 out of 19 were fertile in comparison to 21 out of 22 in the control group) [Chandrashekar et al., 1999].

IGF-1 deficiency is found to have an effect on sperm count [Colombo et al., 1999]. A more recent study found correlation between IGF-1 serum levels and low sperm concentration but not with seminal plasma levels of IGF-1 [Lee et al., 2016]. IGF-1 receptor was absent from seminal plasma from patients with a history of failed fertilization, though IGF1R was present both in fertile and infertile men [Sanchez-Luego, 2005]. IGF-1 levels in seminal plasma from patients with varicocele (common cause of male infertility) was significantly different from that following varicocelectomy and from that of control group, though IGF-1 was not correlated with semen quality [Naderi et al., 2015]. Transgenic mice overexpressing IGFBP-1 showed and alteration of spermatogenesis resulting in lower production and quality of sperm [Froment et al., 2004]. However, a research in stallions on the other hand showed no direct association between IGF-1 and infertility or subfertility [Hess & Rosser, 2001].

MATERIALS AND METHODS

Sample Collection

The study was carried out between March 2017 and September 2017. Sample collection and sperm analysis was performed at Genesis Athens Clinic. Samples were then transferred to Laboratory of Physiology at Medical School of National and Kapodistrian University of Athens. Participants were between 18 and 55 years old. All patients were asked to sign the consent form in order to participate in this study. The consent form and the study were approved by Greek Authority for Personal Data Protection. The samples were collected either in a private room adjacent to the laboratory or transferred to the laboratory within 30 minutes following abstinence of 2 to 5 days.). Sperm analysis was performed at the same day of collection and the samples were then centrifuged twice, once at 2830 rpm (1000g) for 10 minutes and once at 6560rpm (4000g) for 20 minutes. Samples were then stored at -80℃ until ELISA tests were performed.

Seminogram

Seminogram is a diagnostic test used for the assessment of male fertility. Sample is collected in a specially designed room near the laboratory if possible or has to be transferred to the laboratory within 30 min following collection. The parameters evaluated are seminal volume, sperm count, sperm concentration, total motility, progressive motility, morphology, pH and liquefaction.

Sperm count, concentration, total motility, progressive motility and morphology are evaluated using a Mackler counting chamber under a microscope. The counting cambers is constructed from two pieces of optically flat glass, the upper layer serves as a cover glass, with a 1 sq.mm fine grid in the center subdivided into 100 squares of 0.1 x 0.1 mm each.

5 μL of the sample are transferred to the counting chamber. Spermatozoa counted in 10 squares represent the sperm concentration of the sample in millions. Non-motile sperm are counted within an area of nine or sixteen squares in the center of the grid. Moving sperms are then counted and graded. The procedure is repeated in several areas.

Grade a: These are the strongest and swim fast in a straight line.

Grade b: These also move forward but tend to travel in a curved or crooked motion.

Grade c: These have non-progressive motility because they do not move forward despite the fact that they move their tails.

Grade d: These are immotile and fail to move at all.

Grade a and b spermatozoa represent the progressive motility percentage. Total motility is grade a, b and c.

The same method with motility evaluation is used for morphology evaluation but under a light microscope under higher magnification.

According to the results of the seminogram, if abnormal findings occur, one of the following conditions may be diagnosed:

Condition	Description
Oligospermia	Decreased Number of Spermatozoa
Asthenospermia	Decreased Motile Spermatozoa (either progressive motility or total motility)
Teratospermia	Decreased amount of morpohologically normal Spermatozoa
Azoospermia	Absence of spermatozoa but presence of semen

The above conditions may be combined. I.e. oligoasthenospermia means that the patient has both decreased motility and decreased number of spermatozoa.

In case of abnormal findings a second seminogram following 75 days is suggested for improved reliability.

DNA Fragmentation Test

Sperm DNA fragmentation test evaluates the integrity of sperm DNA which is essential for the accurate transmission of genetic information [Agarwal et al., 2003]. DNA Fragmentation rate has been negatively correlated with sperm morphology [Keshteli et al., 2016] and sperm concentration [Irvine et al., 2000]. DNA Fragmentation is also correlated with possible miscarriages [Robinson et al., 2012].

Sperm DNA fragmentation is evaluated via many possible techniques. The most commonly employed laboratory techniques are: single cell gel electrophoresis (COMET assay), Sperm Chromatin Structure Assay (SCSA), In Situ Nick Translation (NT: Nick Translation), Sperm Chromatin Dispersion Test (SCD) and Terminal Uridine Nick-End Labelling (TUNEL assay).

