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"STUDY OF THE EXPRESSION PATTERN OF IGF-1 ISOFORMS IN HUMAN SEMINAL PLASMA"

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ABSTRACT

INTRODUCTION: Nowadays an increased trend of inability of achieving a pregnancy has been observed, with the underlying etiology of infertility to be unclear. This may hinder the optimal management of infertile couples. Albeit the great progress that has been observed, the complex male physiology along with the functioning mechanism of their reproductive system are complex and require intensive and continuous research. Insulin-like growth factor I (IGF-I) has been suggested as a factor that may play a pivotal role in male infertility due to its anabolic effect and differential expression pattern of its three isoforms, as it has been observed in many other pathological situations.

PURPOSE: The aim of the present study is to investigate the occurrence of differential expression of the three IGF-I isoforms between men presenting with abnormal semen parameters in semen analysis and normozoospermic males, as well as a possible association between a different expression pattern of the IGF-I isoforms and male infertility.

METHODS: Forty semen samples from men aged from 32 to 54 years old (43.9 ± 4.6) were collected for the present study. Each sample was evaluated for certain parameters according to the World Health Organization criteria. Written informed consent was obtained from all participants. Four study groups were identified according to the number of defects being identified in their semen analysis: "Normozoospermic", "Single defect", "Double defect" and "Oligoasthenoteratozoospermic (OAT)". First, semen samples were diluted with phosphate-buffered saline (PBS) to $10x10^6$ spermatozoa per mL and underwent osmotic shock to eliminate the non-gamete component. Total RNA was extracted using TRItidy G^{TM} according to the manufacturer's instructions and its concentration and purity was determined using a BioSpec-nano Life Science spectrophotometer. Thereafter, complementary DNA (cDNA) synthesis was performed for each sample using Protoscript II First Strand cDNA synthesis kit. cDNA quantification was then conducted through real time polymerase chain reaction using specific primers for the three IGF-I isoforms. The mRNA level of each sample was normalized against 18s rRNA and expressed as fold change. At the same time, proteins were also isolated from spermatozoa using TRItidy G^{TM} protocol and were used alongside with seminal plasma in western blot for detection of the IGF-IEc isoform at protein level. Statistical analysis was conducted using the R statistical programming language.

RESULTS: IGF-IEc mRNA was present in all 40 samples regardless of their semen pathology, however, the Ec isoform was not detectable at protein level given the amount of protein available. Moreover, IGF-IEa mRNA was not expressed except for a single individual where it appeared to be related to the subject's temperament. Two out of 40 samples may have expressed the IGF-IEb isoform but only sequencing analysis could confirm that. The mean mRNA levels of IGF-IEc in the "Normozoospermic" group were statistically significant lower than those with "Double defect" (5.1 ± 8.5, p-value=0.0117*) and "OAT" (2.9 ± 2.3, p-value=0.0072*) and with a marginally non-significant difference with the "Single defect" group (4 ± 3.9, p-

value= 0.0254). Furthermore, a statistically negative correlation was found in the mRNA levels of IGF-IEc with three of the semen parameters: total sperm count (r=-0.3895225, p-value=0.01298), progressive motility (r =-0.4505146, p-value= 0.003532) and total motility (r =-0.3790446, p-value=0.01586).

CONCLUSIONS: This study reported for the first time the distinct expression of the IGF-I isoforms in spermatozoa. The aforementioned results suggest a possible correlation between IGF-IEc mRNA levels and infertile men carrying two or more sperm defects and also that in spermatozoa the function of IGF-I is mediated mainly through the IGF-IEc isoform. Furthermore, the negative correlation of IGF-IEc mRNA levels with the aforementioned parameters indicates that high levels of IGF-IEc may negatively affect spermatogenesis and maturation process of spermatozoa. However, the conduction of larger, well-designed studies regarding the influence of the locally produced IGF-I isoforms on semen quality and spermatogenesis depending on their expression pattern, are needed in order to strengthen our findings.

ΠΕΡΙΛΗΨΗ

ΕΙΣΑΓΩΓΗ: Η υπογονιμότητα επηρεάζει όλο και περισσότερα ζευγάρια στις μέρες μας, καθιστώντας αναγκαία την ανεύρεση αιτιών και την εφαρμογή της καταλληλότερης θεραπείας. Η φυσιολογία των ανδρών, ωστόσο, και ο μηχανισμός του αναπαραγωγικού τους συστήματος είναι πολύπλοκοι και απαιτούν εντατική και συνεχή έρευνα. Ο ινσουλινο-μιμητικός αυξητικός παράγοντας 1 (insulin-like growth factor – I, IGF-I) έχει προταθεί ως ένας παράγοντας που μπορεί να διαδραματίσει κεντρικό ρόλο στην ανδρική υπογονιμότητα λόγω της δράσης του στον αναβολισμό και του διαφορικού τρόπου έκφρασης των τριών ισομορφών του σε πολλές άλλες παθολογικές καταστάσεις.

ΣΚΟΠΟΣ: Σκοπός της παρούσας μελέτης είναι η διερεύνηση της ενδεχόμενης ύπαρξης διαφορικής έκφρασης των τριών ισομορφών του IGF-I μεταξύ νορμοζωοσπερμικών ανδρών και εκείνων με μη φυσιολογικές παραμέτρους σπέρματος, καθώς και η πιθανή συσχέτιση ενός διαφορετικού προτύπου έκφρασης των ισομορφών με την ανδρική υπογονιμότητα.

ΜΕΘΟΔΟΛΟΓΙΑ: Σαράντα δείγματα σπέρματος συλλέχθηκαν από άνδρες ηλικίας 32 έως 54 ετών(43.9 ± 4.6). Κάθε δείγμα σπέρματος εκτιμήθηκε για την ποιότητά του σύμφωνα τα κριτήρια του Παγκόσμιου Οργανισμού Υγείας. Όλοι οι συμμετέχοντες έδωσαν την έντυπη συγκατάθεσή τους. Σύμφωνα με τον αριθμό των ανώμαλων παραμέτρων που παρατηρήθηκαν στην ανάλυση σπέρματος τους, οι συμμετέχοντες κατηγοριοποιήθηκαν σε 4 ομάδες μελέτης: «Normozoospermic», «Single defect», «Double defect» και «Oligoasthenoteratozoospermic (OAT)». Αρχικά, τα δείγματα αραιώθηκαν με PBS σε τελική συγκέντρωση $10x10^6$ σπερματοζωάρια ανά mL και υποβλήθηκαν σε οσμωτικό σοκ για την εξάλειψη των μη γαμετικών κυττάρων. Το ολικό RNA απομονώθηκε από τα σπερματοζωάρια με το πρωτόκολλο TRItidy G^{TM} σύμφωνα με τις οδηγίες του κατασκευαστή. Η συγκέντρωση και η καθαρότητα του RNA προσδιορίστηκαν χρησιμοποιώντας φασματοφωτόμετρο. Ακολούθησε η σύνθεση του συμπληρωματικού DNA (cDNA) για κάθε δείγμα με το Protoscript II First Strand cDNA synthesis kit. Η ποσοτικοποίηση του cDNA στη συνέχεια διεξήχθη μέσω αλυσιδωτής αντίδρασης πολυμεράσης σε πραγματικό χρόνο (q-PCR) χρησιμοποιώντας ειδικούς εκκινητές για τις τρεις ισομορφές του IGF-I και το18s rRNA ως γονίδιο αναφοράς. Ταυτόχρονα, οι πρωτεΐνες των σπερματοζωαρίων απομονώθηκαν με το πρωτόκολλο TRItidy G^{TM} και χρησιμοποιήθηκαν παράλληλα με το σπερματικό πλάσμα για την ανίχνευση της ισόμορφης IGF-IEc σε πρωτεϊνικό επίπεδο μέσω της τεχνικής Western Blot. Η στατιστική ανάλυση των παραπάνω δεδομένων διεξήχθη στο υπολογιστικό περιβάλλον της R.

ΑΠΟΤΕΛΕΣΜΑΤΑ: Το IGF-IEc mRNA ανιχνεύθηκε και στα 40 δείγματα της μελέτης ανεξάρτητα από την παθολογία του σπέρματός τους, ωστόσο, η ισομορφή IGF-IEc δεν ήταν ανιχνεύσιμη σε επίπεδο πρωτεΐνης, ενδεχομένως λόγω της μη επαρκούς ποσότητας πρωτεΐνης που απομονώθηκε και χρησιμοποιήθηκε κατά την ανάλυση ανοσοαποτύπωσης (western blot). Επιπλέον, το mRNA της ισομορφής IGF-IEa ανιχνεύθηκε σε ένα μεμονωμένο άτομο. Δύο από τα 40 δείγματα φάνηκε να

εκφράζουν την ισομορφή IGF-IEb, αλλά μόνο η αλληλούχιση του προϊόντος της PCR θα μπορούσε να το επιβεβαιώσει. Ακόμα, τα επίπεδα mRNA του IGF-IEc στην ομάδα "Normozoospermic" ήταν χαμηλότερα από την ομάδα "Double defect" (5.1 ± 8.5, p-value=0.0117*) και τους "OAT" (2.9 ± 2.3, p-value=0.0072*). Επιπλέον, βρέθηκε αρνητική συσχέτιση μεταξύ των επιπέδων mRNA της ισομορφής IGF-IEc και τριών από τις παραμέτρους του σπέρματος: ολικός αριθμός σπερματοζωαρίων (r=-0.3895225, p-value=0.01298), προωθητική κινητικότητα (r=-0.4505146, p-value= 0.003532) και ολική κινητικότητα(r =-0.3790446, p-value=0.01586).

ΣΥΜΠΕΡΑΣΜΑΤΑ: Αυτή η μελέτη ανέδειξε για πρώτη φορά την έκφραση των ισομορφών του IGF-I σε επίπεδο mRNA σε σπερματοζωάρια. Ειδικότερα, τα ευρήματα της μελέτης υποδηλώνουν πιθανή συσχέτιση των επιπέδων mRNA του IGF-IEc με την ανδρική υπογονιμότητα, οφειλόμενη σε 2 ή περισσότερες ανωμαλίες στις παραμέτρους του σπέρματος, και φαίνεται ότι στα σπερματοζωάρια οι δράσεις του IGF-I διαμεσολαβούνται κυρίως από την ισομορφή IGF-IEc. Επιπλέον, η αρνητική συσχέτιση των επιπέδων mRNA του IGF-IEc με τον ολικό αριθμό σπερματοζωαρίων, την προωθητική και την ολική κινητικότητα τους υποδεικνύει ότι τα υψηλά επίπεδα της ισομορφής IGF-IEc επηρεάζουν αρνητικά τη σπερματογένεση και τη διαδικασία ωρίμανσης των σπερματοζωαρίων. Ωστόσο, περαιτέρω μελέτες με μεγαλύτερο μέγεθος δείγματος απαιτούνται για να εξακριβωθεί το πρότυπο έκφρασης των ισομορφών και η επίδραση τους στην ποιότητα του σπέρματος και στη σπερματογένεση.

1. GENERAL CHAPTER

I. Male reproductive system

Human reproductive system is one of the most complex organ systems and one of the most well – studied. Even though it does not contribute to homeostasis or the survival of an individual, it still plays a contributing role in the survival and the perpetuation of the species. Reproductive capability revolves around the interactions between the hypothalamus, anterior pituitary, primary reproductive organs, or gonads, and target cells of the sex hormones (Sherwood, 2012).

Male reproductive system consists of a pair of testes which, in their mature form, act for both the gametogenesis, namely the production of spermatozoa and the secretion of sex hormones, specifically testosterone. In addition to the gonads, the reproductive system comprises a reproductive tract and the accessory sex glands. The reproductive tract is actually a system of ducts that carries and accommodates the spermatozoa subsequential to their production while the glands empty their supportive secretions into these passageways. The accessory sex glands, which includes the seminal vesicles, prostate gland and bulbourethral glands (Cowper glands) (Fig. 2), provide the bulk of the semen, the liquid in which the male reproductive system deliver sperm to the female reproductive tract (Sherwood, 2012). The testes are made up of loops of convoluted seminiferous tubules (Fig. 1), in the walls of which spermatogenesis is conducted (Barrett and Ganong, 2010). The inner epithelial layer of the seminiferous tubules comprises of three cell types: spermatogonia, spermatocytes and Sertoli cells. Sertoli cells have a supporting role in the developing sperm (Costanzo, 2018). The sperm then empty into the epididymis, a coiled tube in which sperm is stored and matures. At ejaculation, the sperm is expelled into the ductus deferens, which in turn enlarges into the ampulla of the ductus deferens immediately before the duct meets the body of the prostate gland (Hall and Guyton, 2011). These pairs of reproductive tubes empty into a single urethra, the canal that runs the length of the male reproductive system and empties to the exterior (Fig. 2) (Sherwood, 2012). Between the tubules in the testes are nests of cells containing lipid granules, the Leydig cells (Fig. 1) which secrete testosterone into the bloodstream (Barrett and Ganong, 2010).

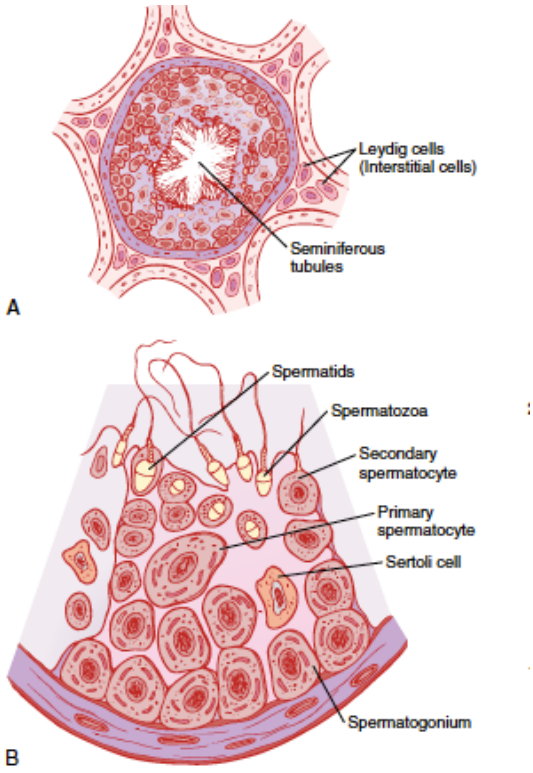

Figure 1. A, Cross section of a seminiferous tubule. B, Stages in the development of sperm from spermatogonia [Hall and Guyton, 2011].

