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# Genetic aspects of male infertility

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## Summary

**Objectives.** Genetic causes can be directly responsible for various clinical conditions of male infertility. In this review, we shall discuss the various genetic causes that have acquired considerable importance from the viewpoint of infertility, whether male and/or of the couple, thus contributing to accomplishing more accurate diagnoses.

**Material and methods.** We performed a review of published in scientific journals and literature using as key words “genetic causes” “male infertility”, “spermatogenesis”, “medically assisted procreation techniques” and similar words.

**Results.** It is known that 10-15% of cases of azoospermia and severe oligozoospermia are genetically-based, represented mostly by Klinefelter’s syndrome (KS) and by the microdeletions of the Y chromosome. The prevalence of KS among infertile men is considerably high: up to 5% in the cases of severe oligozoospermia and up to 10% in cases of azoospermia. The microdeletions of the AZF region, the second most common cause of male infertility, determine a severe primitive testiculopathy with consequent azoospermia or severe oligozoospermia. With regard to the chromosomal alterations, the predominating anomalies are those associated with the sex chromosomes. However, a wide range of structural autosomal anomalies has been identified such as Robertsonian and reciprocal translocations, inversions, duplications and deletions, that can be associated with infertility. Lastly, mutations in single genes can be directly responsible for male infertility, such as the gene CFTR which, once mutated, causes cystic fibrosis and the ‘non-formation’ of the vas deferens; the gene KAL 1, which is responsible for Kallmann’s syndrome; the gene which codifies the receptor of androgens that, once mutated, causes syndromes of insensitivity to androgens and alterations of spermatogenesis and lastly the genes INSL3-RXFP2, the mutations of which are associated with anomalies in the descent of the testicles, as in cryptorchidism.

**Conclusion.** Genetic anomalies have assumed enormous clinical importance, not only because they may cause infertility in the offspring, but also and mainly because they are capable of causing more severe illnesses. The high frequency of genetic alterations in infertile couples demands appropriate and correct diagnosis of these patients in order to reduce the risk of transmitting genetic anomalies to the offspring. Whether to undertake genetic investigations and if so, which in particular, may be indicated through detailed clinical assessment.

## Key words

*Genetic causes • Male infertility • Spermatogenesis • Medically assisted procreation techniques*

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## Introduction

Male infertility represents an ever-increasingly current and complicated problem in view of the fact that many causes may concur to present this condition. Only recently have the biological processes at the base of spermatogenesis been studied, contributing significantly to a wider comprehension of male fertility, emphasizing how genetic and molecular causes can be directly responsible for the different clinical aspects of male infertility.

Current estimated figures indicate that 15% of couples in Western countries have problems with infertility <sup>1</sup>, and in half of these cases the responsibility may be attributed to a male factor <sup>2</sup>. Genetic anomalies are present in a high percentage of infertile males (15%) and females (10%) <sup>3</sup>.

In the male, genetic alterations are most frequently found in those patients with more severely impaired spermatogenesis who are as such candidates for medically assisted procreation techniques <sup>4,5</sup>.

Genetic anomalies acquire significant clinical importance not only because they may cause infertility in the offspring but also, and mainly because they are capable of generating more serious illnesses. In this regard, some studies would seem to indicate an increase in chromosomal alterations in

foetuses and in children conceived following intracytoplasmic injection of the spermatozoan (ICSI). Table I presents the data relating to malformations found in children born after implementation of ICSI, as reported in several follow-up studies <sup>4</sup>.

This explains the importance of identifying genetic factors in routine procedures for an appropriate treatment of infertile couples.

Furthermore, patients affected by some forms of genetic alterations produce more frequently spermatozoa with aneuploidies, that is, spermatozoa carrying numerical chromosomal aberrations due to the presence of a number of chromosomes that differs from the normal condition. The spermatozoon, which is a haploid cell, normally contains a copy of each chromosome, so examples of spermatogenic aneuploidies are nullisomy, disomy or trisomy, etc. Genetic aneuploidies are more frequent in subjects in whom the spermatogenesis is more severely impaired and thus in patients with severe oligozoospermia <sup>5-7</sup>. The presence of spermatogenic aneuploidies, or gametic aneuploidies in general, may lead to the constitution of an uneven chromosomal set in the foetus, giving rise in turn to various clinical conditions, depending on the chromosome involved as well as on the type of aneuploidy. These

Table I. Data relating to malformations found in children born after ICSI <sup>4</sup>.

COUNTRY	AUTHOR	YEAR	SAMPLES	MAJOR MALFORMATIONS %	MINOR MALFORMATIONS %
Belgium	Bonduelle	1996	ICSI (423)	3.3	20.5
Australia/Belgium	Kurinkzuk	1997	ICSI (420) (Naturali 100,454)	7.4 3.8	0.7 0.5
Belgium	Bonduelle	1999	ICSI (1987) IVF (130)	2.3 4.6	- -
Europe	ESHRE	1998	ICSI (807)	2.0	-
Denmark	Loft	1999	ICSI (738)	2.2	1.2
England	Sutcliffe	1999	Natural (123) U.K. Registry ICSI (123)	4.1 5.0 4.9	7.3 - 11.0
Sweden	Wennerholm	2000	ICSI (1139)	3.2	3.5
USA	Palermo	2000	ICSI (3573) FIVET (3277)	1.1 1.7	0.8 1.3
Germany	Ludwig	2000	ICSI (2809) Register	9.1 7.2	- -
Australia	Ansen	2002	ICSI (301) FIVET (837) Natural (4000)	8.6 9.0 4.2	0.3 0.8 0.6
Holland	Anthony	2002	ICSI + FIVET (4224) Register (314.605)	0.7 0.5	1.3 1.1

ICSI: intracytoplasmic injection of the spermatozoan; FIVET: fertilization in vitro and embryo transfer IVF: in vitro fertilisation.

Table II. Frequency of the most common genetic anomalies correlated with male infertility and associated phenotypes <sup>8</sup>.

GENETIC ANOMALIES	PHENOTYPE	PREVALENCE
<i>Chromosomal alterations</i>	From azoospermia to normozoospermia	2-10%
Klinefelter's syndrome	Azoospermia - severe oligozoospermia	5-10% azoospermia
Other alterations of the sex chromosomes	From azoospermia to normozoospermia	2-5% severe oligospermia
Robertsonian Translocations	Azoospermia - severe oligozoospermia	0.1-0.2%
Reciprocal Translocations	Azoospermia - severe oligozoospermia	0.5-1%
		0.5-1%
<i>Deletions and microdeletions of the Y chromosome</i>	Azoospermia - severe oligozoospermia	5-10%
AZFa	Azoospermia - SCOS	0.5-1%
AZFb	Azoospermia - arrest of spermatogenesis	0.5-1%
AZFc	Azoospermia - severe oligozoospermia	3-7%
AZFb-c	SCOS - arrest of spermatogenesis	0.5-1%
<i>Partial Deletions of AZFc</i>	From azoospermia to normozoospermia	3-5%
<i>Genetic mutations</i>		
KAL-1	Hypogonadism hypogonadotrope	60-70% (5% in infertile subjects)
CFTR	Obstructive azoospermia	4-5%
AR	Azoospermia - oligozoospermia	2-3%
INSL3-RXFP2	Cryptorchidism	4-5%
INSL3-RXFP2	Criptorchidismo	

conditions range from abortion to the manifestation of several known syndromes that in this case occur as sporadic syndromes.

The genetic causes of male infertility may include chromosomal alterations and mutations in single genes <sup>8</sup>. The aim of this article is to accurately revise the literature on the known genetic causes (Table II) responsible for the more or less severe clinical conditions of male infertility, that make up the diagnostic course of the infertile patient.

### Chromosomal alterations and male infertility

In about 5% of cases of male infertility, the cause may be attributed to chromosomal alterations; this value reaches 15% of azoospermic males who, in most cases, are represented by patients with aneuploidy 47, XXY (Klinefelter's syndrome – KS). This syndrome was identified by an American clinician, Dr. H.F. Klinefelter, who described this illness for the first time in 1942. Klinefelter's syndrome represents the most common known genetic cause of male infertility. The prevalence of KS among infertile males is very high: up to 5% in cases of severe oligozoospermia and up to 10% in cases of azoospermia <sup>4 5 11 12</sup>.

KS is an endocrine pathology characterised by fibrous testicles of reduced dimensions, bilateral

gynecomasty, hypergonadotropic hypogonadism and altered metabolism of the sex steroids, secondary to poor haematic concentration of androgens <sup>9</sup>, which represents the most common form of male hypogonadism.

In 80% of cases the karyotype that is responsible for KS is 47,XXY. The remaining quota of patients present more numerous aneuploidies such as 48,XXXY or 49,XXXXY, characterised by more severe clinical forms, while a more restricted percentage of patients presents a chromosomal mosaicism 46, XXY/47,XXXY with a highly variable phenotype in proportion to the fraction of cells that present a supernumerary X <sup>13</sup>.

The supernumerary sex chromosome derives from an erroneous disjunction in the gametes of the parents during spermatogenesis or oogenesis, while the mosaics are the result of errors of mitotic origin occurring in the first stages of division of the embryo. Numerical alterations of the chromosomes are often the results of diploidy of the oocyte, in most cases due to advanced maternal age, and may be cause for KS <sup>14</sup>. An important number of cases of KS correlates instead with disomy of the X present in the spermatozoa and according to other authors, the quota of paternal co-responsibility comes close to that of the mother <sup>15</sup>. While some authors consider the age of the parents as a risk factor for KS to be irrelevant <sup>6</sup>, some recent studies correlate the

increase in maternal age with a higher incidence of the syndrome<sup>16</sup>. Most patients with KS are affected with azoospermia<sup>17</sup>.

In the samples analysed by Ferlin et al., it emerged that 72 out of 94 KS patients (76.6%) with no manifestation of mosaicism present complete azoospermia, while the remaining patients present spermatozoa in the ejaculate<sup>8</sup>. Thus it would seem plausible that a percentage of males with KS can present residual spermatogenesis in some seminiferous tubules, as demonstrated by the descriptions in literature of pregnancies obtained through natural conception with relatively young KS partners presenting severe oligospermia<sup>16</sup>. In rare cases some residual foci of incomplete spermatogenesis remain active because they are blocked during the early phases of the first meiotic division<sup>17</sup>, in which meiosis may occur. This is even more frequent in the cases of mosaicism 46,XX747,XXY.

Males with KS produce an elevated number of aneuploidal spermatozoa in response to the altered karyotype of the spermatogones or to meiotic non-disjunctions induced by the particularly hostile and impaired testicular environment typical of this syndrome<sup>13</sup>. In the gametes of a patient affected with KS the frequency of the number of disomies XX ( $4.6 \pm 2.6\%$ ) and XY ( $10.0 \pm 5.9\%$ ) has been found to increase significantly while the disomy YY manifests within a normal range of variability<sup>17</sup>. In cases of residual spermatogenesis, some techniques of assisted fertilization may provide KS patients with the possibility of having children of their own. If some immature gametes are present in the testicles, more specifically in the seminiferous tubules, these may be extracted through multiple testicular biopsies by means of TESE (testicular sperm extraction). The probability of finding spermatozoa with this method in KS patients is between 25 and 40%. Residual gametes are more frequently found in young subjects because the number of spermatozoa diminishes rapidly with the advancing of age. As such, it is fundamental that the diagnosis of KS be made as soon as possible, so as to guarantee cryo-preservation of the semen before complete infertility sets<sup>16</sup>, in view of the fact that any eventual androgen therapy will negatively influence fertility<sup>15</sup>. Since testicular biopsy is a particularly invasive diagnostic technique, it is always advisable to assess the presence of some indicators which suggest residual spermatogenesis. The parameters which may correlate positively with residual fertility are the following: testicular volume above 15 ml, FSH (follicle stimulating hormone) values between 31.2 and 40 U/l, inhibin- $\beta$  lower than

50 pg/ml and no gynecomasty<sup>16 18</sup>, even if in KS patients the only confirmed predicting factor for the success of this technique is that of the result of the histological test.

69% of patients with KS previously administered aromatase inhibitors and hCG (human chorionic gonadotropin) to stimulate the testicular endogenous production of testosterone possess the necessary gonadic spermatozoa for assisted fertilization with the ICSI method. Among these, about 30% achieve full-term pregnancy and a forthcoming progeny with normal karyotype<sup>19</sup>.