ELISA

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. ELISA is being used both for diagnostic and research

purposes. As an analytic biochemistry assay, ELISA involves detection of an analyte (the specific substance whose presence is being quantitatively analyzed) in a liquid sample by a method that continues to use liquid reagents during the analysis. ELISA is performed in specific plates containing 96 wells used for standards, controls and samples. The main detection system for ELISA is colorimetric, using a spectrophotometer, but chemiluminescent and fluorescent detection systems have been developed. There are 4 different types of ELISA: direct, indirect, sandwich and competitive ELISA.

For the assessment of GH levels protocol of sandwich ELISA was used (R&D hGH ELISA kit) and for IGF-1 levels protocol of competitive ELISA was used (Origene IGF-1 ELISA kit).

Sandwich ELISA

The sandwich ELISA quantifies antigens between two layers of antibodies. The capture antibody, which is highly specific for the antigen, is attached to a solid surface. The antigen is then added, followed by addition of a second antibody, the detection antibody. The detection antibody binds the antigen at a different epitope than the capture antibody. As a result, the antigen is 'sandwiched' between the two antibodies. The antibody binding affinity for the antigen is usually the main determinant of immunoassay sensitivity. As the antigen concentration increases, the amount of detection antibody increases, leading to a higher measured response. The standard curve of a sandwich-binding assay has a positive slope. To quantify the extent of binding, different reporters can be used. These reporters can be directly attached to the detection antibody or to a secondary antibody which binds the detection antibody. The substrate for the enzyme is added to the reaction that forms a colorimetric readout as the detection signal. The signal generated is proportional to the amount of target antigen present in the sample. The antibody linked reporter used to measure the binding event determines the detection mode

Competitive ELISA

The central event of competitive ELISA is a competitive binding process executed by original antigen (sample antigen) and add-in antigen. The procedures of competitive ELISA are different in some respects compared with Indirect ELISA, Sandwich ELISA and Direct ELISA.

Primary unlabeled antibody is incubated with sample antigen. Antibody-antigen complexes are then added to 96-well plates which are pre-coated with the same antigen. Unbound antibody is removed by washing the plate. The more antigen in the sample, the less antibody will be able to bind to the antigen in the well. The secondary antibody that is specific to the primary antibody and conjugated with an enzyme is

added. A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.

Statistical analysis

Normality of the distribution in the three groups (normal, asthenospermic and asthenospermic plus at least one more abnormal condition) was tested using Shapiro-Wilks test. Only normal group presented normal distributions. Results of Shapiro-Wilks test were confirmed using Qualitive-Quantitative plots (Q-Q plots). Since not all distributions were normal Wilcoxon rank-sum test (Mann-Whitney U) was preferred instead of Pearson t-test. "Descriptive statistics" was used in order to present the results of the seminograms and the levels of IGF-1 and GH. Spearman's Correlation Coefficient was used to establish statistical dependence between the rankings of two dependents (sperm parameters, levels of GH and IGF-1).

Statistical analysis was performed using R programming language via RStudio development environment interpreter adding the necessary library for Q-Q plots (fBasics). A confidence level of 95% ($p<0.05$) was used in order to establish statistical significance. $0.1>p>0.05$ is widely considered as statistical indication since statistical indication in small samples (such as the sample used in this study) could potentially become statistical significance in larger samples.

RESULTS

Participants were aged between 27 and 55 years with a mean age of 42.3 years, and had at least 3 days of abstinence (optimal abstinence days 2-5). It is important to mention that 90% of our sample had abnormal progressive motility (45 out of 50). Only 5 samples were classified without abnormal parameters in their seminogram. Out of the 50 patients, 16 requested only for a DNA fragmentation test, so there are no data of vitality or morphology based on straining (although 2 samples were checked for morphology as well). The mean values and standard deviations of sperm parameters and GH and IGF-1 levels in seminal plasma are presented in table 1.

Table 1: Mean values of sperm parameters and mean levels of IGF-1 and GH in seminal plasma

	N	Mean	Standard Deviation
Sperm Concentration	50	26 * 10^6	30*10^6
Progressive Motility	50	12.96%	11.68%
Total Motility	50	22.87%	17.75%
Vitality	34	36.91%	32.75%
Morphology	36	9.02%	8.01%
DNA Index	16	0.32	0.12
Volume	50	2.9	1.6
Seminal GH	50	31.28	30.16
Seminal IGF-1	50	40.16	16.86

Table 2: Normal and abnormal findings in each parameter

Sperm Parameters	Normal	Abnormal
Sperm Concentration	22	28
Progressive Motility	5	45
Total Motility	12	38
Vitality	10	24
Morphology	22	14
DNA Index	8	8
Volume	43	7

More detailed results are depicted in the histograms below:

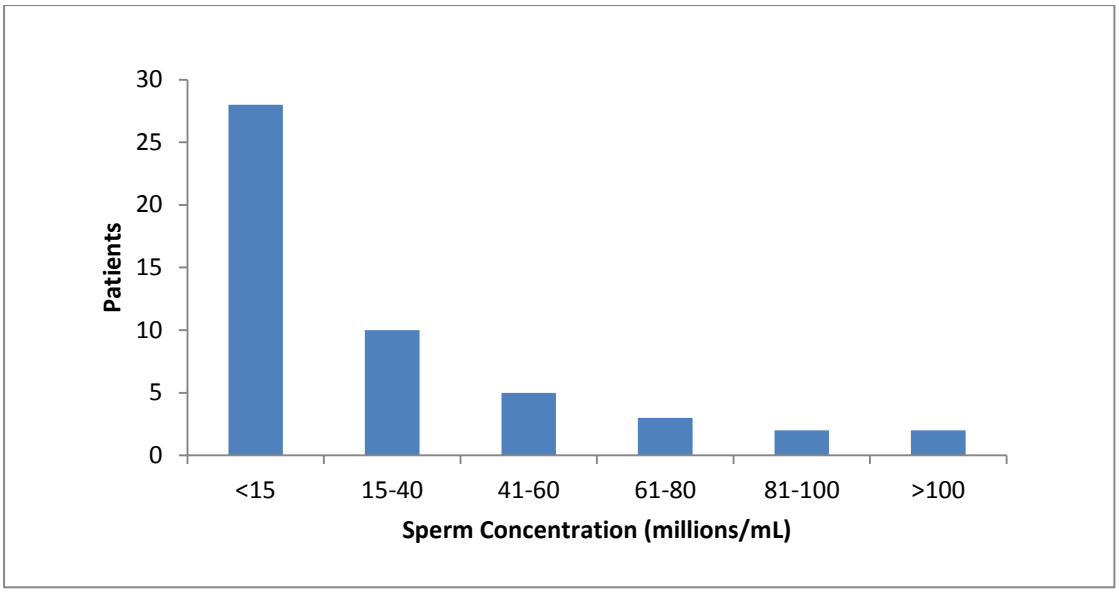

Figure 1: Histogram of sperm concentration in the total sample of participants

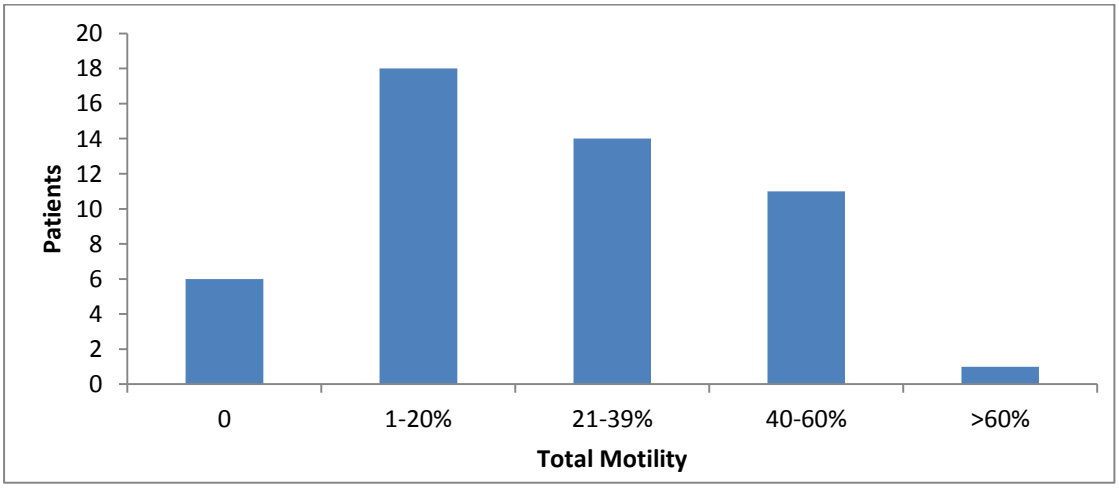

Figure 2: Histogram of Total Motility (percentage of spermatozoa with motility grades A,B and C) in the total sample of participants