The portions of the reproductive system outside the body cavity are known as external genitalia and in men consist of the penis and the scrotum (Sherwood, 2012). Normally, the testes occupy the scrotum, where temperature is maintained at 35 °C –

36 °C (or 1 °C -2 °C below normal body temperature), which is pivotal for normal spermatogenesis (Costanzo, 2018). The penis is the organ used to deposit sperm in the female.

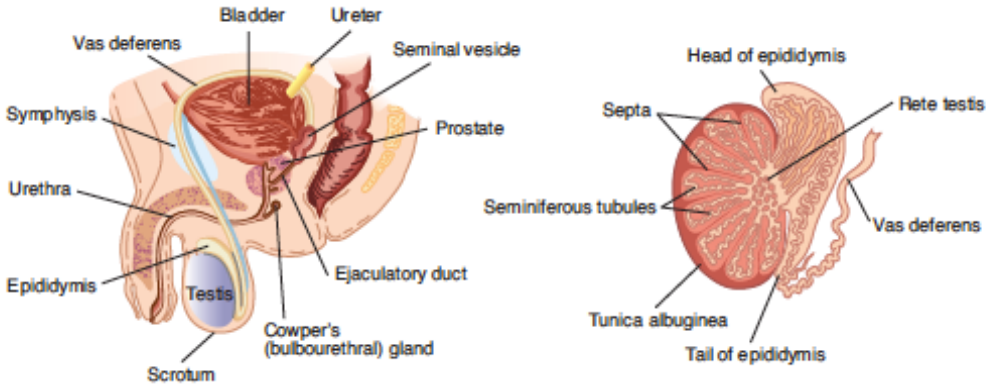

Figure 2. Anatomical features of the male reproductive system. Male reproductive system on the left and duct system of the testis on the right [Ganong, 2010]

II. Spermatogenesis

In order to fully understand the biology of the reproductive system, it is necessary to study the molecular signals, known as hormones that regulate its function. Reproductive hormones signal the reproductive structures to grow and mature while regulating the timing of the events. These in turn are regulated by the brain and the pituitary (Jones and Lopez, 2014).

The hypothalamus-pituitary-testes-accessory glands axis ensures androgenesis, male sexual behavior, and sperm production. Male reproductive hormones include hypothalamic decapeptides (gonadotropin-releasing hormone – GnRH), pituitary gonadotropins (LH, Luteinizing Hormone and FSH, Follicle-Stimulating Hormone), testicular steroids (testosterone and estradiol) and systemically active peptides of the gonads, such as inhibin B (Yen et al., 2014). FSH stimulates spermatogenesis process and the production of inhibin B by the Sertoli cells, whereas LH stimulates the production of testosterone by Leydig cells. Inhibin B and testosterone exert negative feedback over the secretion of FSH and LH. Testosterone is necessary for development and division of the testicular germinal cells, while FSH is necessary for differentiation of spermatozoa Testosterone can maintain spermatogenesis even without FSH, but for the resumption of spermatogenesis both hormones are necessary (Sherwood, 2012).

Sperm is produced in the seminiferous tubules and about 250m of them are packed within the testes. They include germ cells in a variety of stages and Sertoli cells, which support the spermatogenesis. It should be pointed out that, at any particular time, different seminiferous tubules are in different stages of spermatogenesis, meaning that daily a large number of spermatozoa, which can reach up to several hundred million, matures (Sherwood, 2012). The spermatogenic cycle is temporally organized (spermatogenic wave) and that ensures the continuous production of mature

spermatozoa (Costanzo, 2018). The entire period of spermatogenesis takes about 74 days (Hall and Guyton, 2011). The procedure comprises of three major stages: mitotic proliferation, meiosis and packaging (Fig. 4).

II. A. Mitotic proliferation

Spermatogonia locating in the outer-most layer of the seminiferous tubule, undergo continuous mitotic divisions, with every new cell carrying 46 chromosomes identical to those found in the parent cell. Such proliferation provides continuously new germ cells. After a spermatogonium divides mitotically, one of the daughter cells remains as an undifferentiated spermatogonium at the original position of the parent cell, thus maintaining the germ-cell line. The other daughter cell migrates among Sertoli cells toward the central lumen while going through the various steps necessary to form sperm. In humans, two more mitotic divisions of the sperm- forming daughter cell result in the formation of four identical *primary spermatocytes,* each with 46 doubled chromosomes. After the last mitotic division, the primary spermatocytes go into a resting period during which the chromosomes are duplicated without the doubled strands breaking apart until the first meiotic division (Sherwood, 2012).

II. B. Meiosis

During the first meiotic division, each primary spermatocyte forms two *secondary spermatocytes*, each with 23 doubled chromosomes. In the second meiotic division there are four spermatids, each with 23 single chromosomes. Each spermatid is eventually modified to form a single spermatozoon. Because each sperm-producing spermatogonium yields mitotically four primary spermatocytes and each primary spermatocyte subsequently produces meiotically four spermatids, the spermatogenic sequence can theoretically produce 16 spermatozoa. However, yielding is rarely this high cause some cells may be lost at various stages of development (Sherwood, 2012).

II. C. Packaging

Even after meiosis, spermatids still resemble undifferentiated spermatogonia structurally, except for their haploid number of chromosomes. Production of extremely specialized, mobile spermatozoa requires extensive remodeling or packaging of cell elements, a process called spermiogenesis. In sperm cells, unnecessary organelles and most of the cytosol are not crucial for the delivery of the sperm's genetic information and thus they have been extruded. At all stages of spermatogenic maturation, the developing sperm and Sertoli cells exchange small molecules and communicate. Final release of a mature spermatozoa from the Sertoli cell, a process called spermiation, requires the breakdown of the tight and gap junctions between Sertoli cell and spermatozoa. In their final form, spermatozoa have three parts: a head, capped with an acrosome, a midpiece and a tail (Fig. 3) (Sherwood, 2012). The head comprises of the condensed nucleus, which contains the sperm's complement of genetic information. The acrosome, a vesicle loaded with enzymes, is a thick cap on the outside of the

anterior two thirds of the head and is used as an «enzymatic drill» for penetrating the ovum. The whip-like tail provides the spermatozoa motility and has three principal components: (1) a central skeleton composed by 11 microtubules (axoneme), (2) a thin cell membrane which covers the axoneme and (3) mitochondria encompassing the axoneme. The energy for the back-and-forth movement of the tail is provided in the form of ATP (adenosine triphosphate) synthesized by the mitochondria in the body of the tail (Hall and Guyton, 2011).

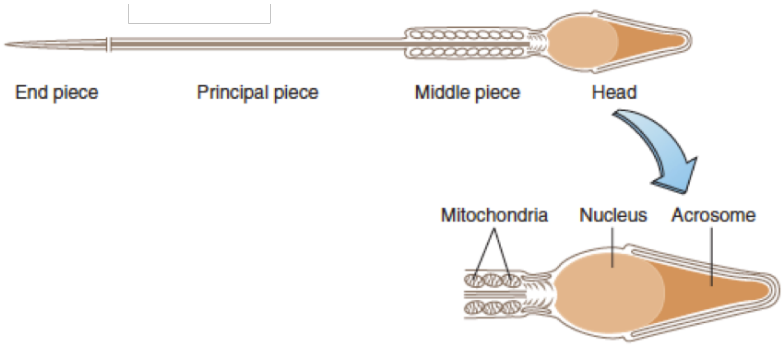

Figure 3. Structure of the spermatozoon [Costanzo 2017]

II. D. Capacitation

The differentiation of spermatozoa is completed inside the male reproductive track, but sperm is not capable of fertilization immediately after ejaculation. They must reside in the female reproductive track for 4-6 hours for capacitation to ensue. During capacitation, inhibitory factors present in the seminal fluid are washed free, cholesterol is withdrawn from the sperm membrane, and surface proteins are redistributed. The inflow of Ca^{2+} into the sperm increases their motility and alters spermatozoa's motion into whip-like. Capacitation also leads to the acrosomal reaction in which the acrosomal membrane merges with the outer sperm membrane. The pores created by this fusion allow hydrolytic and proteolytic enzymes to escape from the acrosome, developing a passage through which sperm is going to penetrate the ovum (Costanzo, 2018).

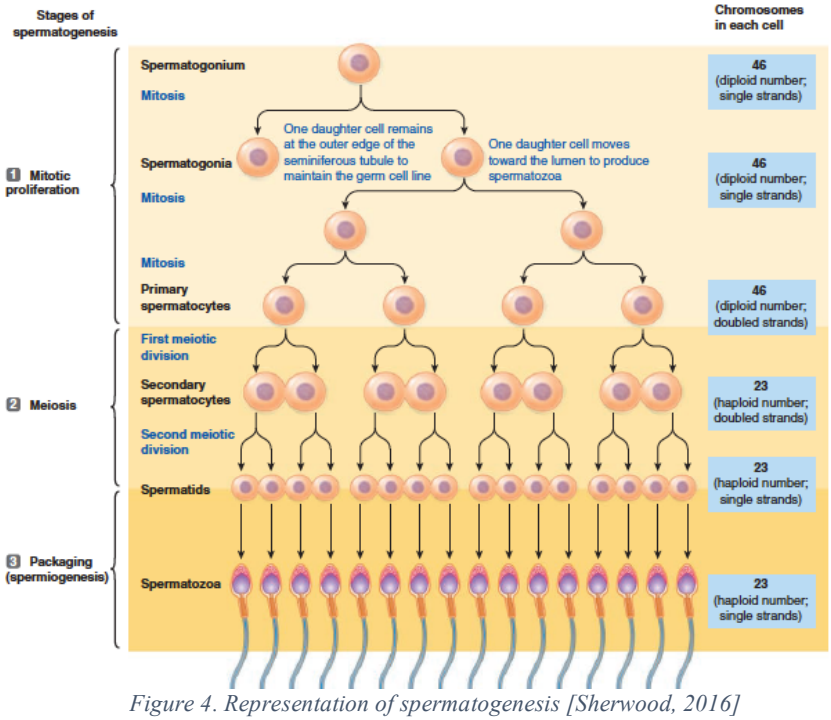

Figure 4. Representation of spermatogenesis [Sherwood, 2016]

III. Semen biology

Semen is a heterogeneous fluid consisting of spermatozoa, other cells (round cells, lining cells of the excurrent ducts, epididymis or accessory glands, spermatogenic cells and migrating leucocytes) and cell vesicles (prostasomes and epididymidosomes). Semen is thus composed of 'cellular' and 'acellular' elements or else seminal plasma (SP) (Rodríguez-Martínez et al., 2011). The chemical composition of the semen is affected by the interactions between the components and thus semen is considered as a functional unit. Biosynthesis of seminal plasma is remarkably complex and significantly different from that of blood plasma or other body fluids (Eliasson, 1982). The seminal plasma is produced by the fluids of both the cauda epididymides and accessory sexual glands. Semen consists of up to 95-98% seminal plasma and therefore, semen composition varies both among individuals and within an ejaculate itself (Rodríguez-Martínez et al., 2011).

The first secretion to the urethra, also known as pre-ejaculate, is that of the urethral and/or bulbourethral glands, a secretion including mostly sialic acid, galactose mucin and salts in a marginally viscous, clearly watery fluid. The release of spermatozoa from the cauda epididymides follows the pre-ejaculate to the urethra accompanied by the emission of the prostate secretions. The initial spurts are the so-called sperm-rich portion of the ejaculate, as most spermatozoa are found there. Along with the spermatozoa, a mix of the ampullar fluids and acidic cauda epididymides are present, together with the zinc-rich and mildly acidic citrate prostate fluid. The former contains specific peptides and proteins [as acid phosphatase and prostate-specific antigen (PSA)]. The following spurts include a gradual prevalence of the seminal vesicles secretions which are plentiful in prostaglandins (PGs), proteins, peptides, fructose etc. The major protein components of the seminal vesicle fluid are primarily semenogelin I but also semenogelin II, with an important role in the gelification of the latter spurts of the ejaculate (coagulum). Products with clear biological functions appear after liquefaction with a huge effect at inhibition of sperm motility and antibacterial activity together with other seminal vesicle proteins including fibronectin, lactoferrin and protein C-inhibitor (Rodríguez-Martínez et al., 2011).

The prostate secretions despite representing only 20–30% of the total SP volume, are the first SP part to meet the cervical canal and are in direct contact with numerous spermatozoa. They comprise three major proteins, all under hormonal regulation: prostatic acid phosphatase, the cysteine-rich prostate-specific protein-94 (PSP-94) and PSA (prostate-specific antigen). The primary function of PSA is the liquefaction of the coagulum through semenogelin's hydrolysis, while PSP-94 and the prostatic acid phosphatase have growth factor and enzymatic action respectively (Rodríguez-Martínez et al., 2011). Another peptide in the prostate fluid is the hormone relaxin. Relaxin is detected in pregnant women (Essig et al., 1982) and appears to maintain high mitochondrial activity levels and low level of apoptosis in spermatozoa, as well as causes an increase in semen capacity for fertilization through the acrosome reaction (Ferlin et al., 2012).