Children born of KS patients with the help of assisted fertilisation do not present a higher incidence of numerical anomalies of the sex chromosomes (even though the risk of this eventuality increases due to the technique utilised), however a statistically significant increase is found in the incidence of production of gametes with disomy 21 in the father<sup>15</sup>. As such, an accurate diagnosis before implantation (where consented) or prenatal diagnosis through amniocentesis are advised, as also genetic counselling for the couple who are trying to conceive.

With regard to chromosomal alterations, the anomalies associated with the sex chromosomes are predominant although a wide range of autosomal structural anomalies have been identified, such as Robertsonian and reciprocal translocations, as well as inversions, duplications and deletions, that may be associated with infertility.

Robertsonian translocations represent the most frequent chromosomal structural anomaly in males and may influence fertility by altering the gene pattern of the spermatozoa in different stages. Robertsonian translocations occur when two acrocentric chromosomes<sup>13-15 21 22</sup> fuse together, thus obtaining a single anomalous chromosome, generally dicentric, which presents the long arms of the two original chromosomes but loses the corresponding short arms. The incidence of Robertsonian translocations is about one in a thousand newborn infants.

A reciprocal translocation, on the other hand, is represented by an exchange of segments between different chromosomes; in general no apparent alteration is found in the phenotype of the carrier.

Carriers of reciprocal translocations present principally spermatozoa with non-balanced chromosomal alterations, while the frequencies of normal spermatozoa or spermatozoa with balanced chromosomal alterations are more scarce. When a spermatozoon carrier of chromosomal translocation fertilizes an egg cell, the resulting embryo will

obviously be a carrier of the translocation, generating a gene unbalance, the associated phenotype of which will depend on the exact chromosomal region involved.

Numerical alterations of the sex chromosomes and in particular Klinefelter's syndrome (47,XXY) represent the most frequently found alterations in patients with azoospermia. Structural alterations of the autosomes (Robertsonian and reciprocal translocations, inversions, duplications and deletions) may instead be present in patients with less severe alteration of the spermatogenesis<sup>20,21</sup>. Chromosomal alterations are very frequent in the male partners of couples undergoing ICSI<sup>20,21</sup>. Gekas and colleagues reported the presence of aneuploidies of the sex chromosomes in 3.7% of cases and anomalies of the autosomes in 2.4%. Considering only the patients with azoospermia undergoing ICSI, the frequency of anomalies of the sex chromosomes increases to 15.9% while anomalies of the autosomes are found in 2.8% of cases. The authors, moreover, highlighted that chromosomal alterations are found in 3% of normozoospermic patients undergoing ICSI (1.4% regarding the sex chromosomes, mostly mosaicist, and 1.6% regarding balanced structural anomalies of the autosomes)<sup>21</sup>. Given the elevated frequency of the numerical and structural alterations of chromosomes in infertile males, and considering the fact that the aneuploidies are a cause for abortion, malformations and mental retardation, thus representing an important genetic risk factor in the human, the karyotype test should be carried out in all patients with azoospermia or severe oligospermia. Furthermore, cytogenetic screening is advisable also for those patients with moderate oligozoospermia and subjects with normal sperm concentration, partners of females with no disorders of the reproductive system who, after a year of targeted intercourse without success, wish to avail themselves of assisted reproduction techniques.

### Chromosomal anomalies confined to gametes

The aneuploidies present in the spermatozoa can be the direct result of constitutional genetic anomalies or they may be caused by errors during the meiotic phases induced by the altered testicular environment that these males present<sup>13,20-21</sup>. An altered testicular environment can, in fact, determine the alteration of the testicular genetic and epigenetic control, giving rise to a greater disposition to meiotic errors in the cells<sup>6-7,10-11</sup>.

A completely new area of study in the field of male

infertility concerns the chromosome set of the spermatozoa in patients with primitive testiculopathy and normal karyotype. Recent research has shown that an alteration in the intra-testicular microenvironment, such as that which occurs in patients presenting damage to the spermatogenesis, negatively influences the mechanisms of chromosomal segregation during meiosis with the formation of aneuploidal gametes<sup>6,10</sup>. Therefore, the presence of a normal karyotype does not exclude patients with oligo-asteno-theratozoospermia from producing spermatozoa with numerical and structural alterations of the chromosomes with the consequent increase in risk of generating progeny with aneuploidies. This has been inferred by recent research which shows that patients with primitive testiculopathy produce a greater number of spermatozoa with aneuploidies compared to subjects with normal parameters of seminal fluid, in spite of the presence of a normal karyotype<sup>6,7,10</sup>.

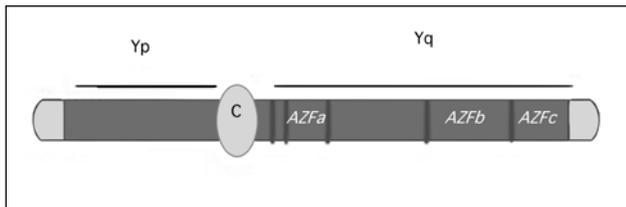
In an ingenious study Calogero et al. described for the first time the presence of an inverse relation between the principal spermatid parameters (concentration, motility and morphology) and the frequency of spermatid aneuploidies, indicating the more severe the testiculopathy, the higher the risk of finding spermatozoa with chromosomal alterations<sup>11</sup>.

Following this the same authors studied the frequency of aneuploidies in a group of patients with oligo-asteno-theratozoospermia (OAT) and in a group of patients in whom the only anomaly of the seminal fluid was the reduction of the number of spermatozoa with normal morphology (theratozoospermia), demonstrating an inverse correlation between rate of spermatid aneuploidies and percentage of spermatozoa with particularly significant normal form, given that the frequency of aneuploidies in OAT patients turned out to overlap with that of the group of theratozoospermic patients. This would suggest that theratozoospermia is the parameter which best associates with aneuploidy<sup>10</sup>.

Other recent data have offered important considerations for reflection regarding the choice and the modalities of assisted fertilisation. There is evidence that shows how morphologically normal spermatozoa of OAT patients present a higher rate of aneuploidies compared to the control group<sup>11</sup>, suggesting the importance of genetic counselling for the couple before carrying out ICSI in order to reduce the risk of transmitting genetic diseases to the progeny.

Moreover, autosomal structural anomalies may be due to deletions, duplications and inversions. Re-

**Figure 1.** Schematic representation of the structure of the Y chromosome with the AZF regions.



search on spermatogenic aneuploidies is thus particularly indicated in the following cases:

1. couples who have failed three or more cycles of treatment;
2. couples with history of multiple abortions;
3. severe male factor;
4. severe male factor after andrological therapy.

### Microdeletions of the Y chromosome and male infertility

The microdeletions of the Y chromosome represent the second most common genetic cause of spermatogenic failure in infertile males after Klinefelter's syndrome<sup>22</sup>.

Thanks to the identification of specific sequences (STS) of the Y chromosome, which have permitted in-depth study of its long arm, three critical regions have been distinguished for spermatogenesis, called AZFa, AZFb and AZFc<sup>23,24</sup>. These regions contain genes and transcriptional units, most of which present a specific testicular expression (Fig. 1).

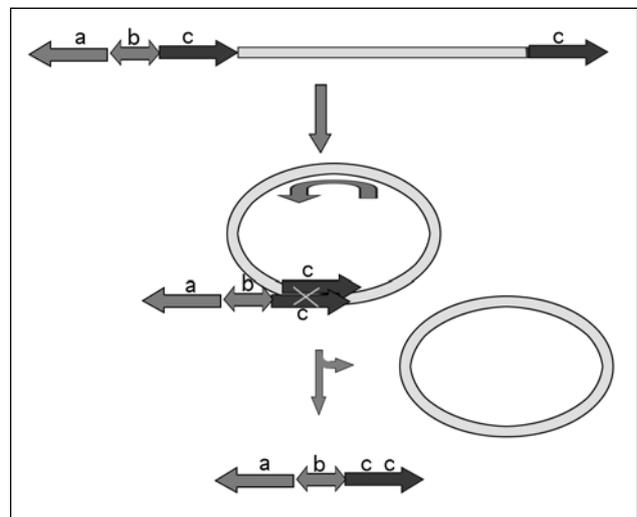
Recent research has shown that the microdeletion of a region of the Y chromosome long arm (Yq), defined "azoospermia factor" (AZF), represents a frequent cause of oligozoospermia and above all of azoospermia<sup>25</sup>.

The microdeletions cluster in three regions which in a proximodistal sense have been denominated AZFa, AZFb and AZFc. Different genes and gene families which seem to be determining for the regular progression of spermatogenesis have been identified in these regions. Nevertheless their precise role is still not completely clear.

The microdeletions of the AZF regions determine a severe primitive testiculopathy with consequent azoospermia or severe oligozoospermia which more frequently involves the AZFc region (about 60%) in comparison with the AZFb region (about 15%) and the AZFa region (about 5%)<sup>25</sup>. In the remaining cases a greater deletion occurs which involves more than one region simultaneously.

In general, the prevalence of the microdeletions of the Yq is around 12% in patients with azoospermia, particularly if of an idiopathic nature, and 3.5% in patients with severe oligozoospermia (spermatic concentration < 5mil/ml)<sup>26</sup>. Thanks to various studies<sup>27-29</sup>, the mechanism underlying the microdeletions involved in these regions is well-known; it is clear that the relatively high incidence of deletions of the Y chromosome is correlated to its structure, rich in repeated sequences which render the long arm particularly susceptible to deletions that arise through intrachromosomal homologous recombination between regions with elevated sequence homology (Fig. 2).

**Figure 2.** Mechanism of intrachromosomal homologous recombination. Two homologous amplicons, oriented in the same direction pair together determining the formation of a loop; this is made up of the region interposed between the two amplicons. The homologous recombination of the amplicons determines the excision of the loop and therefore its removal from the chromosome (by courtesy of Dr. C. Krausz).



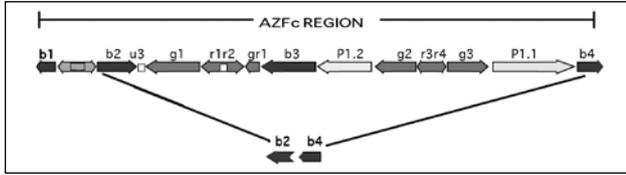
### AZFc microdeletions

The AZFc region includes 12 genes and transcriptional units, each present in varying numbers of copies for a total of 32 copies<sup>30</sup>.

The most frequent deletion in the AZFc region is the one which arises for homologous recombination between the b2 and b4 amplicons (blocks of repeated sequences), that is, deletion "b2/b4"<sup>31</sup> (Fig. 3).

It removes 3.5 Megabases (Mb) that include 21 copies of genes and transcriptional units<sup>31</sup> belonging to 8 gene families (BPY2, CDY1, DAZ, TTY3.1, TTY4.1, TTY17.1, CSPG4LY and GOLGA2LY), of which little is known, excluding the genes DAZ, RBMY, USP9Y

Figure 3. Representation of the molecular mechanism of insurgence of the complete deletion of the AZFc region of the Y chromosome. Intrachromosomal homologous recombination between the sequences 'b2' and 'b4' determines the loss of the whole region interposed between these two sequences (by courtesy of Dr. C. Krausz).



