

Y-Chromosome Microdeletion Analysis in Infertile Men from Upper Egypt

Kamal Z. Shaer, MD*; Essam Nada, MD**; Ramadan Saleh, MD**; Manal O. El Hamshary, PhD***; Ashraf A. Abd El-Latif, MSc**.

* Department of Andrology, Sexology and STDs- Faculty of Medicine- Cairo University; ** Department of Dermatology, Venereology and Andrology- Faculty of Medicine- Sohag University;*** Department of Molecular Diagnostics and Therapeutics- Genetic Engineering and Biotechnology Research Institute- Sadat City University.

ABSTRACT

Objectives: To study the prevalence and patterns of Y-chromosome microdeletions in infertile men from Upper Egypt and to determine the relationship between Y-chromosome microdeletions with clinical and laboratory findings in these patients.

Design: Cross-sectional study.

Patients: Infertile men (n = 210) and a control group of fertile men with normal semen analysis (n = 30).

Methods: Clinical evaluation, standard semen analysis according to the WHO guidelines (WHO, 2010) and serum levels of reproductive hormones were evaluated. Multiplex PCR was done for detection of Y chromosome microdeletions.

Results: AZF deletions were present in 7.14% of infertile men (9.59% in azoospermic and 1.56% in oligo-zoospermic men) with no deletions in the fertile normo-zoospermic men. Complete AZFc was detected in 2.05% of azoospermic men. Partial AZFc deletions were found in 5.7% of infertile men, with gr/gr deletion in 5.24% and b2/b3 deletion in 0.48%. There was no significant difference between patients with AZF deletions and azoospermic men without deletions as regards testicular volume and serum levels of FSH, LH, testosterone and prolactin.

Conclusions: Microdeletions of Y chromosome may play a role in pathogenesis of non-obstructive azoospermia. The testicular volumes as well as levels of FSH, LH, testosterone and prolactin were not correlated with the finding of Y chromosome microdeletions.

Key words: male infertility, Y chromosome microdeletion, AZF regions.

INTRODUCTION

Infertility is defined as the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse⁽¹⁾. It affects approximately 10-15% of couples, even up to 30% in some regions of the world⁽²⁾. Male infertility contributes to more than half of all cases of global childlessness⁽³⁾. At least 15% of cases with male infertility are related to genetic disorders, including both chromosomal and single-gene alterations⁽⁴⁾.

Microdeletions of Y chromosome are the second most frequent genetic cause of male infertility after Klinefelter syndrome⁽⁵⁾. A region on the long arm of the Y chromosome has been identified as the

“Azoospermia Factor” and is subdivided into three regions, AZFa, AZFb, and AZFc⁽⁶⁾. Microdeletions of AZF regions are related to spermatogenic failure⁽⁷⁾. The overall frequency of Y chromosome microdeletions varies from 1 to 55% in the different published studies⁽⁸⁻¹⁰⁾. The most frequent deletion type is the AZFc region deletion (approximately 80%) followed by AZFa (0.5-5%), AZFb (1-4%) and AZFbc (1-3%) deletion⁽¹¹⁾.

Partial AZFc deletions were identified, with gr/gr deletion as the most common, followed by b2/b3 and b1/b3⁽¹²⁾. Although gr/gr deletion removes half of the AZFc gene content, its clinical significance is still a matter of debate, because carriers may exhibit highly variable spermatogenic

phenotypes ranging from azoo- to normozoospermia. Clearly the effect of the deletion is largely dependent on the ethnic and geographic origin of the study population⁽¹¹⁾.

In Egypt; examination of Yq microdeletions in only 33 infertile males with undetectable chromosomal anomalies revealed microdeletions in four men (12%); with two patients had deletions at AZFc, one at AZFa and last one had microdeletion at almost the entire AZF region⁽¹³⁾. Another study carried out in 2010 on 49 patients (28 with azoospermia and 21 with severe oligospermia) reported microdeletions in 24 patients (37%) and the highest frequency of microdeletions was in the AZFb locus (66.7%), followed by the AZFa locus (20.8%) and then the AZFc locus (12.5%)⁽¹⁴⁾.