Generally, seminal plasma function is correlated with the ejaculation of

spermatozoa and their subsequent survival in the female reproductive system (Juyena and Stelletta, 2012). Once spermatozoa are ejaculated in the female genital ducts, they can survive up to 24 to 48 hours at body temperature (Hall and Guyton, 2011). Seminal plasma protects spermatozoa from potential oxidation and provides a regulatory effect to neutralize the acidic vaginal environment. The transfer of sperm to the cervix is facilitated by the combined action of prostaglandin, which stimulates local muscle activity, and relaxin together with other enzymes, which promote sperm motility (Juyena and Stelletta, 2012).

IV. Semen analysis

Semen analysis is the first diagnostic test to examine male infertility and it is the most reliable diagnostic approach so far. Depending on the outcome, further tests may be performed to confirm a pathological condition in the male reproductive system. The analysis is based on specific criteria established and being regularly updated by WHO (World Health Organization, 2010). According to the last update (5th Edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen, 2010) the 3 major steps of a semen analysis is the sample collection, the initial macroscopic examination and the initial microscopic examination, as presenting below.

IV. A. Sample collection

The collection of the sample should be carried out after at least 2 days of sexual abstinence and no longer than 7 days, preferably in a private room close to the laboratory, in order to manage the time between collection and analysis, as well as to limit the exposure of the semen to fluctuations of temperature. The sample should be gathered by masturbation and ejaculated into a clean, non-toxic for spermatozoa wide-mouthed container. Failure to obtain the entire the sample should be reported.

In exceptional circumstances, the collection of a sample may take place at home by masturbation provided that it will be transferred to the laboratory within 1 hour following collection. The same condition applies in the case of sample collection in non-toxic condom

IV. B. Initial macroscopic examination

After ejaculation, the semen starts to liquefy. Liquefaction is ideally completed at 30 minutes at room temperature, but at all cases it should be achieved within an hour after ejaculation. Semen analysis begins with the macroscopic examination, which includes liquefaction time, semen viscosity, appearance of the ejaculate, semen volume and semen pH.

Semen is normally a semi-solid coagulated mass after ejaculation but during liquefaction, the semen becomes more homogenous and quite watery. Rarely the liquefaction may take more than 60 minutes, in which case it should be reported.

The viscosity of the sample can be assessed after liquefaction by mildly aspirating it into a wide-bore plastic disposable pipette, allowing the semen to drop by gravity and observing the length of any thread created. A normal sample should leave the pipette in small separated drops but if viscosity is aberrant, the drop will form a more than 2cm long thread.

A normal liquefied semen sample appears as a homogeneous, grey-opalescent fluid. In case of a very low sperm concentration, the appearance of the semen may be less opaque. In the presence of red blood cells (haemospermia), the color may be red-brown and in a case of a man with jaundice or a man that takes certain vitamins or drugs, the semen may appear yellow.

In terms of sperm volume, precise measurement is substantial when evaluating the semen, since the calculation of the total number of spermatozoa and non-sperm cells in the ejaculate depends on it. The ideal approach for the measurement of the volume is by weighing the sample in the vessel in which it is collected. The lower reference limit for semen volume is 1.5 ml.

The balance between the pH values of the different accessory gland secretions is reflected on the pH of the semen, meaning on the one hand the acidic prostatic secretion and on the other the alkaline seminal vesicular secretion. It is essential for pH to be measured after liquefaction and within an hour after ejaculation since it is under the influence of CO_2 loss that appears after production. For normal samples, the pH is measured by pH paper in the range from 6.0 to 10.0 but for viscous samples, the pH should be measured using a pH meter targeting exclusively viscous solutions. The lower threshold value is set to 7.2.

IV. C. Initial microscopic examination

The microscopic examination requires a phase-contrast microscope and includes the evaluation of the sperm motility, vitality, numbers, morphology as well as the detection of aggregation and agglutination of spermatozoa or cellular elements apart from spermatozoa.

Sperm motility should be assessed in the shortest possible time after liquefaction of the sample so that the harmful impact of dehydration, pH or changes in temperature to be limited. There are categories of sperm movement which serve to separate spermatozoa with progressive (PR) or non-progressive motility (NP) from those that are immotile. The lower reference limit for total motility (PR + NP) is 40% and as for progressive motility (PR) is 32%.

Sperm vitality is critical for samples with less than 40% PR, but in all cases it may be assessed. The test is based on the membrane integrity of the cells as membrane-impermeant stains enter in the non-vital cells or they will not swell in hypotonic solutions. The lower reference for vitality (membrane-intact spermatozoa) is 58%.

The total number of spermatozoa in the ejaculate is measured from the concentration of spermatozoa. To calculate the concentration properly it is essential to determine the use of the proper chambers and dilution to use (the most common chamber is the Neubauer haemocytometer). The lower reference limit for sperm

concentration is 15 x 10^6 spermatozoa per ml. Multiplication of the sperm concentration by the volume of the entire ejaculate results in the total number of spermatozoa per ejaculate. The lower reference limit for total sperm number is 39 x 10^6 spermatozoa per ejaculate.

A haemocytometer can also be used to evaluate the number of non-sperm cells in semen (epithelial cells, “round cells” - germ cells and leukocytes - or isolated sperm heads and tails). Concentration (C) of round cells (10^6 per ml) can be calculated using the following formula C = S Å~ (N/400), where N represents the number of round cells found in the same number of fields as 400 spermatozoa and S is the concentration of spermatozoa (10^6 per ml).

Evaluating the morphology of spermatozoa is based on preparing a semen smear and once it dries, it should be stained to underline details of the spermatozoa. The utilization of the Papanicolaou, Shorr or Diff- Quik stain is recommendable by WHO. With all three stains, the head is stained pale blue in the acrosomal area and dark blue in the post-acrosomal area. The midpiece may appear with some red staining and the tail is stained blue or reddish. Excess residual cytoplasm, typically placed behind the head and around the midpiece, is tinted pink or red with Papanicolaou stain or reddish-orange with Shorr stain. While assessing the sample, the following defects should be noted: head defects, principal piece defects, midpiece and neck defects and excess residual cytoplasm (ERC). The lower reference limit for normal forms is 4%.

Last, the adhesion of either immotile spermatozoa to each other or of motile spermatozoa to mucus strands, non-sperm cells or debris is considered as nonspecific aggregation. On the other hand, agglutination specifies particularly motile spermatozoa sticking to each other, head-to-head, tail-to-tail or in a combined way.

Table 1. The lower reference limits for the parameters that are evaluated during semen analysis [WHO, 2010]

Parameter	Lower reference limit
Semen volume (ml)	1.5
Total sperm number (106 per ejaculate)	39
Sperm concentration (106 per ml)	15
Total motility (PR + NP, 100%)	40
Progressive motility (PR, %)	32
Vitality (live spermatozoa, %)	58
Sperm morphology (normal forms, %)	4
pH	≥ 7.2

IV. D. DNA Fragmentation test

During the past two decades, various novel tests for evaluating semen parameters have been emerged. These rely on measuring sperm DNA fragmentation which may arise out of excessive production of reactive oxygen species (ROS) in the ejaculate, abnormal chromatin packaging during spermatogenesis, defective apoptosis prior to ejaculation, or exposure to environmental and industrial toxins, genetics, oxidative stress, smoking, etc, (Chohan, 2006). Reason behind studying such defects in sperm DNA is to investigate their possible effects on normal embryo and fetal development when the paternal genome suffers from nucleotide or DNA damage (Sakkas and Alvarez, 2010). The tests currently used are TUNEL (TdT-mediated-dUTP nick end labeling assay), COMET (single cell gel electrophoresis assay), cridine orange staining technique (AOT), CMA3, in-situ nick translation, DBD-FISH (DNA breakage detection fluorescence in situ hybridization), sperm chromatin dispersion test (SCD), and the SCSA (sperm chromatin structure assay) (Chohan, 2006; Sakkas and Alvarez, 2010).

Depending on the sperm quality derived from the semen analysis, the condition of each patient is assessed. Hence, each patient may be classified in one or more of the conditions presenting in Table 2.

Table 2.Pathological sperm conditions based on semen analysis [WHO, 2010]

Aspermia	No semen (or retrograde ejaculation)
Asthenozoospermia	Percentage of progressively motile (PR) spermatozoa below the lower reference limit
Teratozoospermia	Percentage of morphologically normal spermatozoa below the lower reference limit
Oligozoospermia	Total number or concentration of spermatozoa below the lower reference limit
Azoospermia	No spermatozoa in the ejaculate
Cryptozoospermia	Spermatozoa absent from fresh preparations but observed in a centrifuged pellet
Normozoospermia	Total number (or concentration) and percentages of PR and morphologically normal spermatozoa equal or above the lower reference limit
Necrozoospermia	Low percentage of live and high percentage of immotile spermatozoa in the ejaculate
Haemospermia	Presence of erythrocytes in the ejaculate

V. Male infertility

Infertility is defined as the inability of a couple to achieve pregnancy after at least one year of unprotected intercourse (World Health Organization, 2010) and affects approximately 15% of couples worldwide (de Kretser, 1997). Conception is conventionally accomplished within 12 months in 80% to 85% of couples who are not using contraceptive methods (Sinclair, 2000). Almost 50% of them achieve pregnancy spontaneously in the second year of unprotected intercourse, and another 14% in the third year. Ultimately, <5% remain childless (Jungwirth et al., 2012). Male factor is the cause of infertility in approximately half of the cases (Dohle et al., 2005). According to the European Urology Association, in 30-45% of male infertility cases, the cause of the abnormal semen parameters remain undetermined (idiopathic infertility) (Jungwirth et al., 2012).

Male infertility is a multifactorial condition resulting from the interaction of genetic and environmental factors, lifestyle (Skakkebaek et al., 2016). There are different causes acting cooperatively and having negative effects in male fertility and as so, an understanding of the cause of infertility is essential to appropriately manage an infertile couple. Responsible for the other 55-70% of the male infertility are the following categories.

V. A. Ejaculatory disorders

Ejaculatory disorders are relatively uncommon but still important factors of male infertility (Colpi et al., 2004). *Aspermia* is either the complete absence of sperm or retrograde ejaculation (World Health Organization, 2010) and it caused mainly by neurological damages (spinal cord injuries)(Ohl et al., 2008). *Retrograde ejaculation* is observed when the semen returns through the bladder to the seminal vesicle (Kalogeropoulos, 2004). Another disorder is *anorgasmia,* in which a man cannot reach orgasm due to psychological causes, and *painful ejaculation* which causes moderate sexual activity and may be caused by seminal vesicular obstruction, urethritis, antidepressant drugs or psychological problems (Colpi et al., 2004).

V. B. Endocrine disorders

A functional endocrine system is prerequisite for male fertility. The most common endocrine disorder is hypogonadotropic hypogonadism (HH), which is caused by gonadotrophin deficiency due to inability to excrete GnRH in the hypothalamus (idiopathic hypogonadotropic hypogonadism) or to inability of gonadotropic cells to respond to GnRH stimulation (pituitary hypogonadism) (Κόικα και Γεωργόπουλος, 2014). Patients with HH present decreased LH and FSH secretion. As a result, Leydig cells do not secrete testosterone and spermatogenesis process is disrupted (Jankowska and Kochman, 2016). Moreover, thyroid gland disorders also seem to exert a negative impact on the male reproductive system. Hypothyroidism negatively affects the quality of the sperm, degrading its volume and impairing the progressive motility of the spermatozoa (Krajewska-Kulak and Sengupta, 2013). In men with hyperthyroidism,

testosterone levels are reported to be low, whereas circulating estradiol is documented high. These changes may lead to gynecomastia, decreased libido, erectile dysfunction, spermatogenic disorders (Trokoudes et al., 2006), decreased sperm count and motility (Jankowska and Kochman, 2016).

V. C. Immunological causes

Immunological factors are considered as one of the main factors of infertility (Hinting et al., 1996). *Antisperm antibodies (ASA)* can be found in both male and female genital track and have significant effects on fertilization capacity of both sexes. These antibodies appear in many biological fluids, particularly, in seminal plasma, and they bind to the surface of spermatozoa. They may originated from a trauma that destroys the blood-testis barrier, the epididymis or the vessels. Their presence has been associated with inflammation, cryptorchidism, varicocele and surgery in the genitals. Antisperm antibodies affect sperm motility, the acrosome reaction and the interaction of the spermatozoa with the zona pellucida of the oocytes. In men diagnosed with ASA, an increased number of agglutination is observed during semen analysis (Bohring and Krause, 2005).

V. D. Urogenital track infections

Many micro-organisms appear to be involved in male infertility in different ways and in varying degrees. Infections in the inferior genital system seem to be of minor importance and rarely cause obstruction. However, infection in other parts of the urogenital system results in microbial colonization that negatively affects semen. For instance, infection with *Neisseria gonorrhoeae* has a significantly negative effect on the male's fertility (Pellati et al., 2008), specifically affecting the concentration and sperm motility (Ochsendorf, 2008). Also, *Chlamydia trachomatis* infection in male genital track causes epididymitis, orchitis, prostatitis and seminal vesicular obstruction, ultimately causing male infertility. Finally, HPV (Human Papilloma Virus) has been found in testis biopsy samples of azoospermic men and when it is detected inside the spermatozoa, it may lead to impaired motility and asthenozoospermia (Pellati et al., 2008).

V. E. Genetic disorders

Various genetic factors of infertility have been documented, mainly including chromosomal abnormalities, isolated gene disorders and multifactorial heredity phenotypes (Shah et al., 2003). Klinefelter's syndrome is the most common chromosomal abnormality caused by aneuploidy and it occurs in 5% of oligozoospermic men (O'Flynn O'Brien et al., 2010) and in 14% of men with azoospermia. The karyotype of men diagnosed Klinefelter's syndrome is 47, XXY and testicular histology shows genital degeneration while testosterone levels are elevated

and FSH and LH are decreased (Zorrilla and Yatsenko, 2013). Another genetic factor is isolated gene disorders, such as cystic fibrosis. In 60-90% of patients suffering from cystic fibrosis caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, there is a congenital bilateral absence of the vas deferens (CBAVD). CBAVD is a type of obstructive azoospermia in which epididymis is no longer in conjunction with the ejaculatory duct (O'Flynn O'Brien et al., 2010). CFTR protein participates in the transport of electrolytes and water along the epididymal epithelium, developing the optimal conditions for maturation and transport of sperm (Pallares-Ruiz et al., 1999). This involvement in sperm maturation suggests that mutations in the CFTR gene can induce infertility through both obstruction and disturbances in the sperm maturation process (Shah et al., 2003).