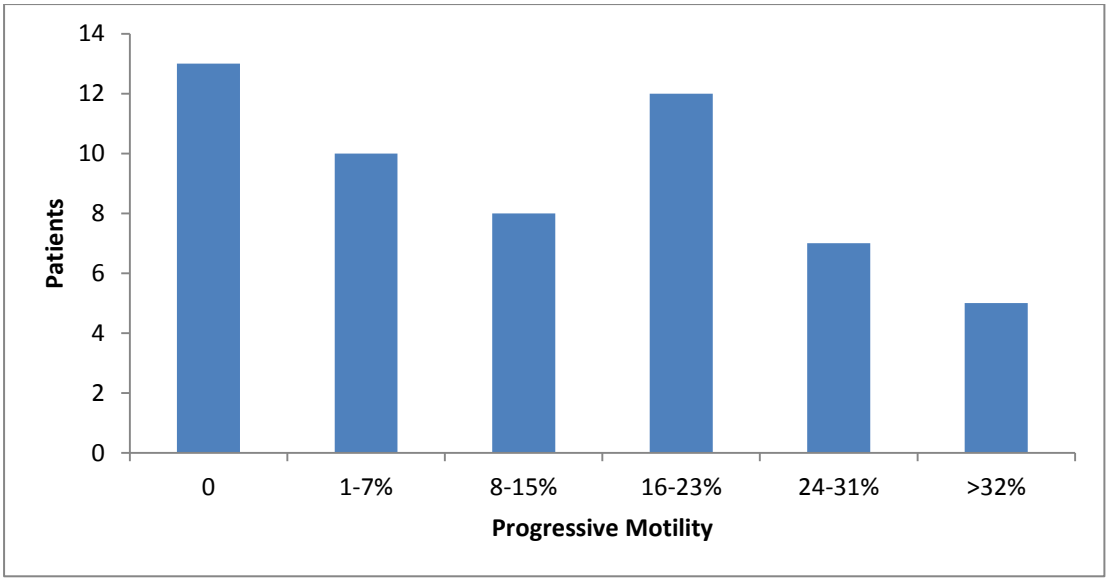

Figure 3: Histogram of Progressive Motility (Spermatozoa graded A and B) in the total sample of participants

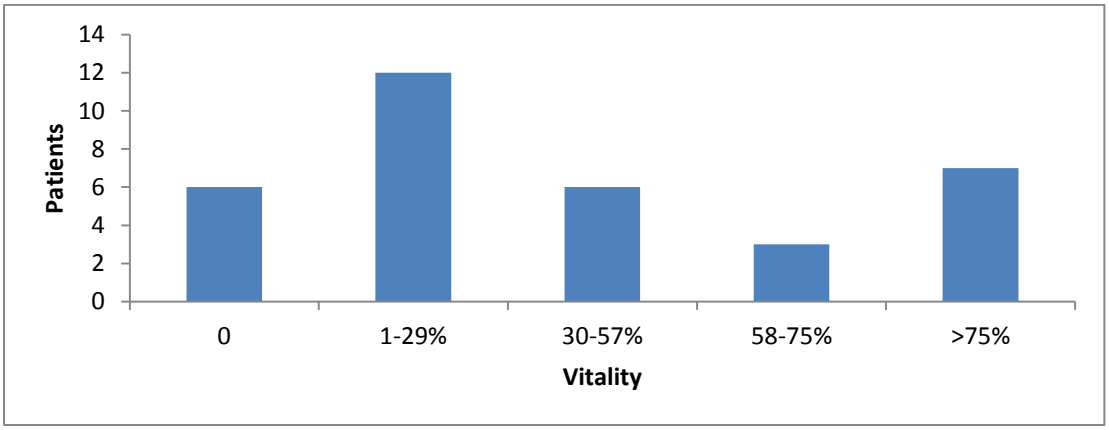

Figure 4: Histogram of Vitality in the total sample of participants (n=34)

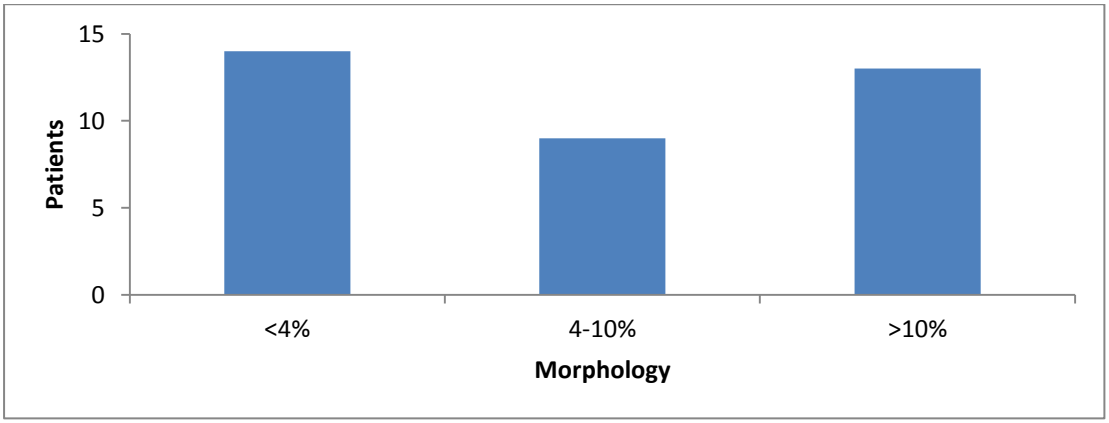

Figure 5: Histogram of percentage of morphologically normal spermatozoa per sample in the total sample of participants (n=36)