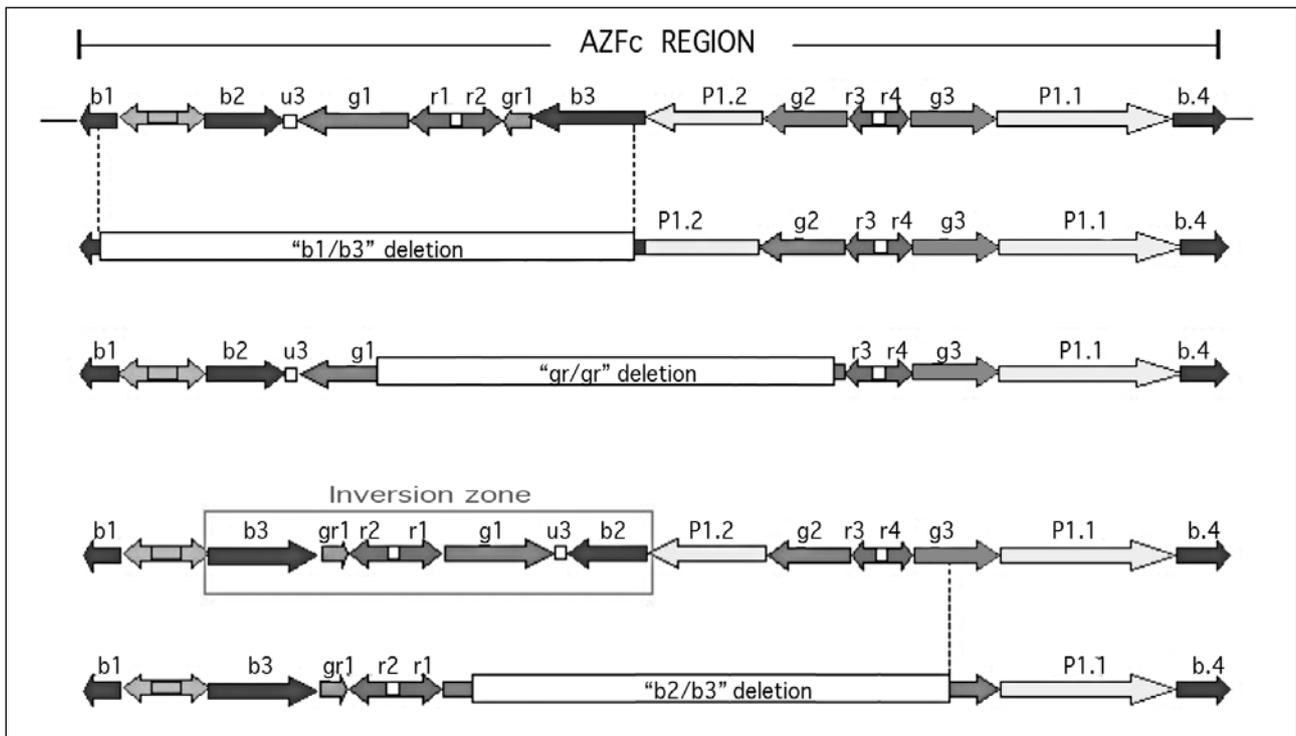
and DBY, and only future research will determine whether these genes are involved in human spermatogenesis or not and whether they should be included in the screening protocol.

The length of the amplicons 'b2' and 'b4' is greater compared to that of the repeated sequences that recombine in the other microdeletions, and this may explain the higher frequency of these microdeletions compared to the others.

The "b2/b4" deletion is associated with histological conditions that vary from SCOS type I (total absence of germinal cells) and type II (presence of some

tubules with normal spermatogenesis) to SGA (spermatogenetic arrest) and ISG (hypospermatogenesis). Moreover, a progressive reduction in the number of spermatozoa has been observed during the course of time. Environmental factors, different genetic backgrounds, or autosomal or X-linked genes that compensate for the absence of genes on the Y chromosome may all contribute to determining the phenotypical variability found in AZFc deletion carriers. As well as the "classical" forms of deletions of the Y chromosome, other deletions may occur which remove more than one AZF region, such as for example the "AZFb+c" deletion<sup>29</sup> and the "AZFa+b+c" deletion<sup>29</sup>, or partial deletions of the AZFc region<sup>29-32</sup>. In fact the "b2/b4" deletion is not the only one regarding the AZFc region, but the presence of numerous amplicons renders this region particularly susceptible also to other types of deletion not as long as the "b2/b4", called 'partial deletions'. Their insurgence mechanism, as for the microdeletions, consists in the intrachromosomal homologous recombination between two sequences that present a high grade of homology (> 99,9%). Recently three types of partial deletion have been described: "b1/b3", "b2/b3" and "gr/gr" (or "g1/g2"<sup>32-35</sup> (Fig. 4).

Figure 4. Molecular insurgence mechanism of the partial deletions of the AZFc region of the Y chromosome. The intrachromosomal homologous recombination determines the loss of the fragment of DNA interposed between the two sequences that recombine (by courtesy of Dr. C. Krausz).



The “b1/b3” deletion derives from homologous recombination between the amplicons “b1” and “b3”; the “gr/gr” deletion derives from recombination between the amplicons “g1” and “g2”; the “b2/b3” deletion involves two steps: an inversion between the amplicons “b2” and “b3”, followed by a recombination between “g1” and “g3”<sup>36</sup>.

These deletions remove about half of the AZFc region, including some genes and transcriptional units specific to the testicle, and may be transmitted to male progeny<sup>31-33 37</sup>.

In both of the partial deletions “gr/gr” and “b2/b3” the loss of two copies of the DAZ gene may be observed (present on the Y chromosome in 4 copies: DAZ1, DAZ2, DAZ3, DAZ4) and a copy of the gene CDY1 (present on the Y chromosome in two copies, CDY1a and CDY1b), which are considered among the most critical genes for spermatogenesis<sup>32 33</sup>.

In the case of the “b1/b3” deletion, on the other hand, two copies of DAZ and two of CDY1 are removed<sup>31</sup>.

Recently a study carried out on the partial deletions of the AZFc region in 556 infertile patients and 487 normozoospermic controls<sup>38</sup> showed an increased frequency of gr/gr deletions in males with spermatogonial defects, compared to normozoospermic males (3.2 vs. 0.4%, respectively;  $p < 0.001$ ), and in particular the highest frequency of deletion was found in oligozoospermic males.

Deletions of the AZFc region are associated with a variable clinical and histological phenotype<sup>36 39-40</sup>. In general, AZFc deletions are compatible with spermatogenetic residue. They may be found in males with azoospermia and severe oligozoospermia, and in rare cases may be transmitted to male offspring<sup>41</sup>.

### AZFb microdeletions

Complete deletion of the AZFb region removes 6.2 Mb which includes 32 genes and transcriptional units, and is the result of homologous recombination between the P5/P1 palindromes<sup>30</sup>.

Complete deletions regarding the AZFb region and AZFb+c deletions are associated with sertoli-cell-only syndrome (SCO) or spermatogenetic arrest resulting in azoospermia<sup>30</sup>.

### AZFa microdeletions

The AZFa region comprises a region of about 1100 kb that contains the single-copy genes DFFRY (or USP9Y) and DBY. Deletions of the whole AZFa

region remove about 792 kb that include both the DFFRY gene and the DBY gene, the only two genes present in AZFa, and they are associated with sertoli-cell-only syndrome (SCO) and azoospermia<sup>42-46</sup>. Deletions involving only the USPY9 or the DBY genes have been described only by one group<sup>47 48</sup> and have not been found in any other study groups<sup>49</sup>. Nevertheless, the mechanism responsible for these partial deletions is still not clear.

Nowadays, genetic examination of the Y chromosome has entered and become part of the routine diagnostic procedure of the infertile patient (azoospermic or severely oligospermic). Using the PCR (polymerase chain reaction) consents the study of the STS with reference to the locus AZF in its three regions (AZFa, AZFb and AZFc), identifying those subjects in whom a microdeletion is present of one or more genes implicated in the spermatogenesis and therefore responsible for male infertility.

The test of the microdeletions of the Y chromosome has been included together with the cytogenetic test in the diagnostic procedure for research of the genetic causes of male infertility as well as in the protocol of preparation for assisted fertilisation, in view of the fact that the microdeletions are transmitted to the male offspring.

As far as the accuracy of the screening is concerned, although it is a genetic test that is relatively easy to carry out, in actual fact a high percentage of laboratories produce erroneous results<sup>50</sup>.

In order to get round this problem and render the results comparable some guidelines have been established for the routine molecular analysis of the microdeletions of the Y chromosome.

These include the amplification of a set of primers relating to specific STS (Table III) and consents the identification of 99% of the deletions of the long arm of the Y chromosome that show to be clinically significant<sup>50</sup>. In accordance with the WHO, the test

Table III. STS utilised for the test of microdeletions of the Y chromosome according to guidelines of the European group<sup>50</sup>.

STS	REGION OF THE Y CHROMOSOME
SY84	AZFa
SY86	AZFa
SY127	AZFb
SY134	AZFb
SY254	AZFc
SY255	AZFc
SRY	
ZFY	

is performed with PCR methodology through amplification of six STS, distributed along the loci AZFa, AZFb and AZFc of the Y chromosome, following indications of the European control group.

The study of the microdeletions of the Y chromosome, as well as having a diagnostic value inasmuch as it defines the etiology of the disorder of spermatogenesis, has also a prognostic value in terms of identifying testicular spermatozoa. In fact, in patients who are carriers of a complete deletion of the region AZFa, or of AZFb, the probability of identifying spermatozoa is virtually zero and as such the multiple testicular biopsy (TESE or testicular sperm extraction) is not recommended. If on the other hand, the deletion regards the AZFc region, the probability of identifying sperm is around 50%<sup>46</sup>.

Lastly, the preventive value of this screening test lies in the possibility of proposing cryopreservation of the seminal fluid to patients who are carriers of microdeletions and affected with oligozoospermia, in order to counteract the progressive reduction of the number of spermatozoa in time.

### Gene mutations and male infertility

Numerous genes are necessary for normal sexual and gonadal development and for spermatogenesis while only some of these have a certain routine clinical importance. Among these are:

- the gene KAL-1, which once mutated, causes the syndrome of Kallmann; patients affected with this syndrome present hypogonadotropic hypogonadism (caused by a deficit of secretion of the hypothalamic GnRH) and anosmia (due to agenesis of the olfactory lobes);
- the gene CFTR which once mutated causes cystic fibrosis and non-formation of the vas deferens;
- the gene which codifies the androgen receptor which, once mutated, causes the syndrome of insensitivity to androgens and alterations of spermatogenesis;
- the genes INSL3-RXFP2, mutations of which are associated with anomalies in the descent of the testicles (cryptorchidism).

### Mutations of the gene KAL-1

The gene KAL-1 is located on the X chromosome and the protein that it codifies for has a central role in the migration of the GnRH-secreting neurons to the hypothalamus.

The study of mutations of the gene KAL-1 is reserved for those patients with hypogonadotropic

hypogonadism and anosmia, irrespective of the fact that they tend to turn to medically-assisted reproduction techniques, given that a simple hormone treatment is capable of restoring natural fertility in these patients with the possible transmission of the gene defect to the offspring<sup>51 52</sup>.

Kallmann's syndrome affects 1 out of 10,000 males and may be inherited as a form linked to the X chromosome in 15% of cases<sup>51</sup> or to the autosomes<sup>52</sup>. The autosomal genes have not yet been identified, therefore genetic screening currently available for the syndrome of Kallmann refers only to the form linked to the X chromosome

### Mutations of the gene CFTR

The gene CFTR is located on chromosome 7 (7q31.1-31.2) and in the homozygous form causes cystic fibrosis, one of the commonest and most severe autosomal recessive diseases in the Caucasian population. It is estimated that 1 out of 2500 individuals is affected and that one out of 25 is an asymptomatic carrier of a mutation.

The presence of mutations that do not completely damage the expression of the gene CFTR causes congenital bilateral absence of the vas deferens (CBAVD) in the male, with consequent obstructive azoospermia that represents a mild or incomplete form of cystic fibrosis.

CBAVD is found in 6% of patients with obstructive azoospermia and in about 2% of infertile patients<sup>53</sup>. Infertility caused by obstructive azoospermia has been found in more than 95% of males affected with cystic fibrosis, while 60-70% of patients with CBAVD present mutations of the gene CFTR also in the absence of other clinical symptoms of cystic fibrosis. Only one mutation of the gene CFTR is identifiable in 50% of patients with CBAVD, two mutations have been found in 20% (compound heterozygosis or homozygosis), while no mutation is identifiable in the remaining 30% of cases<sup>54 55</sup>.

About 70% of the mutations observed in patients with cystic fibrosis is represented by the deletion of three pairs of bases that cause the loss of the phenylalanine aminoacid situated in position 508 in the protein ( $\Delta F 508$ )<sup>56</sup>.

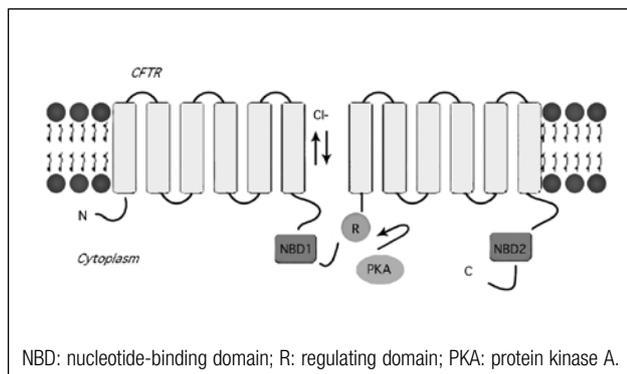
More than 900 mutations have been described for this gene which can lead to:

1. failed synthesis of the protein;
2. alterations in the folding, processing or transport of the protein ( $\Delta F 508$  belongs to this class and constitutes 67% of cases<sup>56</sup>;
3. altered regulation; incapacity to bind and hydrolyse ATP;

4. reduced conductivity (usually less severe phenotype)<sup>56</sup>;
5. reduced quantity of CFTR; mutations in the promoter. Less severe phenotype<sup>56</sup>;
6. altered regulation of other ionic transporters;  $\Delta F 508$  also belongs to this class.