V. F. Physical abnormalities

The physical abnormalities of the male reproductive system are structural abnormalities that harm or obstruct the testes, epididymis, seminal ducts, and ultimately jeopardize fertility (Shah et al., 2003). Such an example is varicocele in which men exhibit elevated scrotum temperature resulting in increased ROS production which negatively impacts sperm quality (Will et al., 2011). In particular, oxidative stress decreases motility and causes DNA fragmentation (Kantartzi et al., 2007). A second physical abnormality of the male reproductive system is cryptorchidism. Cryptorchidism is the absence of one or both of the testes from their normal position in the scrotum (Singh et al., 2012). Men with a history of cryptorchidism are often infertile in adulthood. The latter may be a result of spermatogenic disorders and it exhibits decreased semen concentration, increased levels of FSH, along with decreased levels of Inhibin B in plasma, which is related to decreased testicular volume, demonstrating also the impairement of fertility potential (Foresta et al., 2008).

V. G. Environmental factors and lifestyle

The environment and lifestyle of an individual are factors that need to be taken into account when considering male fertility, as they affect the body and thus the spermatogenesis. For instance, sedentary life, obesity, smoking and exposure to toxic factors adversely affect the growth of testes and their function, which deteriorates semen parameters resulting in infertility (Sharpe, 2010). Another lifestyle factor, the alcohol consumption, has been reported to be associated with male infertility due to the disruption of the hypothalamic-pituitary-gonadal axis feedback mechanisms, resulting in attenuation of LH and FSH production and secretion, and consequently Sertoli cell dysfunction (Gude, 2012).

VI. Management of male infertility

The most common approach for the diagnosis of male infertility is, as previously mentioned, semen analysis. The semen analysis provides the experts with an overview

of the spermatozoa' status and in some cases it can report the underlying cause of infertility. Among with a detailed medical and urological history, physical and hormonal examination (Leaver, 2016), the treatment and management of male infertility can be personalized. However, in most cases of male infertility, treatment needs to be addressed in a multimodal fashion. Current and novel medical therapies for male infertility are conveniently broken down into seven sections:

VI. A. Preventive therapy measures

Lifestyle factors can greatly influence the overall health and well-being, including fertility. The age at which people decide to childbearing is a crucial factor, among others, affecting fertility. Concerning men's age, testosterone level decreases, semen parameters also begin to decline and more DNA damage can be found in the sperm. Thus, attempting pregnancy before the age of 35 for men may provide the highest chances of success. Diet, as lifestyle factor, is also important for the reproductive ability of a men. Following a diet rich in carbohydrates, fiber, folate and lycopene, as well as consuming fruit and vegetables has been reported to improve semen quality. Moreover, antioxidants (i.e. albumin, ceruloplasmin and ferritin) play a pivotal role since they may limit the excess ROS in the seminal ejaculate (Sharma et al., 2013). Healthy nutrition of an individual, combined with exercise, can result in a reduced body mass index (BMI) with significant effects on fertility since obese men are three times more likely to exhibit a reduction in semen quality in comparison to men of normal weight (Magnusdottir et al., 2005). Moreover, conservative measures such as smoking, alcohol and illegal drugs cessation has shown an improvement on the quality of sperm and should be heavily counseled (Pan et al., 2018; Sharma et al., 2013). Another preventive measure is associated with the view that an elevated temperature of the scrotum negatively impacts spermatogenesis and sperm parameters. Therefore, it is important for men to wear loose clothing and underwear and avoid the use of jacuzzis or saunas Besides high temperature, men can be exposed to chemicals and heavy metals while on the job, alongside the several environmental exposures, accompanied with various negative side effects to their reproductive health. As eliminating every possible exposure is unrealistic, identifying and minimizing them may have significantly positive effects on fertility (Sharma et al., 2013). Men who work under such conditions, as well as men who are exposed to radiation, either on their workplace or even due to cancer therapy, are suggested to cryopreserve their sperm for future use. Sperm retrieval for cryopreservation can be done, except for the conventional way of masturbation, with the following approaches: MESA (microsurgical epididymal sperm aspiration), TESE (testicular sperm extraction), TESA (testicular sperm aspiration) and PESA (Percutaneous epididymal sperm aspiration) (Leaver, 2016). All samples, once retrieved, are going to be used in assisted conception. Following the above preventative steps can help men somehow control their own fertility potential and maximize fertility outcomes.

VI. B. Medical treatment

Hypogonadotropic hypogonadism is a very common condition causing male infertility and its treatment relies on direct administration of human chorionic gonadotropin (hCG) with the later addition of hMG or recombinant FSH (depending on the initial testicular volume) to elevate the depressed LH and FSH (secondary hypogonadism) (Jungwirth et al., 2012; Kliesch, 2017; Pan et al., 2018)). Administration of exogenous testosterone often has deleterious effects on sperm production and thus it should be ceased whenever possible. Secondary hypogonadism can be also treated with oral clomiphene citrate, which acts to increase LH and FSH and ultimatelly to stimulate sperm and testosterone production (Pan et al., 2018). It has also been reported that nonsteroidal aromatase inhibitors, such as letrozole, have promising results in treating hypogonadotropic hypogonadism in obese infertile men and in infertile men with abnormal hormonal profiles (Stephens and Polotsky, 2013). Medication is needed in cases of infections in the seminal tract as well. Azithromycin is used commonly for the treatment of Chlamydia, Mycoplasmas and Ureaplasmas but erythromycin and tetracycline also find application. Other common infections of the male reproductive tract are trichomoniasis and Gardnerella vaginalis, which are treated with the administration of metronidazole (Kliesch, 2017).

VI. C. Surgical treatment

Surgical management for infertility varies and is tailored to each patient. Particularly important is the correction of any varicocele present (Pan et al., 2018) if it causes pain and discomfort (Leaver, 2016). Varicocele can be treated using different approaches, including open inguinal, laparoscopic, subinguinal microscopic varicocelectomy and percutaneous radiographic approach (Jungwirth et al., 2012a; Pan et al., 2018). Furthermore, in cases of obstructive azoospermia (OA), depending on where the obstruction is, there may be a need for microsurgical reconstruction of the vas (Jarow et al., n.d.). Stenosis or other obstruction at the level of the vas deferens can be corrected by excising the offending segment and performing a vasovasostomy to reconnect the ends of the vas deferens. Congenital or acquired obstructions at the level of the epididymis in the presence of normal spermatogenesis can be corrected performing a vasoepididymostomy (Jungwirth et al., 2012a). Obstructions found at the level of the ejaculatory ducts are managed endoscopically by transurethral resection of the ejaculatory ducts (TURED) to open the obstructed ducts (Pan et al., 2018). This kind of obstructions are commonly caused by infections of the prostatic urethra and the accessory glands. As a resul, the resection may lead to an increase in semen quality and, occasionally, spontaneous pregnancy (Jungwirth et al., 2012).

VI. D. Assisted Reproduction Technologies (ART)

Only a small proportion of infertile men are amenable to treatment, which often leads to the utilization of intrauterine insemination (IUI) or in-vitro fertilization (IVF) procedures when a specific cause cannot be identified (Adamson and Baker, 2003). The

first-line treatment, in the majority of the cases when considering ART, is IUI (Kim et al., 2014). Indications for IUI concerning male infertility include unexplained and idiopathic infertility, ejaculatory disorders, immunological causes, low sperm count and/or motility (Keck et al., 1997). IUI is usually performed with a fresh semen specimen, but cryopreserved semen can also be used for multiple timed artificial inseminations, in cases of men who cryopreserved their sperm due to chemotherapy or radiation, as well as in cases of a donor sperm (Anger et al., 2003). The IUI procedure has no standard protocols and thus insemination can be done at various periovulatory time points around ovulation (Kim et al., 2014). IUI is the least expensive and least invasive option for assisted reproduction and includes the delivery of sperm cells directly to the uterine cavity of the woman through a catheter (Pan et al., 2018). Prior to insemination, semen has to be prepared and washed using one of the established sperm preparation techniques (Keck et al., 1997). These techniques are necessary for the removal of the seminal plasma in order to avoid prostaglandin-induced uterine contractions and for the separation of the highly motile spermatozoa that will be transferred to the uterus. The most important factor that should be evaluated prior to IUI is semen parameters. It has been reported that pregnancy rates are lower if the semen sample contains less than 10 million sperm in total. Concerning the insemination sample, the recommended lower limit ranges from 3 million motile sperm to 10 million (The ESHRE Capri Workshop Group, 2009).

When IUI is systematically failing, the next step in an attempt to address male infertility is IVF treatment. In vitro fertilization (IVF) has opened a floodgate of possibilities for infertile patients since Louise Brown's birth in 1978. IVF seems to be one of the most effective treatments for male-factor infertility, especially where it has been of long duration(Yates and De Kretser, 1987). Although IVF was first used to deal with cases of tubal and idiopathic infertility (Yates and De Kretser, 1987), later Fishel an Edwars made the first observation that IVF was useful for treating oligozoospermia and oligoasthenozoospermia. Since then conventional IVF has been modified and refined to achieve increased rates of fertilization and conception (Hall and Fishel, 1997). In vitro fertilization (IVF) can be successful when the number of available sperm is limited with low motility (Leaver, 2016). IVF treatment includes extraction of both sperm and oocytes which are coincubated until fertilization occurs with no other intervention (Hall and Fishel, 1997; Leaver, 2016; Pan et al., 2018). The subsequent embryo is cultured until cleavage stage (day 2 to 3) or until blastocyst stage (days 5 to 6) when either the embryo transfer occurs or the embryos are cryopreserved for future use.

As previously mentioned, IVF has been modified over the years to face the challenges arising due to the severity of each patient's status. So, in case of severe male infertility intracytoplasmic sperm injection (ICSI) is performed instead of the conventional IVF. In ICSI procedure, a spermatozoon is injected directly into the oocyte using a micropipette (Pan et al., 2018). More specifically, the oocyte is held in place by suction through the holding pipette and then a single spermatozoon is ejected slowly, close to the beveled opening of the injection pipette, and it is pushed onto the zona pellucida, permitting penetration (Palermo et al., 1995). In cases of azoospermia

or cryptozoospermia, spermatozoa are recruited by the sperm retrieval techniques that have been previously described (Pan et al., 2018). Absolute indications for the employment of ICSI procedure include all groups with non-ejaculated samples (i.e. spermatozoa aspirated from vas deferens, epididymis or the testis) (Hamberger et al., 1998) and also cases where preimplantation genetic testing is required. Apart from primary indicators, ICSI is the preferred option for male patients experiencing various sperm-related problems that include ejaculatory dysfunction, retrograde ejaculation, and complications stemming from paraplegia. ICSI is the most effective means of treating couples with male factor infertility and previous ART fertilization failures (Palermo et al., 2014). Assisted reproduction techniques are just a mean of achieving the ultimate goal of a couple, which is an offspring, and therefore they are better characterized as management of male infertility and not as treatment under the conventional definition of the term.

VI. E. Adoption

ART provides couples with the possibility of having children which are biologically linked to themselves. However, this type of treatment is financially, physically and psychologically demanding when considering the odds of success (Gumus and Lee, n.d.). When a couple wishes a child which is not exclusively biological affiliated and has been through many unsuccessful cycles of IVF treatment, then this couple should be consulted about adoption (Blanchy, 2011). More specifically, in cases, in which there is an absence of sperm due to the severity of the man's condition, or in which fertilization or pregnancy are failed, besides the sperm donation, the couple should then consider adoption. Adoption is under numerous regulations and the couple must be fully informed and supported (Kliesch, 2017).

VI. F. Transcriptomic profiling

It was thought that spermatozoa just serve the function of delivering the male genetic material and have no traces of RNA, as during spermiation the remaining unnecessary cytoplasm is removed from the spermatozoa. However, due to recent studies that employ mostly RT- PCR method , the presence of RNA in sperm is undoubtedly true (Bansal et al., 2015). So, even though transcriptional and translational processes are blocked and most of the RNAs are lost during cytoplasmic extrusion, it seems that some RNA is non-randomly preserved in mature spermatozoa (Jodar, 2019).

The study of sperm transcript may have profound clinical implications in the diagnosis of male infertility and the practice of ART. In fact, differences in the transcriptomic profile between fertile and infertile men could associate specific genes and pathways that are essential in spermatogenesis with infertility (Bansal et al., 2015). Sperm RNA is growing into a valuable tool in the prediction of infertility and study of specific genes could help us determine whether they are related to abnormal semen parameters and therefore affect specific pathways involved in spermatogenesis.

VI. G. Proteomic profiling

Human sperm proteomics is a recent field of investigation and it has been advanced enormously in the last decade especially with the development of the mass-spectrometry-based proteomics pipelines that have been developed. Proteomics is the identification and quantification of all the proteins in a given fluid (or cell) under a specific biological condition. It is known that, proteins form protein-protein interaction (PPI) networks and participate in specific pathways that result in specific functions such as fertilization. The studies performed so far have identified 6198 proteins involved in several cellular pathways relevant to male infertility. In the male gamete, specifically, the most significant pathways are those involved in: metabolism, protein and RNA metabolism, membrane trafficking, apoptosis, meiosis cell cycle and hemostasis (Amaral et al., 2014).