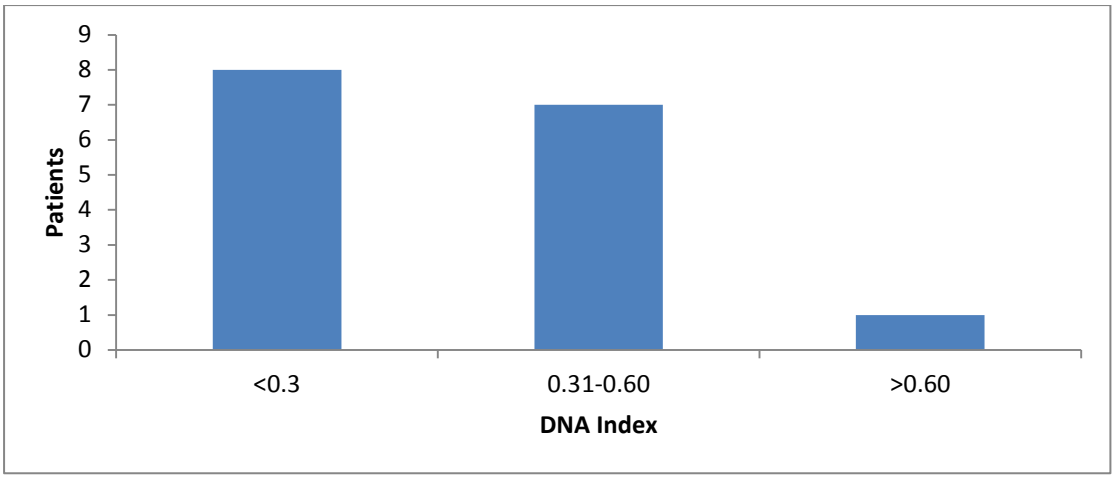

Figure 6: Histogram of DNA Index in the total sample of participants (n=16)

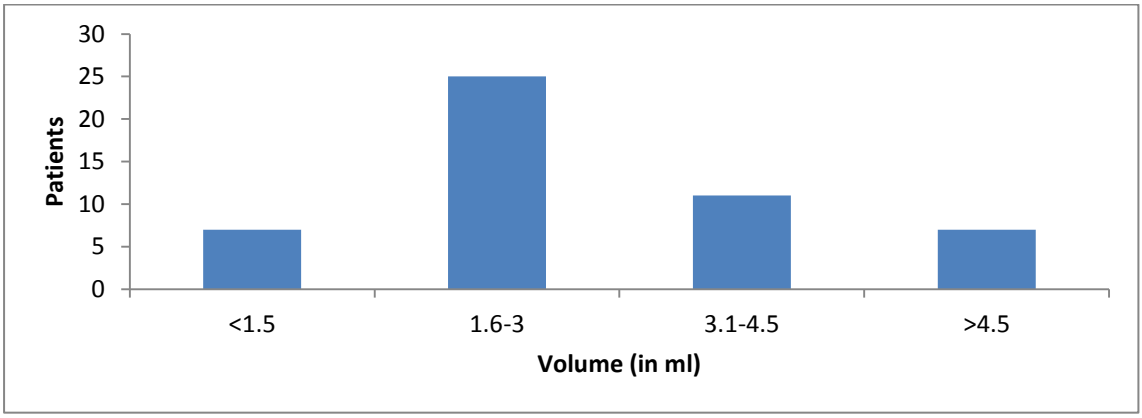

Figure 7: Histogram of sample's volume in the total sample of participants.

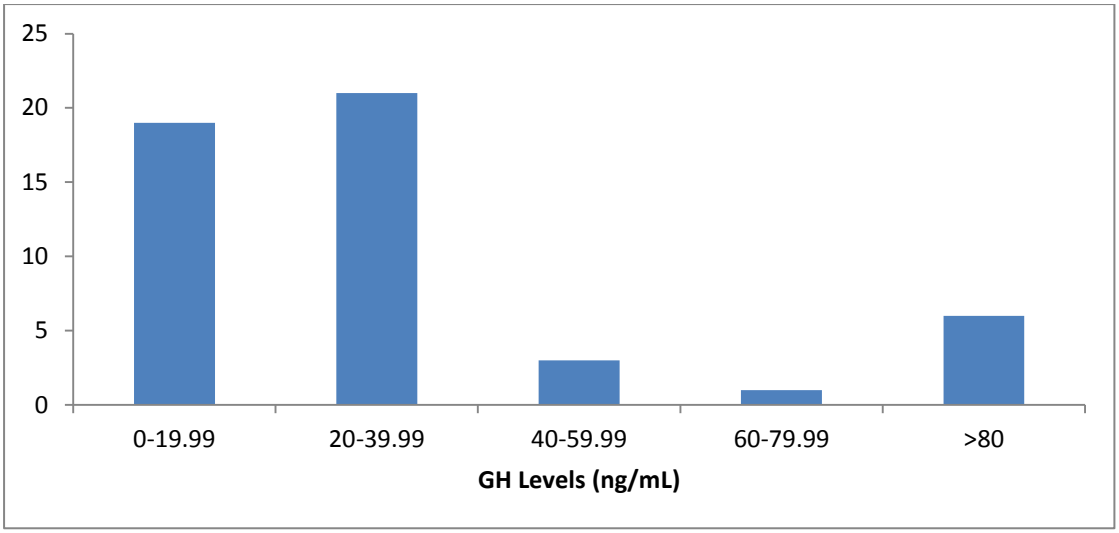

Figure 8: Histogram of GH levels in seminal plasma in the total sample of participants