The protein CFTR is made up of two transmembrane domains, two nucleotide-binding domains (NBD) and a regulating domain R. An agonist, for example, acetylcholine, binds to the cells determining an increase of the cAMP which in turn activates the protein kinase A; this last phosphorylates the CFTR in connection with the R domain, stimulating the opening of the chlorine canal (Fig. 5).

Figure 5. Schematic representation of the CFTR membrane protein and simplified model of the regulation of the CFTR-Cl<sup>-</sup> canal through phosphorylation cAMP dependent on the R dominion.

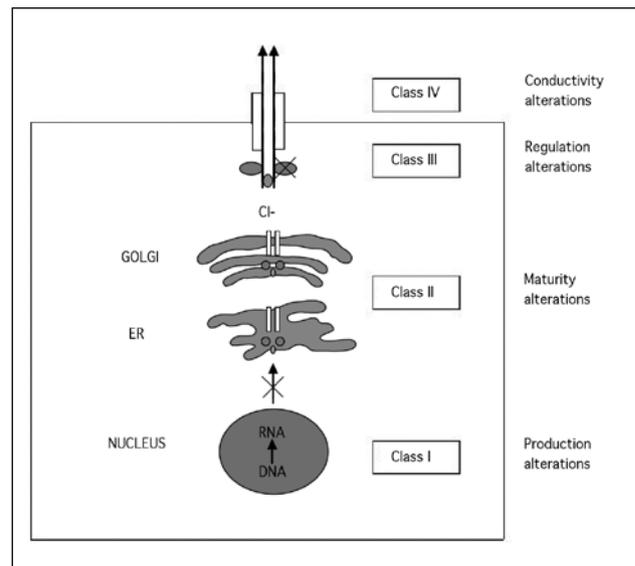


The most frequently found mutation in this gene determines an altered glycosylation of the protein on the Golgi/ER (endoplasmic reticulum), causing degradation before it reaches the cell surface. Other mutations regard the synthesis of the CFTR, of the nucleotide-binding domains, of the R domain and the transmembrane domains.

Welsh and Smith proposed a classification of these anomalies in relation to the function of the Cl<sup>-</sup> canal<sup>57</sup> (Fig. 6).

Molecular anomalies have varying effects on the CFTR protein and on its functionality. Subjects who present mutations of the CFTR gene are good candidates for ICSI, carried out using spermatozoa obtained from the ejaculate, for the testicles or from the epididymis. In these patients spermatogenesis is normal and no increase of aneuploids is found in the spermatozoa. Since there is a risk of manifestation of cystic fibrosis in the offspring of couples in whom the female partner presents mutations in heterozygotes for the

Figure 6. Mutations of the CFTR protein according to the classification proposed by Welsh and Smith in 1993.



CFTR gene, screening is advised for the mutations that involve this gene, before experimenting techniques of medically assisted reproduction.

### Mutations of the genes INSL3-RXFP2

The insulin-like factor 3 (INSL3) is a member of the family of hormones similar to relaxin produced by the Leydig cells. The codifying gene for INSL3 is on chromosome 19 and is composed of two exons with an intron which interrupts the codifying domain for the peptide C.

Only recently have further studies on INSL3 been carried out, following the identification in rodents of a role of this peptide in the transabdominal phase of testicular descent, and subsequently the discovery of its receptor, RXFP2<sup>58</sup>.

In subjects affected with cryptorchidism it has been hypothesized that one of the causes of this alteration may be represented by mutations in the genes INSL3 and RXFP2 which usually lead to replacements of some amino acids<sup>59 60</sup>.

Data in literature report that the prevalence of mutations connected with these two genes is around 4-5% in males with cryptorchidism<sup>61</sup>.

Some of these mutations are actually polymorphisms found with similar frequency in patients and controls, while 7 of these mutations have been found exclusively in subjects presenting alterations in descent of the testicles (Tab. IV)<sup>59</sup>.

All of these mutations are in heterozygotes and no cases have ever been found of patients with two mutated alleles or who present simultaneously

Table IV. Mutations of the genes INSL3-RXFP2 <sup>59</sup>.

INSL3	RXFP2
P49S	T222P
R73X	
P93L	
R102C	
R102H	
N110K	

mutations in heterozygotes in both genes. This evidence is in contrast with that observed in rodents in which homozygotic knock-out mice are affected with cryptorchidism while the heterozygotic ones are normal.

### Gene polymorphisms and male infertility

The polymorphism is a variation in the sequence of DNA, the presence of which in most cases does not produce illness effects. Analysis of the polymorphisms present in the genes involved in spermatogenesis represents one of the most interesting fields of research in the study of the genetics of male infertility.

The polymorphisms or genetic variations in these genes are considered potential risk factors which could influence the severity of the spermatogenic alterations.

Numerous polymorphic variations in association with male infertility have been described. These studies of association often do not lead to univocal results and this is principally due to different important aspects:

- the dimension and the composition of the population being studied;
- the type of polymorphism analyzed and the techniques used for analysis;
- the multifactorial condition and the heterogeneity of the phenotype of male infertility;
- the interindividual variability found in the phenotypical effect of causes which act in the testicles;
- the ethnical and geographical differences which contribute to increasing the genetic variability.

### Polymorphisms of the gene CFTR

More than 300 polymorphisms have been identified in the gene CFTR. A correlation has been established between this malformation and a polymorphous allelic variant of the gene CFTR, known as polymorphism 5T, that shows a frequency about 4-6

times higher in CBAVD subjects compared to the general population.

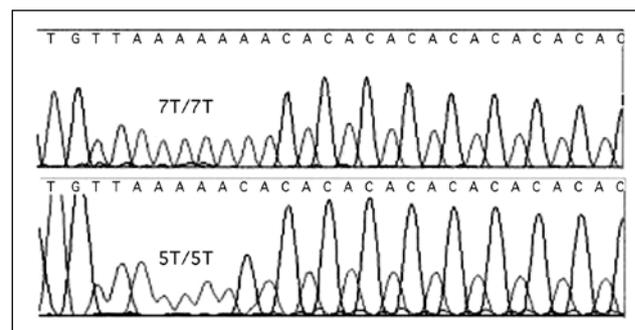
The polymorphism consists of a region of polypyrimidine (polyT) of varying length within the intron 8 of the CFTR gene. The variability presents in the form of three alleles, called 5T, 7T and 9T, depending on the number of thymines present. In particular, the 5T allele is associated with the mutated chromosomes of males with CBAVD.

This intronic polymorphism regulates the molecular mechanism of the process of maturation of the messenger RNA (mRNA) and therefore the quantity of transcribed CFTR within the cell.

The presence of a 5T allele accompanies a synthesis of mRNA that is lacking in exon 9 (ex9-) by about 90%. 7T presents 25% of mRNA ex9-, while in 9T 90% of the mRNA is normal <sup>62</sup>. We hypothesize that a reduced quantity of mRNA is responsible for this incomplete form or variant of FC that is transmitted by heterozygotic parents.

In male infertility, molecular diagnosis of the 5T polymorphism of the gene CFTR is carried out through gene amplification (PCR) and automated sequence analyses for assessment of the 5T polymorphism of the gene CFTR (Fig. 7).

Figure 7. 5T Polymorphism of the gene CFTR (CAVD) Molecular diagnosis of male infertility through gene amplification (PCR) and automated sequence analysis for assessment of the polymorphism 5T of the CFTR gene.



### Mutations of the androgen receptor gene

Androgens and their receptor (AR) are essential for development and maintenance of the male phenotype and of spermatogenesis <sup>63</sup>. The androgen receptor is codified by a gene located on the long arm of the X chromosome (Xq11-12) <sup>62</sup>; it is composed of 8 exons and codifies for a protein of 919 amino acid residues <sup>64 65</sup>. Mutations of the gene codifying for AR cause several different alterations that are denominated collectively with the term

androgen insensitivity syndrome (AIS). This syndrome is a recessive disease that is brought about by mutations of the gene for the androgen receptor. Descriptions of many mutations of this gene <sup>66</sup> are available on the following website: <http://www.mcgill.ca/androgendb/>. Subjects affected with this syndrome may present extremely heterogeneous phenotypes that vary from a totally feminine aspect to male infertility <sup>67 68</sup>.

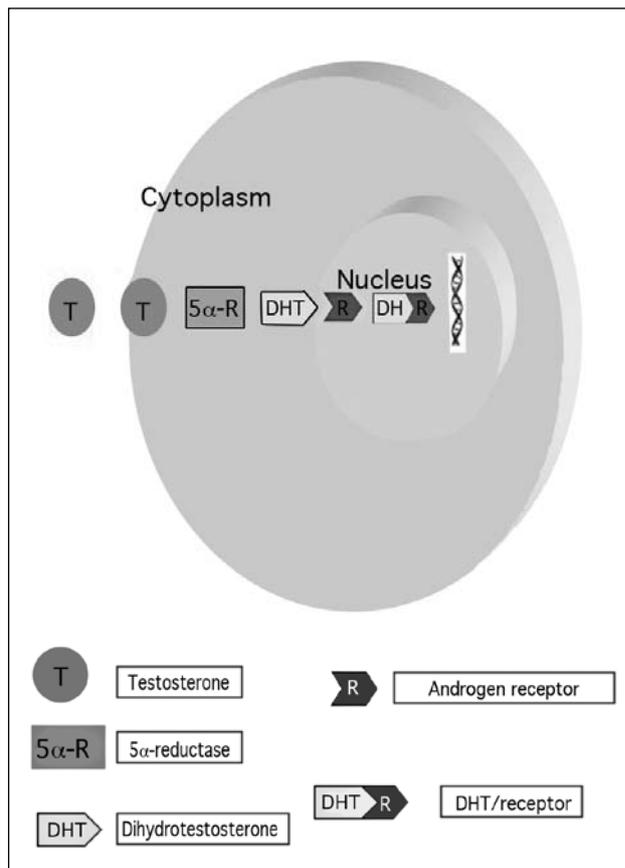
Patients affected with a mild form (MAIS – *mild androgen insensitivity syndrome*) manifest infertility as the first or even only symptom.

Normally the hypothalamic-pituitary-testicular axis regulates the synthesis of testosterone. The testosterone produced undergoes a transformation within the testosterone-sensitive cells and is converted into dihydrotestosterone (DHT) by means of the action of two enzymes called 5 $\alpha$  reductase I (5-AR-I) and 5 $\alpha$  reductase II (5-AR-II). The DHT binds the receptorial complex of the cytoplasmatic androgens, in preparation for its journey to the interior of the nucleus. Here the complex binds to the nuclear DNA, initiating a process which terminates with the synthesis of various proteins that are capable of mediating the biochemical effects of the hormone (Fig. 8).

Infertile males with mutations of the androgen receptor gene present azoospermia or severe oligozoospermia, either as isolated manifestations or associated with other symptoms of reduced sensitivity to androgens (cryptorchidism, hypospadias, gynecomasty, undervirilisation) <sup>69</sup>. The hormone profile of these subjects is characterised by higher than normal LH serum levels and increased or normal testosterone levels. LH-testosterone, called androgen sensitivity index (ASI), may be useful in identifying patients who are carriers of mutations of the androgen receptor gene. In fact, the higher the ASI, the higher the risk of finding a mutation of the gene <sup>70</sup>.

The frequency of mutations of the androgen receptor in patients with oligozoospermia or azoospermia is between 2-3% <sup>70</sup>. Since the same mutation may be associated with varying phenotypes, it is not possible to foresee the clinical consequences in children born with medically assisted procrea-

Figure 8. Schematic representation of the action mechanism of the androgens and the receptor within the cell.



tion (MAP) techniques and the couple should be informed about the risks of worsening of clinical manifestations in offspring who inherit the gene defect. An expansion of the CAG triplet in exon 1 (more than 40 repeats) causes spinobulbar muscular atrophy or Kennedy's disease, characterised by a progressive weakening and atrophy of the muscles associated with undervirilisation, infertility and testicular atrophy. It is not yet clear whether a minor reduction in repetition can be correlated to isolated defects of spermatogenesis <sup>71</sup>.

Screening for mutations of the androgen receptor gene is recommended for patients with high ASI values (> 200Ux nmol/l) <sup>70</sup>.

Table V. Data obtained from study by di Van Steirteghem et al. <sup>78</sup>.