Most sperm comparative proteomic studies aim to characterize potential proteomic anomalies in infertile patients with altered semen parameters and have provided important biomarkers for diagnosis and prognosis of male infertility. These biomarkers constitute a group of multiple interacting proteins across multiple pathways, which is expected as pathogenesis cannot be singled out by only one protein. The variance of different sperm samples at the protein level will help in establishing the elements of the sperm proteome that are required for normal sperm function (Amaral et al., 2014). Once establishing these elements, proteomic profiling of a patient could help in the diagnosis of infertility when semen analysis is failing and thus the treatment could be directed towards each patient's affected pathways.

VII. Insulin-like growth factor I (IGF-I)

Insulin-like growth factor-1 (IGF-1), also referred to as somatomedin C, is a cellular and secreted molecule that is considered to be involved in the developing process of germ cells (Lee et al., 2016; Philippou et al., 2014). IGF-1 is a 7.6-kDa polypeptide consisting of 70 amino acids that mediates the actions of growth hormone (GH) related to cell proliferation (Lee et al., 2016). Thus, IGF-1 is crucial for the normal growth, development and maintenance of the body and also important in numerous biological systems. It should be noted that production of IGF-1 is not under exclusive GH control as even when GH signaling does not occur, IGF-1 can be produced in significant amounts (Barkte, 2000).

The IGF-1 gene is located on the long arm (q) of chromosome 12 (12q23.2 specifically) in humans and spans a region of over 80 kb of genomic DNA (Philippou et al., 2014). IGF-1 is generated mainly by the liver (and locally in various GH target organs), and the pulsatile secretions of GH from the anterior pituitary result in its release (Barkte, 2000). In the peripheral circulation, IGF-1 is mostly obtained from the liver, as well as from skeletal muscle (Philippou et al., 2014) where it circulates mainly in high molecular weight ternary complex with IGF-binding protein 3 (IGFBP-3) and acid-labile subunit (ALS), which expand its half-life in the circulation (Colombo and

Naz, 1999). IGF-binding proteins protect IGF-1 from proteolytic degradation and regulate its bioavailability to the IGF-1 receptors. Actually, its actions are facilitated through its binding to various receptors, such as IGF-1R and IGF- IIR, insulin receptor (IR) and some atypical receptors. Competition between at least six IGFBPs and IGF-1R plays a regulatory role in the biological actions of IGF-1. Normally, IGFBPs have higher affinity to IGF-1 than IGF-1R does. Therefore, by binding to IGFBPs, IGF-1 does not interact with the receptor and, as a result, its actions are suppressed (Philippou et al., 2014). When bound to the IGF-1R and IR, IGF-1 initiates intracellular signaling through tyrosine kinase signal transduction (Colombo and Naz, 1999).

IGF-1 stimulates DNA synthesis in a variety of cells types, including Sertoli cells in men. Plasma IGF-1 levels, which are poor during fetal and neonatal life, escalate during childhood, climax during puberty and then decline gradually throughout adult life (Colombo and Naz, 1999). In the male reproductive system, IGF-1 has been observed in the testes, where it is secreted by Leydig and Sertoli cells (Lee et al., 2016). At the testicular level, the GH acts directly and indirectly via hepatic IGF-1 to promote sperm production, the development of spermatogonia and also, to ensure intact maturation (Magon et al., 2011). Hence, it seems that IGF-1 plays a vital role in the reproductive processes and the regulation of spermatogenesis and steroidogenesis. This role is further supported by the presence of IGF-1 receptors on Sertoli and Leydig cells, secondary spermatocytes, spermatids and spermatozoa (Lee et al., 2016).

IGF-1 gene comprises six exons from which arise heterogeneous mRNA transcripts by a combination of alternative splicing, multiple transcription initiation sites and different polyadenylation signals. Translation of these multiple IGF-1 transcripts yields different precursor polypeptides, which also undergo posttranslational modifications. The structure of the extension peptides (E-peptides) on the carboxy-terminal end and the length of their amino-terminal signal peptides is what distinguishes the IGF-1 protein isoforms. However, they all share the same mature peptide, which is a 70 aa long single-chain peptide coded by exons 3 and 4 (Philippou et al., 2014) and actually exon 4 encodes approximately 70% of the mature IGF-I peptide (Oberbauer, 2013). The mature peptide is composed of four domains, the B amino-terminal domain, C and A domain and D carboxyterminal domain. The mature IGF-1 protein's sequence is highly conserved among primate species and is accountable for binding to the receptors. It binds to IGF-IR with very high affinity, to IGF-IIR with low affinity while remaining capable of interacting with IR. The binding of IGF-1 to IGF-IR results in the activation of the receptor which triggers all of the biological effects of IGF-1 on cell growth, differentiation, invasion and survival (Philippou et al., 2014).

The heterogeneous mRNA transcripts are formed as follows: the different leader sequences give rise to two different classes of IGF-I mRNA variants: class 1 transcripts use exon 1 as a leader exon (promoter 1), whereas class 2 transcripts use exon 2 for their initiation sites (promoter 2). Alternative splicing of exon 5 also gives rise to different mRNA variants containing exon 5, typically described as class B (IGF-IEb), or containing exon 6 and excluding exon 5 described as class A (IGF-IEa). A third variant, the IGF-IEc, includes both exon 5 and 6 in the formed transcript (Fig. 5). In summary, IGF-IEa transcript contains exons 1 or 2 and 3,4,6 of the IGF1 gene, which

serves as the main pro-IGF-1 mRNA derived from the liver. IGF-IEb transcript is a splice variant consisting of exons 1 or 2 and 3,4,5. IGF-IEc mRNA transcript is a splice variant containing exons 1 or 2 and 3,4,5,6, which was originally found in human liver as well. Nonetheless, expression of IGF-IEc is close to 10% compared to the main IGF-IEa transcript (Philippou et al., 2014).

At this time, the biological significance of IGF-I splice variants is unidentified and the underlying mechanism of action is yet unclear. However, the presence of distinct transcripts suggests that the response of cells varies depending on stimuli, indicating that the isoforms of IGF-1 are accountable for the complexity of IGF-1 actions. This implies that the IGF-1 splice variants are differentially transcribed in response to various conditions and pathologies and is probably a function of age. Indications of this differential transcription have been found in exercise-induced muscle damage, cervical cancer, endometriosis and prostate, as well as in cell lines after hormonal treatment. This differential expression is of particular interest, as it specifies the presence of distinct regulatory mechanisms and the biological functions of the IGF-1 isoforms (Philippou et al., 2014). However, it is still undetermined whether the isoforms are of biological significance. It has long been hypothesized, though, that the presence of exon 1 in the transcript leads to an autocrine-paracrine manner of action (Gilmour, 1994; Sussenbach et al., 1992) while exon 2 stands for the secreted endocrine form. Based on this hypothesis, class 1 transcripts may be interfering with secretion due to their longer signal peptide, while class 2 transcript contain a typical signal peptide motif, which is affiliated with effective secretion (Oberbauer, 2013).

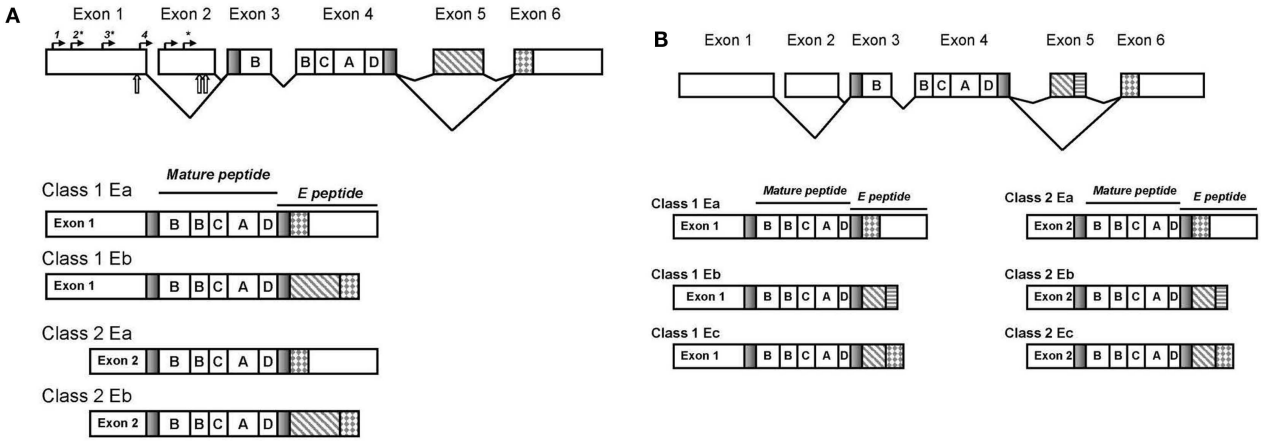

Figure 5. The IGF-1 gene structure [Oberbauer, 2013].

2. SPECIFIC CHAPTER

I. Purpose of the study

Numerous studies have been performed focusing on the association of IGF-I with male infertility. Although IGF-I isoforms' expression pattern have been found to vary in other pathological conditions, there is no scientific evidence to prove this statement regarding infertility. The aim of the present study is to investigate the existence of varying expression of the three IGF-I isoforms between normozoospermic men and men presenting with abnormal semen parameters following routine semen analysis, as well as revealing a possible association of a different expression pattern with male infertility.

II. Materials and Methods

II. A. Sample collection

The present prospective study was carried out between February 2019 and December 2019. Semen samples were collected from the subjects according to WHO criteria for semen collection and analysis (World Health Organization, 2010). The samples were provided by Genesis Athens Clinic. All subjects were informed about the purpose of the study and asked to sign a consent form for their participation. Samples were taken from the volunteers visiting the clinic for routine semen analysis. All samples collected were transferred to Laboratory of Physiology at Medical School of National and Kapodistrian University of Athens where the ejaculated sperm was prepared for spermatozoa isolation. A total of 40 men participated at the present study with a mean age 43.9 ± 4.637 and range from 32 to 54 years old. The samples were stratified into 4 groups: normozoospermic, single defective, double defective and oligoasthenoteratozoospermic (OAT). Normozoospermic group is comprised of 12 individuals, while single defect, double defect and OAT groups are comprised of 9, 12 and 7 individuals, respectively. Semen parameters of each individual are presented below (Table 3).

Table 3. Semen parameters of all participants and their categorization

Sample No.	Volume (mL)	Sperm concentration (million/mL)	Total sperm count (million/ejaculate)	Progressive motility (%)	Total motility (%)	Normal morphology (%)	Group
1	2	40	80	25	50	5	Single defect
2	3.5	35	122.5	40	50	5	Normozoospermic
3	2.5	30	75	20	30	10	Single defect
4	2.5	15	37.5	30	40	1	OAT
5	2	15	30	7	10	1	OAT
6	7	35	245	33	40	10	Normozoospermic
7	3	15	45	17	20	2	Double defect
8	3.5	20	70	15	30	3	Double defect
9	4.5	50	225	40	60	4	Normozoospermic
10	3.5	40	140	35	50	4	Normozoospermic
11	2.8	30	84	15	30	1	Double defect
12	12	18	216	14	20	2	Double defect
13	2.8	40	112	40	50	5	Normozoospermic
14	2	50	100	45	60	2	Single defect
15	3.5	10	35	16	20	3	OAT
16	7	10	70	6	10	1	OAT
17	2	50	100	45	60	5	Normozoospermic
18	6	30	180	27	30	2	Double defect
19	4.5	40	180	39	40	5	Normozoospermic
20	3.5	10	35	13	20	2	OAT
21	5	18	90	34	40	3	Single defect
22	2.8	40	112	47	70	5	Normozoospermic
23	3.5	30	105	15	30	2	Double defect
24	3	25	75	24	30	2	Double defect
25	2	30	60	30	40	2	Double defect
26	3	50	150	35	40	5	Normozoospermic
27	3	60	180	55	60	2	Single defect
28	2.5	25	62.5	11	15	2	Double defect
29	3.5	10	35	15	30	2	OAT
30	2	30	60	30	30	10	Single defect
31	2.5	20	50	35	40	2	Single defect
32	2.5	12	30	15	20	1	OAT
33	3	40	120	35	50	3	Single defect
34	3.5	30	105	35	50	10	Normozoospermic
35	5.5	20	110	12	20	2	Double defect
36	3.5	80	280	75	80	10	Normozoospermic
37	2.5	40	100	20	30	2	Double defect
38	4	15	60	23	30	3	Double defect
39	4	70	280	44	50	3	Single defect
40	2.5	80	200	67	80	10	Normozoospermic

II. B. Sample processing

Prior to spermatozoa isolation, it was determined whether seminal plasma is going to be kept according to the existing volume of each sample. If seminal plasma was to be kept, two aliquots of the original sample were made. One from which seminal plasma was obtained and one from which purified spermatozoa were obtained. For the separation of the seminal plasma an aliquot of the sample was centrifuged at 1000g for 10 minutes at room temperature (RT) followed by another centrifugation of the supernatant of the first centrifugation at 4000g for 20 min (RT). Seminal plasma was stored at -80^{o}C until further analysis. As for the aliquots destined for spermatozoa isolation, they were diluted with Phosphate-Buffered Saline (PBS) at 37^{o}C to a final concentration of 10x10^{6} spermatozoa per mL and underwent osmotic shock to eliminate the non-gamete cell component so as to enable the study of both motile and immotile sperm. Then, aliquots of 1mL of the diluted samples were centrifuged at 1600g for 10 minutes (RT). Pellets were incubated with 1mL of somatic cell lysis buffer (SCLB) (0.1% SDS, 0.5% Triton X-100 in distilled H_2O) for 60 minutes on ice (Paoli et al., 2017) which ensures the purity of the sperm RNA as somatic cells could contribute a significant proportion to the isolated RNA (Mao et al., 2013). Finally, the sperm suspension were again centrifuged at 200g for 15 minutes at 4^{o}C (Goodrich et al., 2007) and the pellets formed were homogenized in approximately 1 mL TRItidy and stored at -80^{o}C until RNA isolation protocol was performed.