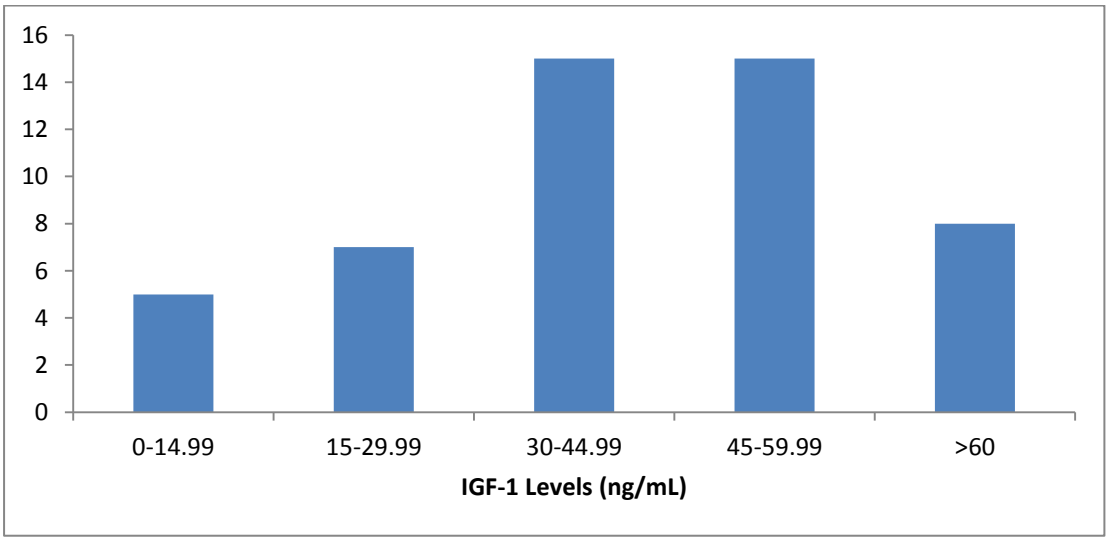

Figure 9: Histogram of IGF-1 levels in seminal plasma in the total sample of participants

Spearman's Correlation coefficient (r) was applied in the total sample of participants as well as, in subgroups defined according to the abnormal characteristicsof the sperm parameters in order to reveal any potential relationships between the hormones and the parameters examined.

Table 3: Spearman's Correlation Coefficient (r) applied in the total sample of participants

	GH	IGF-1	N
Sperm Concentration	-0.023	-0.177	50
Total Motility	-0.018	-0.091	50
Progressive Motility	-0.016	-0.078	50
Vitality	-0.029	-0.130	34
Morphology	-0.038	-0.047	36
DNA Index	0.117	0.413	16
Volume	0.039	0.166	50

Table 4: Spearman's Correlation Coefficient (r) applied in the samples diagnosed with each abnormal characteristic in each specific parameter.

	GH	IGF-1	N
Sperm Concentration	-0.224	-0.276	28
Total Motility	-0.008	-0.140	38
Progressive Motility	0.092	0.048	45
Vitality	-0.141	-0.201	24
Morphology	-0.044	-0.481	14
DNA Index	0.246	0.568	8
Volume	-0.374	-0.136	7

Table 5: Spearman's Correlation Coefficient (r) between IGF-1 and GH applied in samples diagnosed with each abnormal characteristic in each specific sperm parameter

	IGF-1 – GH correlation coefficient	N
Sperm Concentration	0.614***	28
Total Motility	0.319*	38
Progressive Motility	0.270*	45
Vitality	0.644**	24
Morphology	0.588**	14
DNA Index	0.025	8
Volume	0,783*	7

*: statistically indicative ($0.05<p<0.1$), **: statistically significant ($0.01<p<0.05$), ***: statistically significant ($0.001<p<0.01$)

Table 6: Spearman's Correlation Coefficient (r) applied in samples with normal characteristics in each sperm parameter

	GH	IGF-1	N
Sperm Concentration	-0.041	-0.030	22
Total Motility	0.190	-0.278	5
Progressive Motility	-0.4	-0.3	12
Vitality	-0.054	-0.095	10
Morphology	-0.107	-0.304	22
DNA Index	-0.623	-0.116	8
Volume	0.266*	0.010	43