TYPE OF CHROMOSOMAL ALTERATION	ICSI OFFSPRING	NORMAL POPULATION
Aneuploidies of sex chromosomes	0.6%	0.2%
Structural alterations of autosomes	0.4%	0.07%

### Polymorphisms of exon 1 of the androgen receptor

The androgen receptor presents two polymorphic sites in exon 1, characterised by a different number of CAG and GGC repeat triplets that code for polyglutamine and polyglycine repeats of variable length at the N-terminal extremity of the AR<sup>68</sup>. The role of these amino acid repeats seems to be that of modulating the functioning of this receptor.

The number of CAG and GGC triplets varies from 10-35 (with an average of 21-23) and from 4-24 (average 16-17) respectively, in normal subjects<sup>71</sup>.

Recent studies have investigated a possible association between the length of the CAG triplets and male infertility, following the discovery of longer repeats of the CAG triplet being correlated with a diminished transcriptional activity of AR, both *in vivo* and *in vitro*<sup>71-73</sup>. This observation has led to the hypothesis that longer tracts of polyglutamine may be considered a risk factor for male infertility, in consistence with the finding that the polymorphisms identified in the CAG triplets are correlated with the concentration of spermatozoa in normal subjects.

Similarly, the deletion of GGN repeats in 30% of samples leads to a reduction in potential of the transactivation of the receptor<sup>74</sup>.

Recently, the combined effects of variation in length of the CAG and GGC triplets have been studied, suggesting that some haplotypes may modulate the functioning of the androgen receptor and may increase individual susceptibility to infertility<sup>75</sup>.

An analogous study has concluded that the combination of a number of CAG triplets lower than 21 and a number of GGN triplets lower than 23 is associated with a lower risk of infertility while subjects presenting more than 21 CAG triplets and more than 23 GGN triplets are exposed to greater risk<sup>76</sup>.

### Discussion

Many recent studies have led to a greater understanding of the varied genetic causes of male infertility. Widespread use of medically assisted procreation techniques has in fact given rise to a surge of research activity dedicated to studying the genetic causes associated with male infertility, and it is thanks to this that today a genetic cause (chromosomal or gene) for infertility in males may be recognised in a high number of cases.

Normally, the process of natural selection prevents the transmission of mutations or chromosomal alterations associated or not with infertility, although this protective mechanism may be bypassed with more invasive MAP techniques such as intracyto-

plasmic sperm injection (ICSI), which permit the transmission of chromosomal or genetic alterations to the offspring. It is therefore critically essential to include accurate assessment of the chromosome complement in the male as a routine step in the diagnostic course of the infertile couple. In fact, if on the one hand recourse to these techniques allows the couple to resolve problems of infertility that are not treatable with specific therapies, on the other hand this phenomenon has given rise to a degree of apprehension among specialists of this field precisely for the risk of transmission of genetic alterations that it entails<sup>77</sup>.

Genetic anomalies have thus assumed enormous clinical importance, not only because they may cause infertility in the offspring, but also and mainly because they are capable of causing more severe illnesses. In this regard, some studies seem to indicate an increase in the chromosomal alterations in foetuses and in children conceived through implementation of the ICSI technique. In particular, one of these studies has reported a roughly three-fold increase in the incidence of numerical alterations of the sex chromosomes and approximately a six-fold increase in the incidence of structural alterations of the various chromosomes out of about 1500 examinations carried out of the karyotype<sup>78</sup>.

These considerations would seem to suggest, therefore, that the only way to reduce the diffusion of genetic disease that may occur through indiscriminate use of MAP techniques is to implement careful genetic screening of infertile couples, including accurate assessment of the male chromosome complement.

The high frequency of genetic alterations in infertile couples demands appropriate and correct diagnosis of these patients in order to reduce the risk of transmitting genetic anomalies to the offspring. Whether to undertake genetic investigations and if so, which in particular, may be indicated through detailed clinical assessment.

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# Effect of penile rehabilitation on erectile function after bilateral nerve-sparing robotic-assisted radical prostatectomy

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## Summary

**Introduction.** Penile rehabilitation has been regarded as a standard after radical prostatectomy. However, the REINVENT study recently demonstrated that the use of nightly PDE-5 inhibitors failed to increase the potency recovery after.

**Methods.** We collected prospectively the clinical records of all patients who underwent robot-assisted radical prostatectomy for clinically localized prostate cancer at the University of Padua. For the present study, we extracted all consecutive cases receiving a bilateral nerve-sparing technique with a minimum follow-up  $\geq 12$  months.

**Results.** Penile rehabilitation was performed in 151 patients (66%) of 229 patients. Twelve months after bilateral nerve-sparing robot-assisted radical prostatectomy, 142 patients (62%) were potent. The median time to recovery of erectile function was 6 months (IQR: 2.5-11). Specifically, age (hazard ratio [HR]: 1.093;  $p < 0.001$ ), Charlson score (HR: 0.863;  $p = 0.003$ ), baseline IIEF-6 score (HR: 0.954;  $p < 0.001$ ), and penile rehabilitation (HR: 0.800;  $p = 0.018$ ) were predictors of erectile function recovery in univariable analysis. In multivariable analysis, penile rehabilitation did not retain an independent predictive role (HR: 1.663;  $p = 0.188$ ), once adjusted for the effect of age (HR: 1.048;  $p = 0.005$ ), and baseline IIEF-6 score (HR: 0.803;  $p < 0.001$ ).

**Conclusions.** About 60% of the patients were potent 12-mo after underwent robot-assisted radical prostatectomy series. Patients age and preoperative erectile function were the most powerful predictor of erectile function recovery. Adoption of postoperative rehabilitation was not significantly associated with improved postoperative erectile function, once adjusted for the effect of the other covariates.

## Key words

Penile rehabilitation • Radical prostatectomy

## Introduction

Radical prostatectomy (RP) is a common treatment for patients with clinically localized prostate cancer and a life expectancy longer than 10 years<sup>1</sup>. Erectile dysfunction is one of the most important complication after RP. The improvements in the knowledge of the anatomy of periprostatic fascias and cavernous nerves led to significant updates of the nerve-sparing technique improving significantly

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the potency recovery rates following RP. Even after a meticulous dissection to preserve both neurovascular bundles, however, erectile function may take up to 12-18 months to return, as consequence of post-operative neuropraxia. Data coming from referral centres showed 12 or 18 months potency rates ranging from 60 to 80% after nerve-sparing retropubic RP (NSRP) <sup>2,3</sup>; from 45 to 76% after pure laparoscopic RP <sup>4,5</sup> and from 70 to 80% after robot-assisted laparoscopic RP <sup>6-8</sup>. These findings contributed to the development of an increasing interest in the pathophysiology of post-operative erectile dysfunction as well as in its potential prophylaxis and treatment <sup>9</sup>.

Historically, after nerve sparing RP patients have been encouraged during the neuropraxia period to continue waiting for the return of erectile function without any active intervention. With the aim of speed up the recovery of spontaneous erections after RP, since 1997 some Authors proposed the use of specific protocols of penile rehabilitation to prevent the cavernous tissue damage that occurs during the period of neural recovery, providing adequate oxygenation to the cavernous tissues.

Although a lot of experimental and clinical studies supported the use of penile rehabilitation after bilateral or unilateral nerve-sparing RP, the rationale and mechanism for their use in penile rehabilitation programs have not been fully elucidated <sup>10</sup>. Moreover, a recent randomised, double-blind, double-dummy, multicentre, parallel group study conducted at 87 centres across Europe, Canada, South Africa, and the United States showed that in men with erectile dysfunction (ED) following bilateral NSRP, vardenafil was efficacious when used on demand, supporting a paradigm shift towards on demand dosing with phosphodiesterase 5 inhibitors (PDE5-I) in this patient group <sup>11</sup>.

The purpose of the present study was to evaluate the impact of penile rehabilitation on erectile function recovery in a series of patients undergoing robot-assisted laparoscopic radical prostatectomy (RALP).

## Methods

### Surgical technique

All procedures were performed by two surgeons using the same technique (WA and SC).

The ports for the three-arm robot (da Vinci Intuitive Surgical, Inc., Sunnyvale, CA, USA) and the traditional laparoscopic tools were placed as previously

reported. The anterolateral surface of the prostate was dissected following intrafascial or interfascial planes, and the lateral pedicles were controlled using monopolar forceps, using electrocautery as little as possible and as far as possible from the neurovascular bundles <sup>12</sup>. Specifically, in the intrafascial dissection, the plane between the prostatic capsula and the thin surrounding periprostatic fascia was developed, while in the interfascial dissection, a plane on to the periprostatic fascia was developed <sup>13</sup>. Intrafascial dissection was our preferred approach in most of the cases, where interfascial dissection was selected intraoperatively when the intrafascial plan was not easily developable. Vesicourethral anastomosis was performed using a running suture, as described by Van Velthoven et al. <sup>14</sup>. In patients with intermediate or high risk according to the D'Amico classification, bilateral internal iliac and obturator lymphadenectomy was performed <sup>15</sup>.

The prostate specimen was formalin fixed in the standard manner. The whole-mount sections were identified consecutively with capital letters, always starting from the section closest to the apex, making the whole specimen available for histologic examination. The *en face* section was then processed as a single section. Specifically, the paraffin-embedded specimen was examined histologically in the form of 4-mm-thick, whole-mount, hematoxylin and eosin-stained sections. Positive surgical margin was defined as a tumor being present at the inked margin.

Every patient was evaluated for the following preoperative parameters: age at diagnosis, body mass index, comorbidity according to Charlson score <sup>16</sup>, preoperative total prostate-specific antigen, prostate volume estimate during preoperative transrectal ultrasound, biopsy Gleason score, clinical stage according to the 2002 TNM system <sup>17</sup>, and risk groups according to D'Amico et al. <sup>15</sup>. Moreover, the following pathologic parameters were extracted from the database: Gleason score, perineural and endovascular invasion, pathologic extension of the primary tumor according to the 2002 TNM system, and presence of positive surgical margins.

Urinary continence at follow-up was evaluated using the International Consultation of Incontinence Questionnaire-Urinary Incontinence short-form instrument. The questionnaire was completed by the patient before surgery and at the 12-month follow-up. All of the patients reporting no leak for the question "How often do you leak urine?" were defined as continent. Erectile function was evalu-

ated preoperatively and at 12 months after surgery using the International Index Erectile Function 6 (IIEF-6) questionnaire. Patients were invited to complete the questionnaire and return it by mail. A third person who was blinded for the preoperative characteristics of the patients inserted the score in the database. In this study, we define potent patients as those with an IIEF-6 score  $\geq 18$ , regardless of the use of phosphodiesterase type 5 inhibitors (PDE5-Is). Patients with full erection only after penile prostaglandin E1 injection were considered impotent.

All patients were invited to undergo a penile rehabilitation protocol 15 days after catheter removal; PDE-5Is and different schedules and drugs were used according to the attending urologist.

Institutional review board approval is not usually needed in Italy for nonexperimental studies, such as the present one; however, all of the patients signed an informed consent form authorizing data collection for scientific purposes.

### Statistical analysis

Mean and standard deviation were used to report continuous normally distributed variables, and me-

dian and interquartile range (IQR) were used for the non-normally distributed ones. The Pearson chi-square and Mann-Whitney U tests were used to compare categorical and continuous variables, as appropriate. Logistic regression was used to perform univariable and multivariable analysis.

A two-sided  $p < 0.05$  was considered statistically significant. All statistical analyses were performed using Statistical Package for Social Sciences software v.16.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Descriptive analysis

Table 1 summarized the characteristics of 229 cases evaluated in this study (Table I). Specifically, mean age was  $61.3 \pm 6.4$  years. Median Charlson score was 2 (IQR 2-2). According to D'Amico risk groups, 157 cases (69%) were low risk, 58 (25%) were intermediate risk, and 14 (6%) were high risk. All patients were preoperatively continent. The baseline median IIEF-6 score was 22 (IQR: 19-24). A bilateral, intrafascial nerve-sparing technique was performed in 167 cases (73%). In the remaining 62 cases (27%),

Table I. Association of penile rehabilitation with clinical and pathologic characteristics of 229 patients treated with robot-assisted laparoscopic radical prostatectomy.