II. C. Sperm RNA isolation and quality analysis

The spermatozoa suspended into the TRItidy were thawed in order to proceed with the RNA isolation protocol. Total RNA from spermatozoa was extracted using TRItidy protocol. The pooled sperm pellet was homogenized by pipetting the suspension up and down several times followed by a short incubation at room temperature which improves the separation of RNA/protein complexes. An amount of 200 μl was added to the lysate and mixed vigorously. Incubation of 10 minutes at room temperature follows. The lysate was centrifuged at 12.000g for 15 minutes at 4^{o}C. Following centrifugation, the mixture contained three well-formed phases. RNA remained exclusively in the aqueous, upper phase and transferred to a new reaction tube carefully, without disturbing the interphase. RNA was precipitated by adding the same volume of isopropanol to the aqueous phase and incubated for at least 15 minutes. Centrifugation at 12.000g for 15 minutes at +4^{o}C followed. Further, the pellet was washed twice with 1 mL of ethanol 100% followed by a centrifugation at 7500g for 5 minutes at +4^{o}C each time. The pellet was air-dried and dissolved in DEPC-treated water by gently pipetting. The organic phase/interphase with the DNA/protein were kept at +4^{o}C until the isolation was complete and then stored at -80^{o}C until protein isolation occurs. RNA concentration and purity were determined using a spectrophotometer (Shimadzu Corporation BioSpec-nano Life Science).

II. D. cDNA synthesis

Following RNA isolation, reverse transcription reaction was performed using the standard protocol of Protoscript II First Strand cDNA synthesis kit. In the process of reverse transcription, an RNA-dependent DNA polymerase called reverse transcriptase is necessary. This enzyme uses RNA as template to generate complementary DNA (cDNA) and it was discovered in retroviruses (Baltimore, 1970). The role of reverse transcriptase in retroviruses is related to the need of the RNA virus to integrate its genetic material into the host DNA chromosomes to replicate their genomes and thus complete its life cycle. Therefore, it needs both a template and a primer for its action (Hu and Hughes, 2012; Warren et al., 2009). The protocol used indicated that the RNA sample was mixed with primer $d(T)_{23}$ VN in sterile RNase – free microfuge tubes. The amount of each RNA sample was 0,5μg and the amount of $d(T)_{23}$ VN (50 μM) was 2 μl. To the mixture was added nuclease – free H_2O to a total volume of 8 μl. The RNA/ $d(T)_{23}$ VN was then denaturated for 5 minutes at 65^{o}C, was spinned briefly and put promptly on ice. The final volume of each mixture was 20 μl as 10 μl of Protoscript II Reaction Mix (2X) and 2 μl of Protoscript II Enzyme Mix (10X) were added. Enzyme mix included reverse transcriptase and RNase inhibitor while reaction mix contained dNTPs and an optimized buffer. The 20 μl of the cDNA synthesis reaction mix were incubated at 42^{o}C for 1 hour and then at 80^{o}C for 5 minutes for the inactivation of the enzyme. The cDNA product was stored at -20^{o}C till further processing.

II. E. Quantification of cDNA using Real-time polymerase chain reaction (qPCR)

II. E. 1. qPCR principle

The polymerase chain reaction (PCR) is a revolutionary method in the field of molecular biology since its discovery in 1983 by the biochemist Karry Mullis. It is an enzymatic method that allows in vitro proliferation of certain fragments of the original DNA sequence in multiple copies in a short period of time. Since *Escherichia coli* DNA polymerase I was replaced by a thermostable DNA Polymerase purified from a thermophilic bacterium, *Thermus aquaticus* (Taq), that can survive high temperatures (95^{o}C), the procedure has been simplified and automated. PCR reaction is performed in three major steps that make up each cycle in a PCR reaction, namely denaturation, annealing and extension. During the melting process of denaturation, the two strands of DNA are being separated in high temperature (usually 95^{o}C). The hydrogen bonds between the complementary bases of the double stranded DNA break and therefore single strands are produced and will be used for the synthesis of new DNA molecules. At the stage of annealing, temperature is lowered to allow the primers to hybridize to their complementary target sequences. Extension is the final step of the reaction, where DNA polymerase binds to the primers and then extends the primes under the optimal temperature of approximately 72^{o}C. The above steps are repeated 20-35 times in a Thermal Cycler, a device with a heated plated that can switch temperatures rapidly and accurately. The standard PCR technique allows the analysis of the product only at the

end of the reaction via agarose gel electrophoresis. PCR reagents include a set of primers for the targeted gene, DNA polymerase, deoxynucleotides (dNTPs), a buffer for the DNA polymerase and sterile water.

Real-time PCR is a variation of the traditional PCR technique in which a particular DNA (or RNA) sequence is simultaneously amplified and quantified using fluorescent probes or fluorescent DNA-binding dyes. Fluorescent DNA-binding dyes, such as SYBR Green, bind to any amplified product whereas probes bind more specifically. SYBR Green is a fluorescent monomeric cyanidine dye with high affinity for double-stranded DNA (Skeidsvoll and Magne Ueland, 1995). If a particular sequence is present in the sample, then at some point depending on its abundance in the sample, amplification will be observed. The fluorescence increases proportional to the amount of the amplicons produced and is measured by a machine (Fig. 6) which combines an excitation source to a thermal cycler (Pabla and Pabla, 2008).

Figure 6. A typical thermal cycler machine for RT-PCR

II. E. 2. Primer design

In the study, three sets of primers (Table 4) were used to amplify three different target mRNAs, IGF-1Ea, IGF-1Eb & IGF-1Ec. The design of these primers ensured specific detection of only one of the IGF-1 transcripts as they include sequences from different exons. These primers also assured the avoidance of amplification of a similar-sized product from infectious genomic DNA. 18S ribosomal RNA (rRNA) was used as a housekeeping gene. The specificity of the primers targeting the three isoforms is undoubtful since in previous study (Philippou et al., 2009) all q-PCR products were identified by sequencing analysis.

Table 4. Primers used for the target mRNAs and the housekeeping gene

Target mRNA	***PCR Primer Sequence***	***Product Size (bp)***
IGF-1Ea	5′-GTGGAGACAGGGGCTTTTATTTC-3′ 5′-CTTGTTTCCTGCACTCCCTCTACT−3′	*251*
IGF-1Eb	5′-ATGTCCTCCTCGCATCTCT-3′ 5′-CCTCCTTCTGTTCCCCTC−3′	*411*
IGF-1Ec	5′-CGAAGTCTCAGAGAAGGAAAGG-3′ 5′-ACAGGTAACTCGTGCAGAGC-3′	*150*
18s rRNA	5’ TCA AGA ACG AAA GTC GGA GG 3’ 5’ GGA CAT CTA AGG GCA TCA CA 3’	*449*

II. E. 3. RT-PCR

Depending on the number of samples (n) that were each time amplified, a (n+2)X reaction mixture was prepared as described on Table 5. The final volume of the reaction mixture for each sample was 20µl. After all samples were loaded, the plate was sealed with a film cover. The samples were amplified in a Thermal Cycler (Bio-Rad iCycler Thermal Cycler IQ5 Multicolor Real-Time PCR Detection System). A negative control with no template was used in each qPCR plate.

Table 5. Reaction mixture for RT-PCR

1X Reaction Mixture
10µl iTaq Universal SYBR Green Supermix (Bio-Rad)
1 µL forward Primer
1 µL reverse Primer
3 µL nuclease-free (NF) H_2O
5 µL cDNA (200ng)

The protocol used is as seen on the Table 6.

Table 6. Protocol used to amplify the target mRNAs in RT-PCR

	Stage	Temperature (^oC)	Time	Number of repeats
Cycle 1	Initial denaturation	95	4 minutes	1X
Cycle 2	Denaturation	95	12 seconds	45X
	Annealing	61	30 seconds	
	Extension	72	30 seconds	
Cycle 3	Final Extension	72	5 minutes	1 X
Cycle 4	Dissociation	65-95	12 seconds	61 X

II. F. Electrophoresis of RT-PCR products

II. F. 1. Electrophoresis principle

Agarose gel electrophoresis is a very common and effective laboratory practise used to separate nucleid acids based on their size. Nucleic acids are negatively charged due to phosphate chain, so they move through the agarose matrix toward the positive charged anode once electric field is applied. The shorter the nucleic acid is, the faster it will migrate through the matrix. Agarose is isolated from the seaweed Gelidium and Gracilaria, which

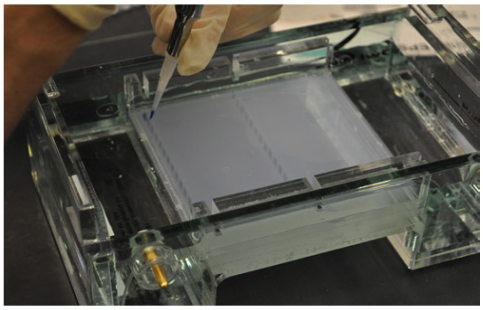

Figure 7. DNA sample is being loaded into a well in the agarose gel (Lee et al., 2012).

consists of L- and D- galactose subunits. During polymerization, these subunits form pores through which the fragments move. The size of the pores is contingent on the concentration of the agarose. The migration of the molecule through the agarose gel not only depends on its size but also on the agarose concentration, the voltage applied, the presence of ethidium bromide and the buffer. Samples are loaded with fluorescent dye in order to visualize them using a UV light source (Fig. 7). Along with the samples, an appropriate ladder should always be loaded as it allows the determination of the sizes of samples bands (Koontz, 2013; Lee et al., 2012).

II. F. 2. Electrophoresis of RT-PCR products

Amplification products were electrophoresed on a 2% w/v agarose gel. The gel was prepared by adding 1 gr agarose to 50 mL 0.5X TBE solution, a buffer containing Tris base, boric acid and EDTA (Ethylenediaminetetraacetic acid). To allow the visualization of the DNA, 1 μl of ethidium bromide was added to the gel prior to its solidification. The ladder of known molecular weight used was the Quick-Load Purple 100bp DNA ladder (New England BioLabs). The DNA samples were mixed with loading dye with a 5:1 ratio. Samples were electrophoresed at 100 Volts for 30-35 minutes at a electrophoresis apparatus (Biorad PowerPac Basic). DNA was observed by exposing the gel to a chamber with ultraviolet light (GelDoc – It^2 Imager).

II. G. Protein isolation from isolated spermatozoa

In order to proceed with western blotting technique, the remaining organic phase/interphase with the DNA/protein from the RNA isolation protocol was used to isolate proteins from spermatozoa. Thus, the residual upper aqueous supernatant was removed and 300 μl of ethanol was added and the reaction tube was repeatedly inverted and incubated for approximately 5 minutes at room temperature. The mixture was then centrifuged at 2.000g for 5 minutes at +4^{o}C and proteins were precipitated from the phenol/ethanol mixture (supernatant) resulted from the centrifugation. The double sample volume was added to the supernatant and an incubation time of 10 minutes at room temperature followed. The mixture centrifuged at 12.000g for 10 minutes at +4^{o}C and the protein precipitate was washed with 2 mL of 300mM guanidine hydrochloride in 95% ethanol and then incubated for 20 minutes at room temperature. Following incubation period, the mixture was centrifuged at 7.500g for 5 minutes at +4^{o}C. The stages from washing till the centrifugation was repeated two more times. The final step was the air-dry of the protein precipitate after removal of the supernatant and the dissolution of the precipitate in 1% SDS. The extracts were analyzed for total protein concentration using the BCA (bicinchoninic acid) Protein Assay kit and measured spectrophotometrically. The proteins were stored at -20^{o}C.

II. H. Western blot

II. H. 1. Western blot principle

Western blotting (or else known as immunoblotting) is a procedure used for detection and identification of proteins. Five are the major steps of this technique: (1) the separation of the protein mixture based on their molecular weight through SDS – polyacrylamide gel electrophoresis (PAGE), (2) transfer of the separated proteins to an appropriate adsorbent blotting membrane, (3) blocking of the unreacted sites of the membrane with non-fat dry milk, (4) incubation of the membrane with antibodies specific to the protein of interest and (5) develop of the film for the visualization of the protein band. The membranes mostly used are nitrocellulose and PVDF (polyvinylidene difluoride) membranes. The thickness of the band, meaning the intensity of the signal produced, corresponds to the abundance of the protein on each sample tested (Kurien and Scofield, 2006; Yang and Mahmood, 2012).