Table 7: Spearman's Correlation Coefficient (r) between IGF-1 and GH applied in samples with normal characteristics in each sperm parameter

	IGF-1 – GH correlation coefficient	N
Sperm Concentration	-0.141	22
Total Motility	0.136	5
Progressive Motility	0.5	12
Vitality	-0.057	10
Morphology	0.266	22
DNA Index	0.371	8
Volume	0.117	43

Due to the presence of asthenospermia in all patients with abnormal parameters, patients were further divided into two groups, with respect to motility, normal, asthenospermic and asthenopermic plus at least one more abnormal parameter. Mean levels of seminal GH, IGF-1 and their deviations are presented in table 8.

	Normal (n=5)		Asthenospermic (n=12)		Asthenospermic plus more abnormal parameters (n=33)	
	Mean	Sd	Mean	Sd	Mean	Sd
GH	28.16	8.74	23.37 *	12.93	34.18	37.71
IGF-1	36.90	15.31	39.99	18.56	40.94	16.15

*: statistic indication (0.1>p>0.05) using Wilcoxon test (p=0.063)

Table 9: Spearman's Correlation Coefficient (r) between IGF-1 and GH in each group

	IGF-1 and GH correlation		
	Normal (n=5)	Asthenospermic (n=12)	Asthenospermia plus at least on more abnormal finding (n=33)
Coefficient	0.5	-0.133	0.366**

**: statistically significant (0.01>p>0.05).

DISCUSSION

Infertility is a common disorder affecting 7% of the male population worldwide [Lotti & Maggi, 2015]. It is a multi-factor disorder that has been the topic of many studies. Common aims of those studies were to either investigate possible new biomarkers and/or causes of infertility, , or to test new therapeutic treatments. The purpose of the present study was to investigate possible associations between GH, IGF-1 and male infertility.

In this study, a marginally non-significant difference between asthenospermic (group B) and normal group was revealed, while group C (i.e., asthenospermia plus at least one more abnormal finding) presented no statistically significant difference in comparison to any other group. The lack of statistical significance in the differences between normal group and group C may be due to the fact that 4 samples in the latter group showed extremely high levels of GH, increasing the inter-individual variability. Thus, it could be speculated that this variability might have been smoothened if the total number of participants was larger and potentially might reveal significant differences between the normal group and group C.

The correlation found between GH and sperm motility is in accordance with the literature. Specifically, GH has been shown to improve motility, but not sperm concentration, in asthenospermic and oligospermic men, with pregnancies reported in the asthenospermic group but not in the oligospermic group [Ovesen et al., 1996]. In addition, treatment with recombinant-hGH was found to increase motility in half of the male subjects with idiopathic oligospermia [Radicioni et al., 1994].

In addition, GH treatment has been found to raise seminal volume in oligospermic patients [Kalra et al., 2008] but to our knowledge no study has been conducted for oligo-astheno-teratozoospermic patients. In our study, a correlation between GH and seminal volume in samples with normal-ranged volume marginally failed to reach statistical significance, while such correlation was not established neither in patients with low seminal volume nor at aggregation of both groups. The reason for that may be the fact that all patients with low seminal volume had abnormalities in all other sperm parameters examined.

GH has been proposed as a novel therapy for both male and female infertility with promising results [Magon et al., 2011]. Especially in females, GH has been used to enhance ovarian stimulation in poor responders during IVF/ICSI cycles. Administration of GH increases the number of MII oocytes retrieved and the number

of embryos obtained [Yu et al., 2015]. Moreover, administration of low-dose GH along with standard gonadotropin protocol improves embryo quality and pregnancy rates in poor responders [Lattes et al., 2015]. Ovarian stimulation associated with GH has been proposed for poor responders with history of assisted reproduction failures [Hazout et al., 2009]. Co-administration of GH and aspirin, along with the GnRH antagonist protocol, has been found to increase the rates of retrieved oocytes, promote the maturation of oocytes and improve the fertilization rate in IVF [Guan et al., 2007].

However, other studies have reported controversial results.GH co-administration along with GnRH antagonist protocol did not increase pregnancy rates, although it did increase the number of retrieved oocytes [Eftekhar et al., 2013; Kucuk et al., 2008]. Another study contradicted the above-mentioned results, concluding that GH supplements improved pregnancy and live birth rates but did not improve oocyte retrieval [de Ziegler et al., 2011].