VARIABLES	CASES (%)	PENILE REHABILITATION		P VALUE
		PERFORMED (N = 151, 66%)	NOT PERFORMED (N = 78, 34%)	
Mean age (years)	61.3 $\pm$ 6.4	60.1 $\pm$ 6.4	63.8 $\pm$ 0	< 0.0001
Mean BMI	26.1 $\pm$ 2.8	25.8 $\pm$ 2.8	26.5 $\pm$ 2.6	0.093
Charlson score (%) (median and IQR)	2 (2-2)	2 (2-2)	2 (2-2.5)	0.007
D'Amico risk groups (%)				0.107
• low	157 (69%)	100 (64%)	57 (36%)	
• intermediate	58 (25%)	44 (76%)	14 (24%)	
• high	14 (6%)	7 (50%)	7 (50%)	
Baseline IIEF-5 score (median and IQR)	22 (19-24)	22 (19-24)	21 (18-24)	0.039
Definitive Gleason score (%)				0.118
• $\leq 6$	112 (49%)	76 (68%)	36 (32%)	
• 7	97 (42%)	66 (68%)	31 (32%)	
• 8-10	20 (9%)	9 (45%)	11 (55%)	
Pathological stage (pT)				0.382
• pT2	148 (68%)	101 (68%)	47 (32%)	
• pT3-4	81 (32%)	50 (62%)	31 (38%)	
Positive surgical margins (%)	73 (32%)	46 (63%)	27 (37%)	0.551
Median follow-up, mo (IQR)	14 (12-16)	14 (12-15)	15 (12.7-20)	0.009
Potent at follow-up (%)				0.002
• Yes	142 (62%)	105 (74%)	37 (26%)	
• No	87 (38%)	46 (53%)	41 (47%)	

we performed an interfascial nerve-sparing technique. The median catheterization time was 5 days (IQR: 4-7), and the median in-hospital stay was 6 days (IQR: 5-8). The recommended penile rehabilitation protocol was performed in 151 patients (66%). The remaining 78 cases (34%) used PDE5-Is on demand.

The patients undergoing penile rehabilitation were significantly younger, with lower Charlson comorbidity index and higher preoperative IIEF-5 (all  $p$  values  $< 0.005$ ) (Table I).

### Prognostic factors predictive of 12-month potency recovery

Twelve months after bilateral nerve-sparing RALP, 142 patients (62%) were potent. The median time to recovery of erectile function was 6 months (IQR: 2.5-11).

Table 2 summarizes the results of univariable and multivariable analysis to predict erectile function recovery 12 months after RALP.

Specifically, age (HR: 1.093  $p < 0.001$ ), Charlson score (HR: 0.863;  $p = 0.003$ ), baseline IIEF-6 score (HR: 0.954;  $p < 0.001$ ), and penile rehabilitation (HR: 0.800;  $p = 0.018$ ) were predictors of erectile function recovery at univariable analysis.

At multivariable analysis, penile rehabilitation did not retain an independent predictive role (HR: 1.663;  $p = 0.188$ ), once adjusted for the effect of age (HR: 1.048;  $p = 0.005$ ), and baseline IIEF-6 score (HR: 0.803;  $p < 0.001$ )

## Discussion

Potency recovery after RALP is significantly related to nononcologic preoperative characteristics of patients. Our study showed that age of patients and, above all, preoperative erectile function were the most important predictors of potency recovery 12 months after surgery, while the use of penile rehabilitation did not have an independent predictive role for return to erectile function once adjusted for the effect of these covariates.

The goal of penile rehabilitation is to prevent the cavernous tissue damage that occurs during the period of neural recovery providing an adequate oxygenation to the corpora cavernosa. Moreover, restoring nocturnal erections must be considered an alternative way to increase oxygenation of the cavernosal bodies using oral PDE5-I<sup>18</sup>.

The potential options for early treatment of erectile dysfunction following nerve-sparing radical prostatectomy encompass pharmacologic and non-pharmacologic agents. The first group includes the oral PDE5-I (sildenafil, vardenafil and tadalafil), intracavernous agents (alprostadil, papaverine and phentolamine) and intraurethral agents (alprostadil). The main non-pharmacologic tools used for early penile rehabilitation are vacuum constriction devices (VCDs)<sup>18</sup>. Montorsi et al. first demonstrated the advantages of penile injection of Alprostadil as an early intervention strategy in a randomized, controlled study, including 30 patients undergoing bilateral nerves-sparing retropubic RP<sup>19</sup>. One month after surgery, 15 patients were randomized to intracavernous alprostadil 2-3 times/week for 12 weeks, while 15 received no treatment. After a minimum follow-up of 6-month, 8 of 12 patients (67%) had spontaneous erections sufficient for intercourse, compared to 3 of 15 (20%) of those who were not injected. Moreover, penile Doppler ultrasonography revealed veno-occlusive dysfunction in only 2 of 12 patients (17%) included in the treatment group, compared to 8 of 15 (53%) in the control group<sup>19</sup>. Although this study must be considered as one of the most original contribution in the literature, it suffers of some important limitations, including lack of blinding procedure and placebo control, small number of patients enrolled, non intention-to-treat analysis and, finally, lack of further validation.

Although several preclinical studies supported the opportunity to use also PDE5-I in the penile rehabilitation following RP, however, the clinical pieces of evidence were limited and of poor methodological quality until the recent publication of the

Table II. Univariate and multivariate analyses for 12-month potency recovery in 229 analyzed consecutive cases.

VARIABLES	UNIVARIABLE ANALYSIS			MULTIVARIABLE ANALYSIS		
	HR	95% CI	P VALUE	HR	95% CI	P VALUE
Age (year) (continuous)	1.093	1.044-1.145	$< 0.001$	1.048	1.015-1.083	0.005
Charlson score (continuous)	0.836	0.743-0.941	0.003	1.036	0.681-1.576	0.869
Baseline IIEF-6 score (continuous)	0.954	0.939-0.969	$< 0.001$	0.803	0.741-0.869	$< 0.001$
Penile rehabilitation	0.800	0.665-0.963	0.018	1.663	0.780-3.545	0.188

Recovery of Erections: Intervention with Vardenafil Early Nightly Therapy (REINVENT) study<sup>11</sup>. The study was a randomized, double-blind, double-dummy, multicenter parallel-groups study that randomized 628 patients after nerve-sparing RP to a 9-mo treatment with nightly vardenafil plus on-demand placebo, on-demand vardenafil plus nightly placebo, or nightly plus on-demand placebo. After the 9-mo double-blind treatment period, the patients entered a single-blind placebo washout period, which was followed by a 2-mo open-label treatment with on-demand vardenafil. According to the primary end point of the study, the percentages of subjects with an IIEF-EF score  $\geq 22$  after the end of the 2-mo washout period were similar in the three treatment arms (28.9%, 24.1%, and 29.1% of patients for placebo, vardenafil nightly, and vardenafil on demand groups, respectively)<sup>11</sup>. In other words, nightly vardenafil was not shown to be more effective than vardenafil on demand, as theoretically hypothesized. Moreover, the efficacy of vardenafil on demand in the open label phase of the study was similar in the three study arms regardless of the previous treatments, indicating that, even in patients who did not take any PDE5-Is during the first 12 mo after surgery, the efficacy of the drug was not reduced due to fibrosis within the corpus cavernosa and to cavernous veno-occlusive dysfunction<sup>20</sup>. The figures of the present study, however, reconfirmed the results of the REINVENT study.

The major strength of the present study is the accurate methodology for prospective data collection and standardized reporting, which fulfills most of the criteria recently suggested by Mulhall<sup>21</sup>. Moreover, the patients were treated by only two surgeons using the same surgical technique, which had the peculiarity of being clipless. The major limitations of the study are the number of evaluated patients, which was not particularly large, and the median follow-up, which was only 14 months.

## Conclusions

About 60% of the patients were potent after RALP series. Patients age and preoperative erectile function were the most powerful predictor of erectile function recovery. Adoption of postoperative rehabilitation was not significantly associated with improved postoperative erectile function, once adjusted for the effect of the other covariates.

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# Intra-plaque injection of betamethasone and verapamil using a new plunger mechanism syringe with ergonomic leverage handling for the treatment of Peyronie's disease

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## Summary

We report a review of the literature and our experience in the intra-lesional therapy (medical therapy) for the treatment of Peyronie's Disease.

We use, since 1997, a plunger mechanism syringe with ergonomic leverage handling named Citoject. This syringe develops a high hydraulic pressure, exerting both a pharmacological and a mechanical effect on plaques.

The main endpoints of this study is to compare our results with all the data reported in the recent literature about this issue.

From June 2002 to December 2007, we treated 67 men with Peyronie's disease by administering 10 weekly intra-lesional injections, alternating between betamethasone (4 mg) and verapamil (5 mg). 50 patients (89%) reported plaque-related symptoms during erection. Palpable plaque was present in all patients. Recurvatum was present in 45 patients (80%).

At a mean follow-up of 30 months, we observed rapid resolution of the symptoms in 48/50 patients (96%). A significant reduction in plaque size was noted in 43% of cases (24/56;  $p = 0.001$ ). Recurvatum improved only in a small percentage of cases (26.6%), with no progression of recurvatum.

This device represents a good choice for intra-lesional drug administration in Peyronie's disease. Minimal side effects or adverse reactions were reported by patients, and there was minimal involvement of the normal tissue surrounding the plaque.

## Key words

*Intra-lesion therapy • Penile recurvatum • Peyronie's disease*

## Introduction

The incidence of Peyronie's disease (PD) is about 3-8.9% among the male population. PD is commonly found in men between 40 and 70 years of age; the mean age at presentation is about 55-57 years<sup>1-3</sup>.

Surgical correction is still the gold standard for treating curvature. Since spontaneous resolution of the plaque occurs in 3-13% of patients with PD and surgery for PD is recommended only after stabilisation of the disease<sup>4,5</sup>, a conservative approach is commonly adopted for at least one year after diagnosis. Treatments selected for PD de-

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pend on the stage of the disease, and the main goal of the conservative approach is to reverse, interrupt or attenuate the disease process itself as well as to reduce deformity and to improve sexual function<sup>6,7</sup>. The most common treatments are: ESWT (extracorporeal shock-wave therapy)<sup>8</sup>; radiotherapy<sup>9</sup>; iontophoresis<sup>10,11</sup>; oral agents such as vitamin E<sup>12</sup>, colchicine<sup>13</sup>, tamoxifen<sup>14</sup> and Potaba<sup>15</sup>; and intralesional injection of various compounds, including collagenase<sup>16</sup>, steroids<sup>17</sup>, interferon alpha-2B<sup>18</sup> and verapamil<sup>19,20</sup>.

The goal of our multicentre study was to evaluate the efficacy of intra-plaque therapy delivered using Citoject, a plunger mechanism syringe with ergonomic leverage handling, and to compare our results with the results reported by other authors in the recent literature.

## Patients and Methods

Between June 2002 and December 2006, we treated 67 patients affected by PD: we enrolled 55 patients from the University of Perugia Department of Urology and Andrology (Italy) and 12 patients from the Urology Department at the University Children's Hospital in Belgrade (Serbia).

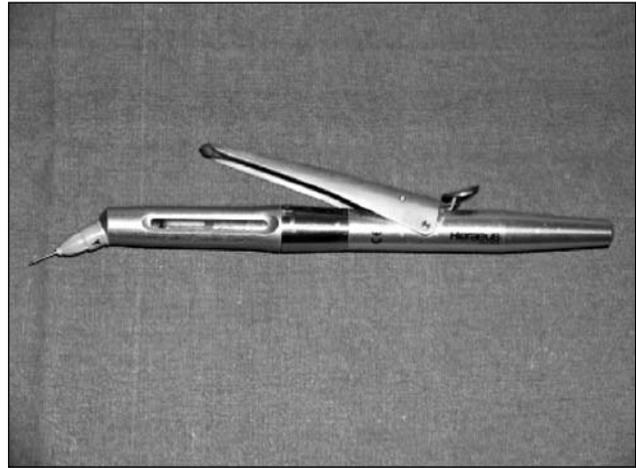
We report here complete and detailed data on 56/67 patients before and after treatment: 11 patients excluded, 3 dropped out during treatment and 8 did not return for follow-up after their treatment was completed.

The mean age of the patients was 54 years (range: 30-67 years). All patients were treated with 10 intra-plaque injections (1 injection/week), alternating betamethasone 4 mg (2 ml) and verapamil 5 mg (2ml). Drugs were injected directly inside the plaque using a plunger mechanism syringe with ergonomic leverage handling (Citoject; Heraeus Kulzer, Hanau, Germany) attached to a short but very hard needle (Carpule 0.4/8 mm, 27 G) (Fig. 1). Using this method, it is possible to create high pressure inside the plaque (the maximum pressure achievable by the Citoject is about 1784 pounds per square inch).

Furthermore, easy modulation of the depth of the injection is possible using this technique, thus reducing damage to surrounding cavernous tissue.