More specifically, the molecules loaded to a porous matrix (gel) are negatively charged and thus migrate toward the anode (+) when an electrical field is created. The main reagents of a polyacrylamide gel are acrylamide and bisacrylamide, Tris, a polymerization initiator, APS (ammonium persulfate) and a polymerization catalyst, TEMED (tetramethylethylenediamine). APS and TEMED, due to their function, must be added last in the gel preparation. The polyacrylamide gel is a discontinuous gel meaning that consists of two stacked layers of gel, the separating gel and on top of that, a stacking gel. For the preparation of the separating gel, a high concentration solution of Acry/Bis - Acrylamide is used whereas the stacking gel is made of a lower concentration of Acry/Bis. These two layers of gel have different pH also. The pH of a stacking gel is 6.8 and its purpose is to collect all the proteins into a single band while the pH of the separating gel is 8,8 with the function to separate proteins by size. The samples are loaded into the wells which are created with a use of a comb prior to the polymerization of the gel. The proteins are loaded in the gel by the addition of a loading buffer consisting of a sample buffer and a reducing agent. A reducing agent is actually a detergent, SDS, that denaturates the proteins and gives them negative charge and β-mercaptoethanol which reduces the disulfide bonds of the proteins, creating thus linear chains of polypeptides. Therefore, the addition of the reducing agent ensures the separation of the proteins according to their molecular weight. The sample buffer is used for the visualization of the samples during their migration through the matrix. Among the samples in the gel, a marker of known molecular weight is used to define the size of the proteins running. When the gel is prepared, it is placed on a electrophoresis apparatus and then running buffer (Tris, SDS, Glucine) is added. The gel runs under the appropriate conditions until the dye reaches the bottom of the gel. The next step is to transfer the proteins from the gel to a surface where the binding site of the protein is exposed. The surface is a membrane, usually a nitrocellulose or a PVDF (polyvinylidene difluoride) membrane. The transfer occurs under electrical field (electrotransfer) where proteins from the gel migrate to the membrane and they are both immersed in transfer buffer (Tris, Glucine, Methanol) (wet transfer). Methanol is used

in the transfer buffer to facilitate the binding of the proteins to the membrane by detachment of the SDS molecules from the proteins. Once the electrotransfer is done, proteins are supposed to have moved on the membrane. To avoid nonspecific binding of the antibodies and thus the background signal, the membrane is incubated in a blocking solution after transfer. The blocking solution (nonfat dry milk, casein, bovine serum albumin) binds to the non-specific antibody binding sites of the membrane. Following the blocking step, a primary unlabeled antibody is first used to bind directly against the target protein and then a species-specific secondary antibody coupled with a fluorescent or chemiluminescent molecule binds to the primary antibody. However, after primary antibody probing, the wash of the membrane is necessary to remove excess antibody. A low concentration non-ionic detergent solution, such as Tween-20 in TBS (Tris-buffered saline) is used and the washing is repeated at least 3 times for 5 minutes per wash. The last step of western blotting is the detection that depends on the type of secondary antibody that has been used. The most commonly used enzymatic detection system is chemiluminescence, based on horseradish peroxidase (HRP)-labeled antibodies. After the addition of a luminol peroxide detection reagent, the HRP catalyzes the oxidation of the luminol and generates light emission. The light signal is detectable on X-ray film that must be digital for further computer analysis ("Western Blotting Principles and Methods," n.d.).

II. H. 2. Western blot analysis of seminal plasma and spermatozoa

Seminal plasma and isolated proteins from spermatozoa were loaded with loading buffer on polyacrylamide gel, consisting of 20% separating gel and 4% stacking gel. The final volume of the sample loaded was 40 μl. Several different quantities of seminal plasma were loaded starting from 100 μg, then 150 μg, 300 μg, 600 μg, 750 μg, 850 μg and at last 900 μg. Likewise, proteins isolated from spermatozoa were loaded in four different quantities, namely 6.8 μg, 8.6 μg, 11 μg & 13 μg. The quantity of positive control, namely Ec peptide, was 50 ng. Two different ladders were used, the Precision Plus Protein™ Dual Color Standards (Bio-Rad) and the Color Prestained Protein Standard, Broad Range (11–245 kDa) (New England BioLabs).
The gel run on a vertical electrophoresis apparatus for about 4 hours and 30 minutes. Following electrophoresis, a sandwich was created in which the gel was in direct contact with a PVDF membrane, covered with filter papers and sponges to help compress the sandwich. The PVDF membrane was merged in methanol for 1 minute for its activation and then washed with distilled H_2O and transfer buffer. The sandwich cassette was placed in a tank filled with transfer buffer where a magnet was placed. The whole tank was put on a magnetic stirrer to circulate the buffer during transfer and connected to the power supply at 100V for 1 hour and 45 minutes at $+4^oC$. Blocking procedure occurred for 1-2 hours in 5% milk in TBS-T. Following blocking, anti-IGF-IEc primary antibody was used in 5% milk in TBS-T (1: 10.000 dilution) for the detection of the corresponding isoform. An amount of 10 μl of the HRP anti- rabbit secondary antibody was added in each membrane after 3 washes of the primary antibody with TBS-T (10 minutes/wash). The secondary antibody was an Anti-Rabbit

antibody specific for the primary antibody used. The ratio of the secondary antibody was 1:3.000 in 5% milk in TBS-T. Two washes of 10 minutes each with TBS-T were followed and one of 10 minutes with TBS-1X prior to the detection which occurred in a dark room with the help of ECL (SuperSignal™ West Pico PLUS Chemiluminescent substrate, Thermo Scientific). A positive control was used each time in the western blot technique, namely 30 μg of total proteins from human muscle.

II. I. Statistical analysis

The statistical analysis was conducted using R programming language for statistical computing version 3.6.1 (R Core Team, 2019). Normality test Shapiro-Wilk was initially performed to examine if the data follow the normal distribution. Non parametric Kruskal – Wallis test was used to compare the four groups due to lack of normal distribution of the variables. The Dunn's test used to analyze the specific pairs of groups. For the correlation of the semen parameters with the mRNA levels of IGF-I Ec, Spearman's (rho) rank and Kendall's tau correlation coefficient were used. The level of statistical significance was set as p-value= 0.05, meaning there is 5% risk that the observed difference does not actually exist.

III. RESULTS

III. A. Expression of IGF-I isoforms at mRNA level

Real-time PCR revealed expression of IGF-IEc mRNA at the spermatozoa level whereas IGF-IEa & IGF-IEb mRNAs were not detectable, except for one individual that showed expression of IGF-IEa mRNA. The amplification charts for IGF-IEc and 18S are seen in the figures 8A & 8B respectively. The statistical analysis revealed a statistical significant difference of the expression between the groups "Normozoospermic" and "Double Defect" (p-value = 0.0117*) and between "Normozoospermic" and "OAT" (p-value = 0.0072*) (Fig.9), showing that expression of IGF-IEc mRNA in "Normozoospermic" men were lower than the other three groups (Table 7).

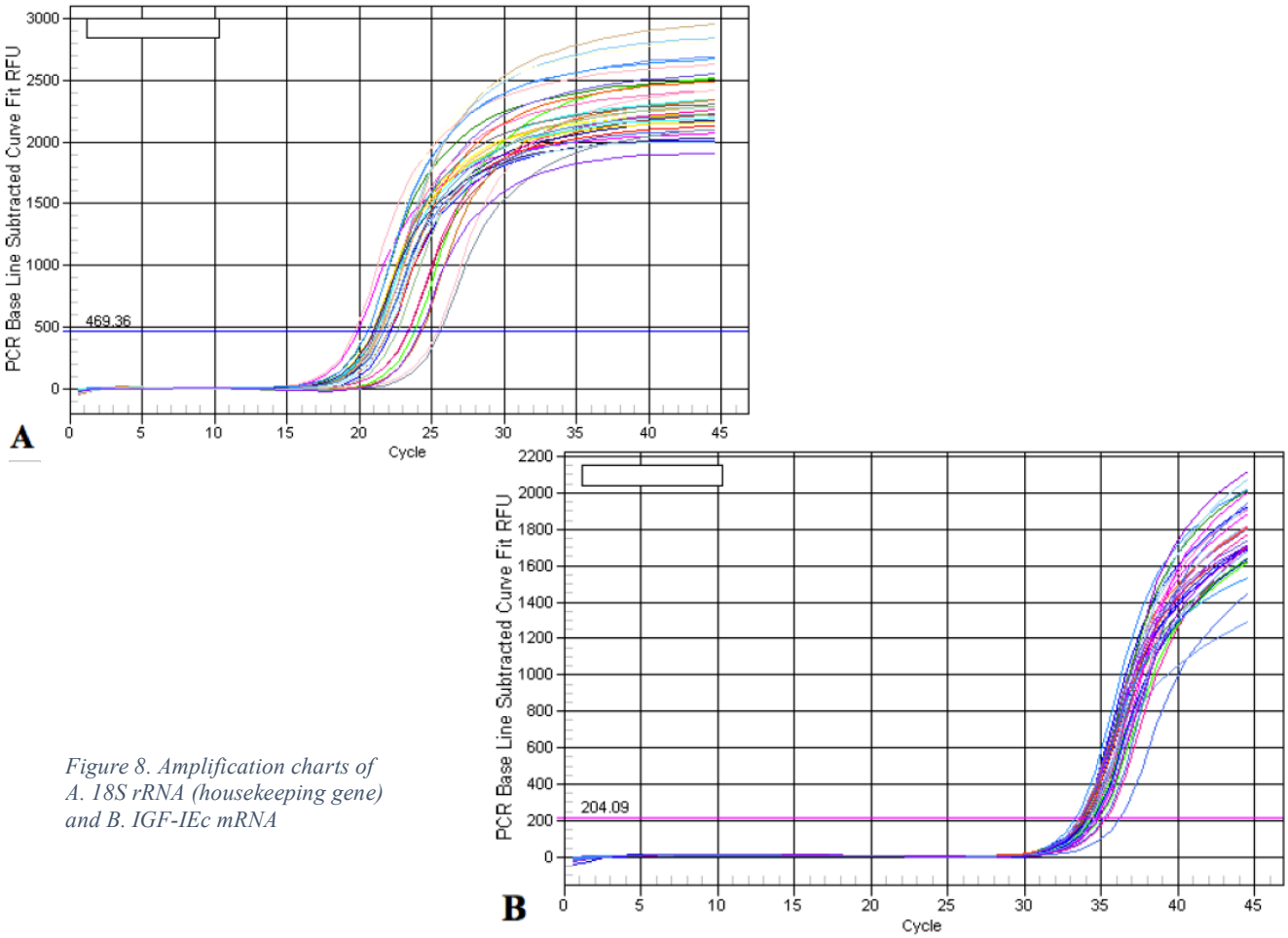

Figure 8. Amplification charts of A. 18S rRNA (housekeeping gene) and B. IGF-IEc mRNA

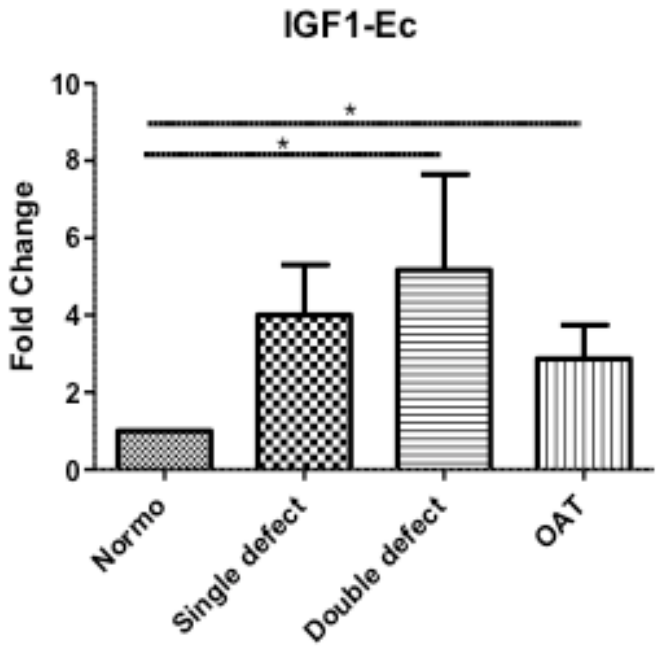

Figure 9. Histogram of IGF-IEc mRNA levels in each group

Table 7. Mean fold changes of each group studied

Groups	Mean fold change ± SD
Normozoospermic	1
Single Defect	4 ± 3.9
Double Defect	5.1 ± 8.5
OAT	2.9 ± 2.3

Moreover, IGF1-Ec mRNA levels were analyzed in relation to semen volume, sperm concentration, total sperm count, progressive & total motility, and morphology. A statistically significant negative correlation was found between IGF-IEc mRNA

levels and total sperm count (Fig. 10A), progressive motility (Fig. 10B) and total motility (Fig. 9C). No additional correlations were found between the levels of IGF-1Ec transcript and other conventional sperm parameters (Table 8).

Table 8. Results of the correlation analysis. The levels of statistical significance are defined as (*) when p=value is below 0.05, (**) when p-value is below 0.01 and (***) when p-value is below 0.001

Variable	IGF-IEc mRNA	p-values
Volume (mL)	**r = -0.1289675** **τ = -0.105276**	**0.4277** **0.3756**
Sperm concentration (million/mL)	**r = -0.2620722** **τ = -0.1934128**	**0.1023** **0.09999**
Total sperm count (million/ejaculate)	**r = -0.3895225** **τ = -0.2453872**	**0.01298*** **0.03213***
Progressive motility (%)	**r = -0.4505146** **τ = -0.3138845**	**0.003532**** **0.006393****
Total motility (%)	**r = -0.3790446** **τ = -0.2732595**	**0.01586*** **0.02303***
Normal morphology (%)	**r = -0.305244** **τ = -0.2107348**	**0.05545** **0.08546**