According to the evidences of the present study and the literature reports, GH plays a role in the physiology of reproduction. However, further research is required in order to investigate which subgroups of infertile men and women may be benefited by its use as therapeutic intervention, and at which dose it will be beneficial. Nevertheless, reduced fertility was found in transgenic mice overexpressing GH [Bartke et al., 1999]. More specifically, in Bartke's study [1999], both GH-resistant (GHR-KO) and GH-overexpressing mice had reduced fertility, interestingly however it was concluded that GHR-KO mice subfertility was mild in comparison to IGF-1-KO. Thus, overall the study suggested that IGF-1 production in reproductive tissues is independent of GH. This is in agreement with the findings of other research groups that concluded that IGF-1 production in testicular tissues is stimulated by gonadotropins [Grizard, 1994] and more specifically by FSH and testosterone [Itoh et al., 1994].

In our study, though, a strong positive correlation was found between IGF-1 and GH in patients with low sperm concentration, with low vitality and abnormal morphology spermatozoa, as well as a marginally significant correlation in patients with low progressive, total motility and low volume. Such correlations do not exist in the total sample of participants or in healthy groups for each of the above aspects. The indicative correlation between GH and IGF-1 in patients with low motility (progressive and or total) may be a false positive indication, since all our patients suffered from asthenospermia. A further analysis after the subdivision of samples in normal, asthenospermic and asthenospermic plus at least one more abnormality (group C) showed asignificant correlation between GH and IGF-1 in patients with asthenospermia and at least one more abnormal condition, however there was not statistically significant correlation between GH and IGF-1 in asthenospermic patients. ($p<0.05$).

To the best of our knowledge, there are no studies correlating GH and IGF-1 in seminal plasma or male reproductive tissues in general. The fact that this correlation occurred only in subfertile patients requires further research, in order to investigate and delineate a possible biological significance of this relationship and to characterize the nature and mechanisms of this association. This could be a novel research topic and its conclusions might contribute to the explanation of the controversial results reported in the literature and, subsequently, to the better understanding of male infertility.

Seminal IGF-1 levels showed no statistically significant association with any of the sperm parameters, which is in accordance with another recent study [Lee et al., 2016]. However, in that study there were statistically significant associations between serum IGF-1 levels and male infertility. Another study in stallions showed no direct association between IGF-1 and infertility or subfertility [Hess & Rosser, 2001]. These results contradicted the findings of a previous study that found statistically significant differences in seminal IGF-1 levels in patients with low sperm concentration compared with normal subjects [Colombo et al., 1999]. The reason of these conflicting results could be the role of IGF binding proteins (IGFBPs) in the regulation of the IGF-1 bioavailability, or the absence of IGF-1 receptor [Sanchez-Luengo et al., 2005]. IGF-1 levels in seminal plasma derived from patients with varicocele (common cause of male infertility) were significantly different compared with that following varicocelectomy and that of control group, though IGF-1 levels were not correlated with semen quality [Naderi et al., 2015]. Transgenic mice overexpressing IGFBP-1 showed alterations in spermatogenesis, resulting in lower production and quality of sperm [Froment et al., 2004].

IGF-1 has been investigated in the scope of female infertility, embryo growth and Assisted Reproduction Techniques (ART) as well. IGF-1 and IGFBP-3 serum levels increased during ovarian stimulation in females with polycystic ovaries syndrome, undergoing GnRH antagonist protocols without the supplement of GH.. Patients whose IGF-1 levels decreased from day 3 to day of hCG had a higher number of oocytes retrieved, but less mature oocytes than patients whose IGF-1 levels were elevated. Moreover, an increase in IGFBP-3 levels during stimulation was correlated with a higher number of pregnancies [Dragisic et al., 2005]. Levels of IGF-1 in follicular fluid were found to be directly correlated with embryo quality and pregnancy outcome [Mehta et al., 2013]. This is in agreement with a recent study that concluded that follicular fluid level of IGF-1 may have a predictive value in IVF outcome, since IGF-1 levels were positively correlated with higher pregnancy rates [Faraj et al., 2017].

IGF-1 deficiency is associated with intrauterine growth restriction, implying the major role of IGF-1 in normal fetal and placental growth and differentiation. Intrauterine growth restriction (IUGR) is the second most frequent cause of perinatal morbidity

and mortality, defined as the inability to achieve the expected weight for gestational age. [Martin-Estal et al., 2016]. IGF-1 levels were higher to women with a live birth in comparison to women with spontaneous abortion, ectopic pregnancy or them who were not pregnant. That study concluded that maternal serum levels of IGF-1 and IGFBP-1 were positively associated with the likelihood of a live birth, whereas levels of IGF-2 were negatively correlated with live birth [Ramer et al., 2015].

To conclude, IGF-1 and GH appear to have a pivotal role both in the physiology of reproduction and in assisted reproduction. In our study, a possible association between GH and asthenospermia was revealed. IGF-1 seminal levels had no significant association with the sperm parameters examined. The novel finding of this study is the significant correlation between IGF-1 and GH in patients with asthenospermia and at least one more abnormal sperm parameter. This finding requires further investigation in order to establish a possible biological significance, the functionality of this association and possibly a better understanding of male infertility.

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