During the medical history, 2 patients were found to have Dupuytren's contracture in addition to PD. One patient had a positive family history for PD. Ten patients reported the onset of the disease after penile trauma during sexual intercourse, two patients developed PD after a radical retropubic prostatectomy and one patient developed PD after endoscopic

Figure 1. Citoject: plunger mechanism ergonomic leverage handling syringe with its disposable blistered needle (0.4 x 8 mm 27 G) and the internal phial for the drugs delivery.



surgery for TURP (transurethral resection of the prostate).

The mean time between onset of the disease (symptoms and/or curvature) and diagnosis was 4.3 months (range: 1-18 months). At presentation, all patients showed at least one palpable plaque, with 46 patients (82%) presenting with a single palpable lesion, 9 patients presenting with 2 lesions, 1 patient presenting with 3 lesions, and 1 patient presenting with 4 plaques. Fifty patients (89%) reported plaque-related symptoms during erection, such as pain, tension or some other kind of disturbance, whereas 6 patients had no symptoms. Forty-five patients presented with penile curvature (80%). Using Kelami classification<sup>21</sup> we found 23 patients to have mild curvature, 17 patients to have moderate curvature and 5 patients to have severe curvature at baseline. Details regarding plaque site and penile recurvatum are described in Tables I and II. Erectile dysfunction was present in 15 patients (26.7%), with 8 patients reporting complete erectile dysfunction and 7 patients reporting mild erectile dysfunction.

We performed a single cycle of injections (10 injections) in 52 patients (93%). Five patients underwent two series of injections – four because of incomplete resolution of the symptoms and one for treatment of a new plaque site.

Before starting treatment, all patients provided a complete medical and sexual history and underwent a routine physical examination and detailed genital examination to localise plaque sites. All patients also underwent penile ultrasound (US) at baseline to assess their plaques (number, size, site, involvement of the septum, presence of calcification) and

Table I. Plaque sites.

PLAQUE SITE	DORSAL	VENTRAL	LEFT SIDE	RIGHT SIDE	SEPTUM
Proximal	8	1	0	0	0
Medium	28	2	3	1	2
Distal	7	2	1	0	1

Table II. Penile recurvatum (45 patients).

RECURVATUM	DORSAL	VENTRAL	LEFT SIDE	RIGHT SIDE	COMPLEX
Number of patients (%)	32 (71.1%)	3 (6.6%)	6 (13.3%)	1 (2.2%)	3 (6.6%)
Mean curvature angle (range)	39.5° (10-90°)	16.6° (10-20°)	30.8° (10-45°)	20°	

penile colour Doppler US during erection (induced using 10 µg of alprostadil) to assess their erections and to measure curvature or other deformities. Patients who did not respond to 10 µg of alprostadil (11 patients, who thus had an incomplete erection) were re-assessed after a several days using a dose of 20 µg of alprostadil. Sexual history, physical examination, penile US and colour Doppler US during erection were repeated 3 months after the first injection to assess any changes in plaque morphology or penile curvature. Only 4 patients required a second alprostadil injection to achieve erection after the PD treatment was completed. Pre-treatment and post-treatment US evaluation was performed by the same physician and with the same ultrasound device in the Italian group.

Patients were enrolled in the study if they met one or more of the following inclusion criteria: plaque-related symptoms during erection (such as pain or tension) and/or the presence of progressive curvature. Patients with plaques that were found to be calcified on US were excluded from the study.

We received the approval of an internal review board for this study. The main endpoints of the study were to examine change in plaque size [the major diameter of the plaque (width, length or depth) was measured using US] and complete resolution of plaque-related symptoms. We also evaluated changes in penile curvature and overall sexual satisfaction as secondary endpoints.

### Treatment technique

The penis is manually grasped at the level of the lesion, and a single puncture is performed. The needle is then advanced into the plaque and the drugs are slowly injected (Fig. 2). After several pushes of the plunger, a decrease in the resistance of drug down flow can easily be perceived – a clear sign that the needle is inside fibrotic tissue. Once inside the

plaque, the injected fluid forms a space between plaque's fibres.

In patients with large plaques, the needle may be removed and the injection repeated at several sites in the same lesion. No local anaesthesia is required for the treatment. After the injection has been completed, patients are advised to hold pressure at the site of injection for 2 minutes to avoid the risk of ecchymosis and/or haematoma. During each of the 10 treatment sessions, we changed the site of intra-plaque injection in order to approach the plaque from every side. In this way, every part of the plaque received a good dose of the drugs.

### Statistical analysis

Statistical analysis was performed using the Wilcoxon test for paired data. The threshold for statistical significance level was set at  $p < 0.05$ . All data analyses were carried out using Prism 4 for Windows, release 4.00 (GraphPad Software Inc., San Diego, CA, USA, 2003).

Figure 2. Treatment of the plaque using Citoject.



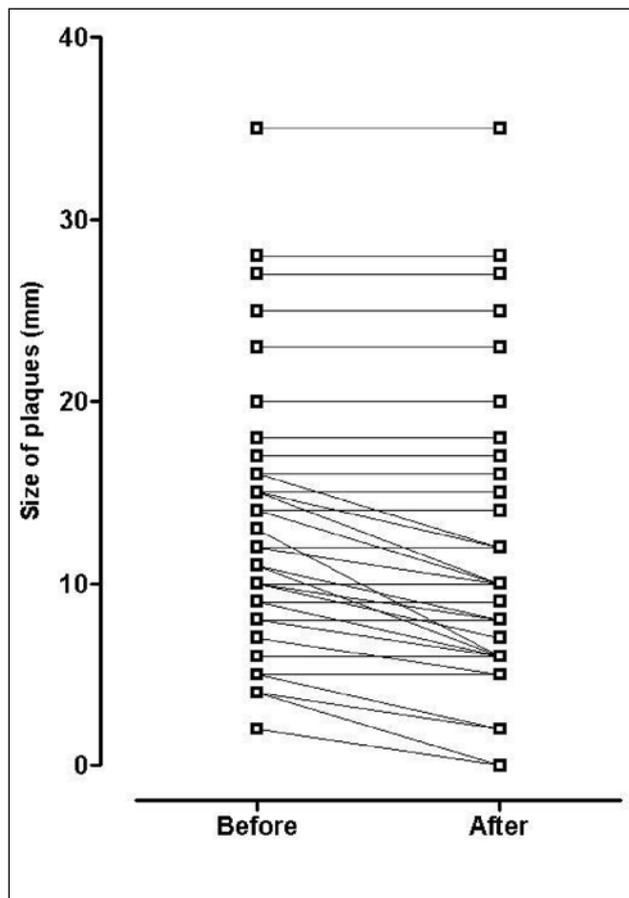
Table III. Treatment results 3 months after diagnosis.

	PRE-TREATMENT MEAN (STD. DEVIATION)	POST-TREATMENT MEAN (STD. DEVIATION)	P
Plaque size	12.46 mm (6636)	11.13 mm (7216)	0.001

**Results**

The mean follow-up of 56 patients included in this study was 30 months (range: 3-57 months). We observed complete resolution of plaque-related symptoms in 48 of 50 patients (96%), and 2 patients reported only partial pain relief. A mean of 4 injections (range 2-10) was required for resolution of clinical symptoms. Three months after the first injection, a reduction in plaque size was observed in 24/56 patients (43%), while in 57% of patients, the plaques remained stable. Results were significantly better among patients with plaques smaller than 2 cm (p = 0.001; Table III; Fig. 3).

Figure 3. Statistical analysis shows the plaques size variation before and after treatment (better results were obtained in plaque < 2 cm).



A reduction of recurvatum was reported in a small percentage of patients (26.6%). Reduction in recurvatum was assessed using pictures drawn by the patients and by physical examination during erection performed 3 months after the treatment. None of the patients reported an increase in penile curvature. During the sexual interview, 47 of 56 patients (84%) reported an improvement in overall sexual satisfaction, most of them because of the resolution of symptoms.

Only 7 patients (12.5%) underwent surgery after medical treatment. Three patients underwent corporoplasty with buccal mucosa grafting, two underwent corporoplasty with saphenous vein grafting and two underwent corporoplasty with saphenous vein grafting and Virilis I prosthesis implant (soft prosthesis) placement. The surgeons who performed these surgeries did not experience any technical difficulty during the dissection of the penile fascia (Buck's fascia) from the tunica albuginea or note the presence of any inflammatory reaction or scar tissue in any patient who underwent surgery.

Only 3/67 patients dropped out of the study during treatment due to pain (4%). No patients experienced ecchymosis and/or haematoma in the site of the injection, and no patients experienced side effects and/or adverse reactions to the drugs.

**Discussion**

PD is an acquired disease of the tunica albuginea, which presents as a palpable fibrous plaque or a nodule (suggesting a localised reduction of penile elasticity). It can then cause penile curvature, painful erections, shortening of the penis and, in some cases, erectile dysfunction. The aetiology of PD is still unknown, and its molecular pathology is poorly understood. However, according to the theories proposed by several authors, a single traumatic event or repeated micro-traumas during sexual activity<sup>22</sup> may cause a prolonged inflammatory reaction of the fibres of the tunica albuginea and lead to a low-level autoimmune response<sup>23</sup>. This reaction involves several mechanisms, such as micro-vascular injury, the accumulation of inflammatory cells

and fibrin and the overexpression of cytokines and growth factor which stimulate the production of a proteins matrix (elastic and collagen fibres) that can ultimately lead to plaque formation <sup>24</sup>.

As reported by several authors, intra-lesion injection of several different kinds of drugs can successfully be employed to treat PD <sup>16-20</sup>, especially when patients are in the early, painful progressive stages of the disease, do not have uncalcified plaques, and have a short case history.

Many different drugs are currently used for intra-lesional therapy, but corticosteroids and verapamil are the most common, as reported in a survey of German urologists <sup>25</sup>. The rationale for using steroids is based on the anti-inflammatory effect of these drugs and their capacity to decrease collagen synthesis. At present, only one single-blind, placebo-controlled study has been performed using corticosteroids, which showed no significant effects on disease characteristics but good results in terms of disease-related symptoms <sup>17</sup>.

The rationale for using verapamil in the treatment of PD is based on evidence that the function of fibroblasts (cell proliferation, extracellular matrix protein synthesis and secretion, collagen degradation) can be changed when treated with a calcium antagonist. In vitro models and several clinical trials have shown that calcium antagonists (e.g., verapamil) can slow, prevent or even reverse the process of plaque formation and growth <sup>26 27</sup>.

Intra-lesional drug injection is still one of the most common treatments used in patients with early-stage symptomatic PD. This choice is based on studies that have shown satisfying results.

However, little evidence is available from randomised prospective studies. The first randomised single-blind study proposed by Rehman et al. including 14 patients (7 patients treated with ve-

rapamil vs. 7 untreated who comprised the control group) showed a reduction of plaque dimensions in the verapamil-treated group compared to the control group. Unfortunately, no significant difference was noted between the groups with regard to improvement of recurvatum ( $p < 0.07$ ) <sup>28</sup>. In a recent non-randomised study by Levine et al., which included 156 patients with PD treated with intralesional verapamil injection, the authors reported a reduction in pain in 84%, decrease of curvature in 60% and improvement in sexual function in 71% of patients <sup>19</sup>. More recently, in another non-randomised study, Bennet et al. treated 94 patients with a total of six intralesional verapamil injections that were administered on alternating weeks. In this series, 18% of patients showed reduction of recurvatum and all patients had improvement in penile pain (100%) <sup>20</sup> (Table IV).

In our study we used steroids and verapamil together in order to combine the anti-inflammatory effect of betamethasone, which leads to early resolution of plaque-related symptoms, and the anti-fibrotic effects of verapamil, which serves to prevent or reverse plaque growth. Citoject offers the ability to inject the drugs into the plaque, which is ideal, because the extreme rigidity and hardness of plaque commonly obstructs the downflow of drugs when a normal plastic syringe is used. The high pressure achieved inside the lesion using a Citoject syringe leads to a detachment of several anatomic planes of the plaque and to its progressive thinning.

We obtained a reduction in plaque size in a good percentage of patients (43%), with complete plaque stabilisation in the remainder of patients (67%;  $p = 0.001$ ). Furthermore, statistical analysis showed a strong correlation between plaque dimension and treatment efficacy: the larger the plaque, the worse the result of the treatment. We also observed

Table IV. Comparison of our data with other large series.