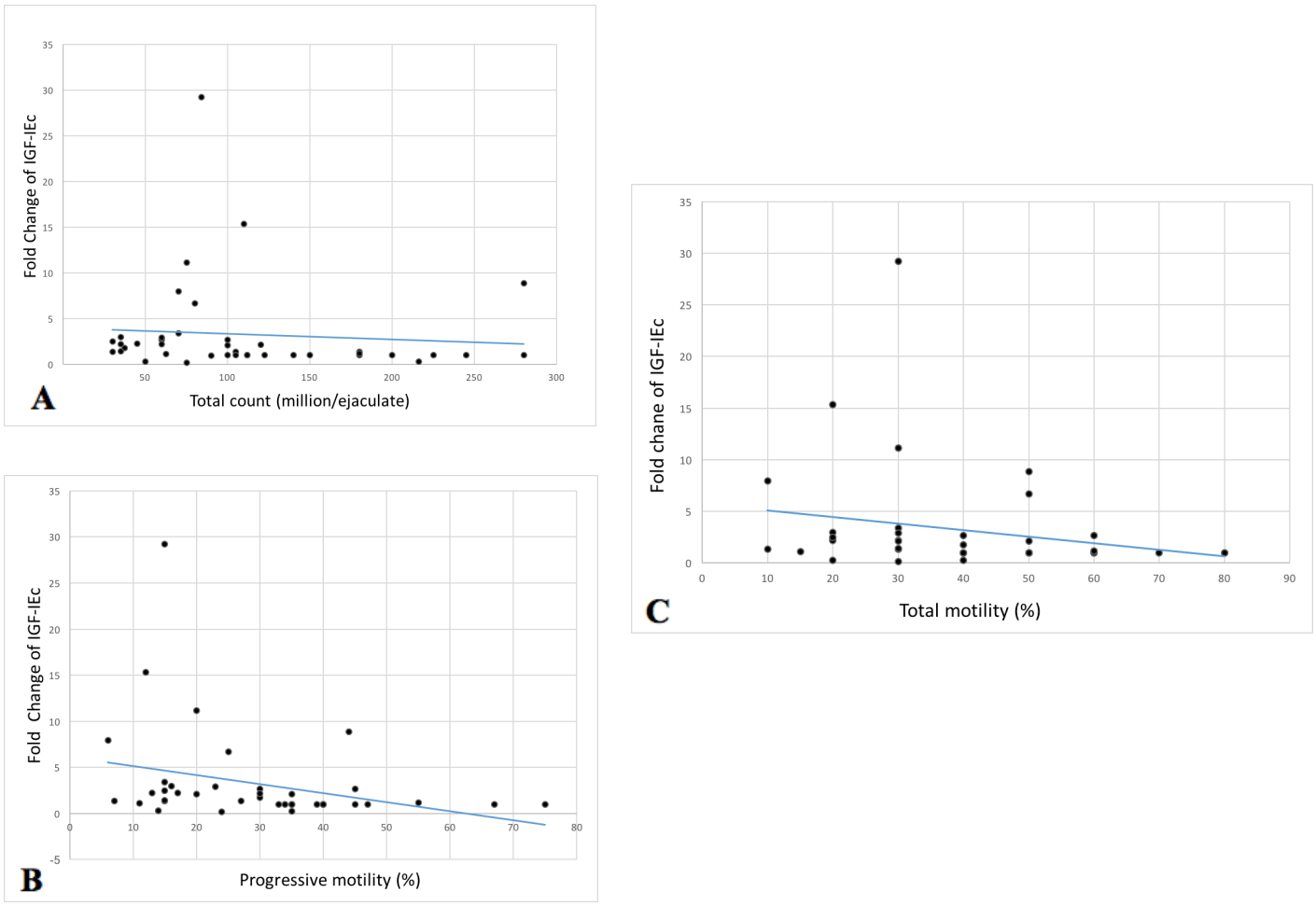

Figure 10. Correlation analysis of sperm IGF-IEc mRNA levels with total sperm count (A), progressive motility (B) and total motility (C).

III. B. Electrophoresis of RT-PCR products

Electrophoresis of the qPCR products confirmed the expected sizes of the amplified cDNAs, namely the 18S rRNA and IGF-I Ec (Fig. 11A). In the Figure 11B, it is clear that the size of the product of individual that seemed to have expressed IGF-IEa mRNA, indeed corresponds to it. Two different individuals coming from different pathologies (Normozoospermic and OAT) seem that may have expressed the IGF-IEb mRNA, athough the band is quite faint to ensure that the amplification was specific (Fig. 10C).

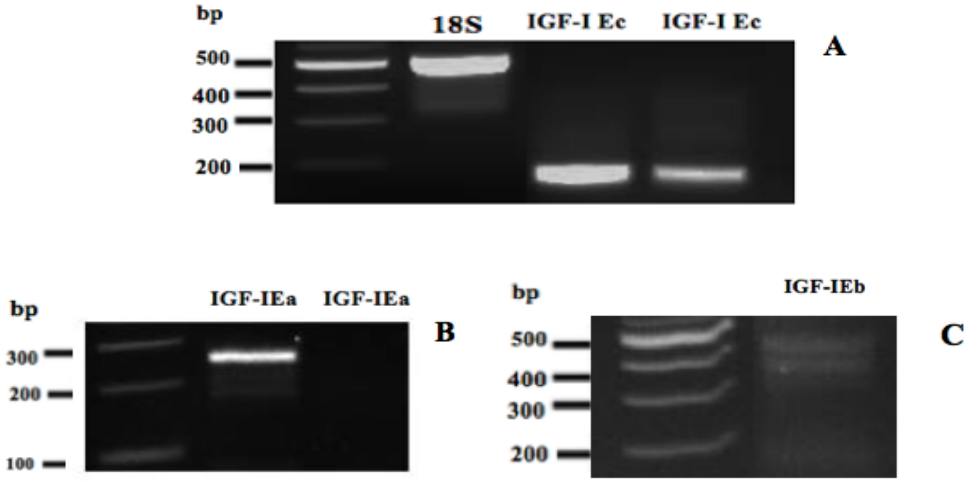

Figure 11. Electrophoresis analysis of the qPCR products

III. C. Expression of IGF-I isoforms at protein level

Although we tried different quantities of protein loading, western blotting of the proteins derived from spermatozoa (6.8µg, 8.6µg, 11µg and 13µg) and the seminal plasma (100µg, 150 µg, 300 µg, 600 µg, 750 µg, 850 µg, 900 µg) using the anti-human Ec polyclonal antibody did not reveal any band. However, proteins derived from human muscle (30µg) disclosed a band which was assumed to correspond to pro-IGF-1Ec and among Ec peptide (50ng) was used as a positive control in this procedure. Ec peptide was detected in a band of molecular weight below 11kDa (Fig. 12).

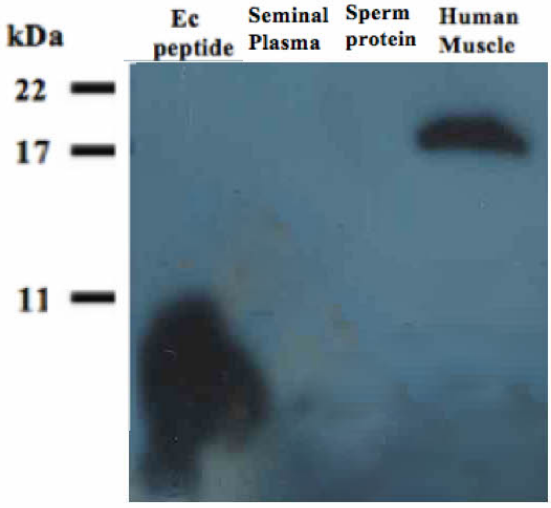

Figure 12. Western blot analysis showing detection of IGF-IEc isoform in Human Muscle (30µg) and of Ec peptide (50ng) but no signal at seminal plasma (900µg) and sperm protein (appr. 10µg)

DISCUSSION

Infertility affects 12-15% of couples of reproductive age worldwide, while in 50% of the cases are due to male factor (Vander Borght and Wyns, 2018). Nonetheless, physiological processes regarding male reproductive system are complex and require intensive and continuous research to discover the underlying causes associated with male infertility. For nonce, clinicians are managing male infertility mostly depending on semen analysis while studies are being performed in the field of genetics (e.g. Microarray-based SNP analysis), metabolomics (e.g. formation of reactive oxygen species and its impact on spermatogenic abnormalities) and proteomics (e.g. Heat shock protein 2) to identify novel biomarkers for male infertility (Kovac et al., 2013).

During the last two decades, IGF-I has been studied a lot for its anabolic effects on the male reproductive system. IGF-I's involvement in the development of germ cells, its influence in steroidogenesis, cell proliferation and differentiation and the regulation of spermatogenesis have been widely reported (Colombo and Naz, 1999). Although IGF-I is synthesized mainly in the liver, it is also found in the testis where it is secreted by Leydig and Sertoli cells and stimulates spermatozoa maturation (Lee et al., 2012). Thus, the role of IGF-I in fertility is unequivocal and its deregulation may cause infertility. Regulation of IGF- I expression is sensitive since it includes the alternative splicing of mRNA generating three isoforms of the original IGF-I molecule (Brązert and Pawelczyk, 2015).

Due to the involvement of IGF-I in the male reproductive system, several studies have investigated the association of its levels in seminal plasma and/or serum with semen quality. However, the conclusions appear to be contradictory. According to the study conducted by Colombo and Naz, IGF-I was detected in the seminal plasma of both fertile and infertile men. Infertile men with oligospermia had the lowest levels of IGF-I, indicating a correlation between low levels of IGF-I and oligospermia, while infertile men with antisperm antibodies had the highest levels of IGF-I. A possible explanation is that a defective IGF-I system could amend spermatogenesis through impaired cellular growth and differentiation resulting in oligozoospermia (Colombo and Naz, 1999). Another study performed by Lee and colleagues (Lee et al., 2016), found that IGF-I levels in seminal plasma did not differ significantly between the group with normal parameters and the groups with abnormal motility, abnormal morphology and with 2 or more abnormal parameters. However, serum IGF-I levels of the above three groups were found to be significantly lower in relation to the group with normal parameters. A third study showed that levels of IGF-I in seminal plasma did not seem to be associated with either of the semen parameters (Simopoulou et al., 2018). Yet, a significant correlation between IGF-I levels in seminal plasma and the morphologically normal spermatozoa and their concentration was reported by Glander and colleagues (Glander et al., 1996), possibly indicating that IGF-I functions as a marker of

differentiation of the male germ cells. Another study (Hassan et al., 2009) found a correlation between low levels of IGF-I and male infertility in men presenting with oligoasthenoteratozoospermia, leading to a recent case report (Mora Rodríguez et al., 2019) which showed that daily intradermal injections of IGF-I for 2 months significantly improved the quality of semen parameters in an OAT patient. Contrary to the study conducted by Hassan and colleagues (Hassan et al. 2009), patients with varicocele showed greater levels of seminal IGF-I compared to controls and when these patients underwent varicocelectomy, the levels of IGF-I diminished significantly while the semen parameters demonstrated vast improvement (Naderi et al., 2015). Furthermore, research by Baxter and colleagues (Baxter et al., 1984) reported about 60% lower IGF-I leves lower in azoospermic individuals and in men who had ejaculatory duct resection compared to those with normal semen parameters. Complementary to the previous study, seminal levels of IGF-I were found greatly reduced after vasectomy, indicating once more that a high proportion of IGF-I is of testicular origin (Ovesen et al., 1995).

Although there are few studies investigating IGF-I levels and its correlation with semen quality and male infertility in general, there is no study examining the expression of insulin-like growth factor –I isoforms in human spermatozoa. Despite this gap in the literature regarding the expression of IGF-I isoforms in semen and their association with male infertility, it was reported an association of the IGF-I isoforms and their expression in endometriosis (Milingos et al., 2006) and patients presenting with PCOS (Brązert and Pawelczyk, 2015), thus concerning the female reproductive system. The existing literature reports a different pattern of expression of IGF-I isoforms in various pathological conditions in humans such as endometriosis, colon and cervical cancer, skeletal muscle and prostate damage (Oberbauer, 2013; Philippou et al., 2014).

In light of the above findings, the present study was conducted to investigate the expression of IGF-I isoforms in human semen and whether there is a differential expression between the three isoforms that could possibly interpret with different defects in the semen parameters and thus male infertility. The results revealed that IGF-IEc mRNA is present in all 40 men included regardless their semen pathology, however, the Ec isoform was not detectable at protein level given the amount of protein available, and this may be due to low expression of the total IGF-1 in semen. Moreover, IGF-IEa mRNA was not expressed except for a single individual which appears to be related to the subject's temperament as any possible technical problems were excluded. A larger sample size and more patient background information is required in order to clarify whether amplification of IGF-IEa is random or a specific pattern of expression exists. The third isoform, Eb, does not seem to have been expressed in any of the subjects. A faint band was present in the agarose gel though specific amplification of IGF-IEb isoform is uncertain.

As far as IGF-IEc concerns, it was found an overexpression in the groups “Single defect”, “Double defect” and “OAT” in comparison to “Normozoospermic”. Results were statistically significant between the group with double defect and normozoospermic men as were between normozoospermic and OAT group. A marginally nonsignificant difference between the “Single defect” group and the normozoospermic was also reported. The fact that difference only occurred in men with 2 or more abnormal parameters, as reported from the semen analysis, requires further research to investigate the specific semen parameters affected by the overexpression of the IGF-IEc and enlighten the particular processes in which IGF-IEc is involved. Furthermore, the levels of IGF-IEc are negatively correlated with statistical significance with total sperm count, progressive and total motility, indicating that high levels of IGF-IEc mRNA could negatively affect spermatogenesis and maturation process of the spermatozoa.

IGF-IEc seems to be the most abundant isoform in spermatozoa and probably mediates action of total IGF-I. A limitation of this study is the lack of proof of fertility for subjects in the normozoospermic group. Thus improved fertile male individuals may be needed for comparison of IGF-IEc levels. Furthermore, it is common knowledge that protein expression differs from gene expression and mRNA does not always translate into protein. As mRNA levels were examined in this study, possible differences on proteomic level were not discoverable. Future studies must focus more on proteomic analysis as it has higher potential to provide clinicians with tremendous infertility-specific biomarkers. To the best of our knowledge, there has been no other study identifying the different IGF-I transcripts in human spermatozoa and the present study has been the first in this field of research.

In conclusion, this study demonstrates the expression of IGF-I isoforms at mRNA level for the first time. IGF-IEc transcript is present in all of the subjects and its mRNA levels in the “Single defect”, “Double defect”, “OAT” groups are elevated compared to the normozoospermic group. IGF-IEc may mediate all functions of IGF-I in sperm. Moreover, IGF-I mRNA levels are negatively correlated with total sperm count, progressive motility and total motility. The exact pathophysiologic function and the molecular mechanism of IGF-I isoforms regarding male infertility are in need of further investigation. Research on the influence of the locally produced IGF-I isoforms on semen quality and spermatogenesis is also required for the idea of obtaining novel biomarkers to identify and diagnose male factor infertility to become more realistic.

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