	LEVINE 2002 <sup>19</sup>	BENNET 2007 <sup>20</sup>	OUR SERIES
N° of patients	156	94	56
Drugs	Verapamil	Verapamil	Verapamil/betamethasone
Dosage	10 mg every 2 weeks	10 mg every 2 weeks	10 mg/4 mg (alternating weekly)
Number of injections	12	6	10 (5+5)
Pain reduction %	84	100	96
Improved curvature %	60	18	26.6
Improvement of sexual function %	71	26	84
Plaque size reduction %	Not available	Not available	43

early resolution of plaque-related symptoms and, consequently, an improvement in overall sexual satisfaction.

Finally, the localised pharmacological action of the drugs is strictly limited to the plaque, preventing inflammatory involvement of the surrounding tissues and avoiding scar reaction. Thus, intra-lesional therapy stabilises fibrotic plaques and therefore does not affect surgical success if surgery is needed in the future.

## Conclusions

The larger part of current literature, about conservative treatment in Peyronie' disease, is so confused and the results are not so easy to understand because all the Authors used different parameters to evaluate efficacy of the different treatments.

Furthermore all the authors used different schedule to treat patients and different drugs, so it is impossible, at present state, to establish which of them is the most useful.

However, in our experience, Citoject syringe simultaneously delivers a high concentration of drug inside plaques and exerts a mechanical effect on them, has minimal side effects and/or adverse reactions for the patients, and leads to minimal involvement of the tissues surrounding the plaque. The combined use of betamethasone and verapamil, injected in the deep part of the plaque, leads to rapid resolution of pain after few injection.

This device represents a good option for intra-lesional drug administration in the treatment of PD.

Surely the development of new technologies and new therapeutic strategies is necessary to obtain better results in the treatment of PD.

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# L-carnitine fumarate, L-acetyl-carnitine and other components in male infertility: a pilot study of nutraceuticals on sperm motility

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## Summary

The aim of this study was to evaluate any possible effect a combination of L-carnitine, fumarate, acetyl-L-carnitine, fructose, citric acid, selenium, co-enzyme Q10, vitamin C, zinc, folic acid and vitamin B12 (Proxceed<sup>®</sup>) on sperm motility in a group of patients with unexplained asthenozoospermia.

Thirty two patients received 1 sachet/day of Proxceed<sup>®</sup> for three months. Sperm parameters were studied before, during and after this treatment. Motility was also studied by the means of a computerized analysis (CASA system).

The results of this study indicate that Proxceed<sup>®</sup> is able to increase sperm motility, both in a quantitative and qualitative manner. We conclude that oral administration of Proxceed<sup>®</sup> may improve sperm quality, at least in patients with idiopathic asthenozoospermia.

## Key words

L-carnitine • Male infertility • Sperm motility

## Introduction

In spite of the progress in knowledge of the physiological mechanisms of spermatogenesis, of its endocrine stimulation and of intratesticular paracrine control, the possible therapies and applications resulting from this knowledge are still scarce. In fact, to date a therapeutic approach of oligoasthenozoospermia (OAT) based on an etiopathogenetic diagnosis is possible only in a small percentage of cases. This type of approach, which is obviously the most correct, has limitations partly due to not understanding fully the mechanisms at stake in determining infertility, especially as far as damage to sperm maturation is concerned, and partly due to the fact that often at the moment of diagnosis the *noxa patogena* is no longer evident nor identifiable and thus not resolvable. The OAT therapy is often based on attempts to correct the symptomatology of the seminal fluid.

Symptomatic treatment of idiopathic dyspermia is used both in cases where an etiological diagnosis is impossible and also in cases of permanent dyspermia after resolving the basic *noxa patogena*. The following are among the substances that have been used: L-carnitine

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and acetyl-carnitine, co-enzyme Q10, L-arginine, callicreine, pentoxifilline, phosphatidylcholine, vitamins (above all A., C and E) <sup>1</sup> and glutathione <sup>2 3</sup>.

L-carnitine (LC) is a water-soluble molecule, present both in plasma and in the tissue where it can be found free-moving or bound to fatty acids in the membranes in the form of acylic derivatives.

Carnitine is an essential cofactor in the transfer of lipids into the mitochondrion where they are metabolized for energy in the beta-oxidation process. The role of carnitine in cellular metabolism is as such better known in the mitochondrions where the interaction between the metabolism of the fatty acids and the glucose is fundamental for the production of cellular energy. Moreover, the activity of carnitine has been proposed also for the antioxidising systems. Male germ cells have low levels of molecules and enzymes with antioxidising functions and present a particular structure of polyunsaturated fatty acids in the membrane. These fats are particularly susceptible to the lipoperoxidative phenomena associated with forms of OAT <sup>4</sup>.

In the last few years, preparations based on dietary supplements, vitaminic complexes and nutritional principles have come to be used also in the treatment of infertility, following the latest indications of nutraceuticals. By this term we mean the use of nutritional substances added to a normal diet in order to promote a healthy and active way of life. Nutraceuticals research, of French origin, is based on the principle of nutritional synergy: "the wider the range of nutrients taken, the greater will be the assimilation and efficacy of these, while at the same time, lesser quantities will be needed of each specific nutrient". Recently in Italy a product called Proxeed NF® (Sigma Tau) has been commercialized, which is made up of the following: L-carnitine fumarate: 250 mg, acetyl-L-carnitine hydrochloride: 75 mg, fructose: 250 mg, citric acid: 50 mg, selenium: 50 µg, coenzyme Q10: 20 mg, vitamin C: 90 mg, zinc: 10 mg, folic acid: 200 µg and vitamin B12: 1.5 µg. The L-carnitine and the acetyl-L-carnitine participate in important metabolic processes involved in the production of energy and the upkeep of normal cellular functioning. Fructose and citric acid are essential for energetic metabolism. The coenzyme Q10 is a natural fat-soluble antioxidant which protects against lipidic peroxidation and oxidative damage to the DNA. Selenium is an essential component for cellular protection and for defence against the free radicals of oxygen. Zinc is a micro-element that is essential for cellular division and protection, as well as being a scavenger of the free radicals of oxygen. Vitamin C

has an antioxidising action and promotes the use of microelements such as zinc. Folic acid and vitamin B12 are essential elements for energetic metabolism and cellular division. The various components of this product taken singly show a positive action on the sperm parameters, particularly on the total and forward motility.

The aim of our work was to assess the efficacy of this important combination of components on the kinetic parameters of the spermatozoa in an open study.

## Materials and Methods

We studied a group of 32 infertile patients, aged between 20-40 years with the following seminal characteristics: sperm concentration 10-60x10<sup>6</sup>/ml, total motility 5-40%, forward motility.

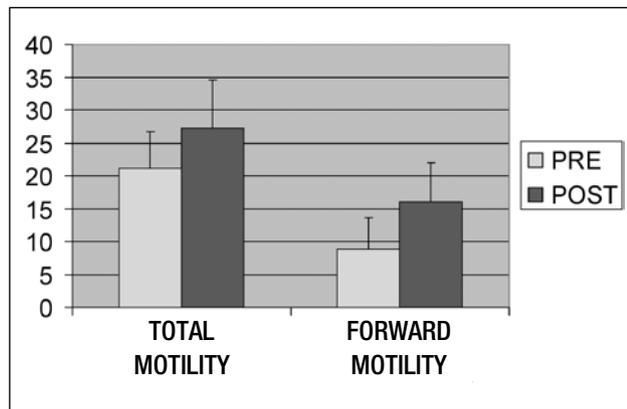
≤ 15% and atypical forms ≤ 90%. None of the patients presented antispermatozoa antibodies, systemic or endocrine illnesses, infections of the genital tract, mono or bilateral testicular hypotrophy, varicocele with testicular hypotrophy, cryptorchidism and mump orchitis in the pubescent phase, and none had undergone surgical operations in the genital area.

The patients were administered Proxeed NF® at a dose of 1 sachet/day for three months. The study included analysis of seminal fluid and sperm kinetics with the CASA system at baseline and after three months of therapy. Analysis of the seminal fluid was carried out according to WHO criteria (1999). By means of the CASA system the following kinetic parameters were assessed: mean curvilinear velocity, linearity, maximum width of lateral beats of the head (ALH – amplitude of lateral head) and frequency of beats (BCF – beat cross frequency). The mean and the standard deviation was calculated for all the seminal variables.

## Results

The results of the analysis of the semen showed a statistically significant increase in total and forward motility after three months of treatment; in fact the average total motility before and after treatment was 21.2 ± 5.6 % and 27.2 ± 7.4% respectively, while the forward motility was 8.9 ± 4.7% before and 16.1 ± 5.9% after therapy (Fig. 1). As far as the computerized study of sperm kinetics is concerned, the data show a statistically significant increase in mean velocity and linearity of the spermatozoa; also the ALH and BCF values showed an increase, although these last did not reach statistical significance.

**Figure 1.** Means and SD of total and forward motility pre and post treatment.



## Discussion

Nutraceuticals is a new term which derives from the combination of the words 'nutrition' and 'pharmaceuticals'; its principal aim is to study the combined nutrient and pharmaceutical properties of foods that have beneficial effects on human health. It has been developing over the last years as a complementary sector to the field of pharmaceuticals, as an adjuvant in the maintenance of physiological homeostasis and as prevention of cellular stress from free radicals. It is worthy of note that nutraceutical supplements must be based on actual necessity, just as pharmacological ones, in order to not risk causing damage rather than benefits to the patient. As such, it is fundamental to increase clinical studies and trials in order to identify the actual needs of each single individual and consequently formulate the most appropriate nutraceutical response. Among the substances in this group of nutraceuticals are included the following: L-carnitine, Acetyl-L-carnitine, Fructose, Citric acid, Selenium, Coenzyme Q10, Vitamin C, Zinc, Folic acid, Vitamin B12. These have been used in various clinical trials, either singly or in combination, in the therapy of alteration of seminal parameters, especially that of sperm motility. Our group carried out two controlled studies on the use of carnitine and its metabolites in the treatment of selected forms of male infertility. The first was a double-blind crossover trial, using carnitine and placebo. One hundred infertile patients were selected aged between 20-40 years with the following seminal characteristics: sperm concentration 10-20x10<sup>6</sup>/ml, total motility 10-30%, forward motility <15% and atypical forms <70%. The patients were administered L-carnitine at doses of 2 gr/die or placebo. The study was organized in phases of two months of run-in, two months of therapy/placebo, a further two months of wash-out and two months of

therapy/placebo. A statistically significant increase in concentration values and in total and forward motility was found in patients who were administered therapy compared to those treated with placebo. Improvement of sperm motility was more marked in those patients who presented a lower concentration of normo-kinetic spermatozoa before therapy (number of spermatozoa with total motility <10x10<sup>6</sup> and forward motility <5x10<sup>6</sup> / ejaculate) <sup>5</sup>.

The second was a double-blind randomized study the aim of which was to determine the efficacy of combined therapy of L-carnitine and L-acetyl carnitine. Sixty infertile patients aged between 20-40 years were selected according to the following criteria: sperm concentration 10-40 x 10<sup>6</sup>/ml, forward motility <15%, total motility 10-40%, atypical forms < 80%. The patients were administered a combined therapy of L-carnitine (2g/die) and L-acetyl carnitine (1g/die) or placebo. The study was organized in phases of 2 months of run-in, 6 months of therapy or placebo followed by 2 months of follow-up. Also in this study the most significant increase of forward motility was observed in patients with initially lower kinetic values (number of spermatozoa with total motility < 5x10<sup>6</sup> and forwards motility < 4x10<sup>6</sup>/ejaculate) <sup>6</sup>.

Other studies have demonstrated the efficacy of this therapy in patients with OAT associated with varicocele <sup>7</sup> and in patients with prostate-vesicular-epididymitis <sup>8</sup>.

The data published with reference to controlled studies of efficacy show that treatment with carnitine and its derivatives is able to improve sperm motility, especially in groups of patients with more marked asthenozoospermia.

The current study has confirmed the positive effect of these substances on sperm motility; despite the limited number of patients, a statistically significant increase in sperm motility (both total and forward) was found after therapy with Proxeed NF<sup>®</sup>, associated with a statistically significant increase in the kinetic parameters, velocity and linearity, assessed with the CASA system. These results appear particularly significant and interesting in the light of the fact that our group of patients was characterised by an idiopathic asthenozoospermia.

In conclusion, this study has demonstrated a clear positive effect of the oral administration of Proxeed NF<sup>®</sup> on the kinetics of the spermatozoon in a selected group of subjects affected with asthenozoospermia *sine causa*. Thus, we propose this type of therapeutic approach as a possible technique for treating selected forms of male infertility, also

in view of the serious lack of treatments that are efficacious on the mechanisms of activation and maintenance of sperm kinetic parameters. This therapy could also be indicated in the preparation of the male partner in couples following intrauterine insemination programmes or other assisted fertilization techniques.

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