Microdeletions were reported in 20.4% (11/54) of azoospermic men with 10 patients (90.9%) having AZFc deletion and one case (9.1%) had partial AZFa+b deletion. Among the 10 cases who had AZFc deletions, five cases had gr/gr subtype (50%), and the other 5 cases had b2/b4 subtype⁽¹⁵⁾. Another study found Y-chromosome microdeletions in 10.3% (11/107) of azoospermic patients and the highest frequency of microdeletions was in the AZFc locus, followed by the AZFb and the AZFa loci⁽¹⁶⁾.

Identification of a particular AZF deletion can provide valuable prognostic information. Complete deletions of the AZFa or AZFb regions indicate that sperm will not be found at the time of testicular sperm extraction (TESE), whereas deletions in AZFc indicated a 50% likelihood of finding sperm on microTESE⁽⁷⁾. Testing of Yq microdeletions is also an essential prerequisite for infertile men undergoing intra-cytoplasmic sperm injection to rule out the possibility of transmission of the same deletions to their male offspring, who could also experience infertility⁽¹⁷⁾.

The objectives of this study were to:(1) explore the patterns of Y-chromosome microdeletions in infertile men from Upper

Egypt, and(2) determine the relationship between Y-chromosome microdeletions with clinical, laboratory and histopathological findings in these patients.

PATIENTS AND METHODS:

This cross-sectional, study included 210 infertile men attending Andrology clinic at Sohag University Hospitals. A group of 30 fertile men with history of having at least one child in the last 2 years and normal semen analysis was included as a control. The study was approved by Ethical and Research committees at Sohag Faculty of Medicine. An informed consent was obtained from all participants.

Exclusion criteria:

Ÿ Patients with evidence of obstruction of seminal tract.

Ÿ Patients with evident varicocele.

Ÿ Patients with defective spermatogenesis secondary to genital infection, trauma or torsion.

Ÿ Patients with history of testicular maldescent.

Ÿ Patients having numerical chromosomal anomalies.

Ÿ Patients who were treated with chemotherapeutic agents or radiotherapy.

Methods: Patients were evaluated as follow:

I- Initial evaluation: Personal data (age, residency, occupation and special habits) were obtained from all participants. Marital and sexual histories were obtained from all participants. The family history, including data on the fertility status of parents, and relatives was reported.

General examination was done to detect eunuchoid features. Careful genital examination was performed to detect abnormality of penis, testes or epididymis. The spermatic cord was examined for presence of vas, nodularity or varicocele.

II- Laboratory investigations:

1) Semen analysis:

Semen analysis was performed according to World Health Organization 2010 guidelines⁽¹⁸⁾. According to sperm concentration, participants were classified into three categories: normozoospermic (\geq

15 millions/ml), oligospermic (<15 millions/ml), or azoospermic (complete absence of spermatozoa even after centrifugation at 3000g for 15 minutes for at least 2 times, 2 weeks apart).

2) Hormonal profile:

Four ml of venous blood of the patient was drawn from the cubital vein in the morning from 08.00 a.m. to 10.30 a.m. and was incubated at 37°C water bath for 10 minutes, and centrifuged at 3000 g for 10 minutes. Serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), total testosterone and prolactin were measured using an enzyme linked fluorescent immunoassay with a mini-VIDAS device (BioMERIEUX SA). The normal male reference ranges for these hormones were: FSH (1.7-12.0 mIU/ml), LH (1.1-7.0 mIU/ml), total testosterone (3.0-10.6 ng/ml) and prolactin (3.0- 25.0 ng/ml).

3) Y-chromosome microdeletion analysis:

3.1- DNA Extraction:

Genomic DNA was extracted from peripheral leukocytes collected from a venous blood sample. The DNA was extracted using the wizard genomic DNA purification kit according to the manufacturer's instructions.

3.2- DNA Amplification:

Polymerase chain reaction (PCR) was done on MJ-research-100 machine. The long arm of the Y chromosome DNA was subjected to PCR using 8 sets of Y specific sequence tagged sites (STSs). A two-step approach was employed to identify the partial AZFc deletions. First screening was performed using six pairs of primers to screen and exclude large deletions of the AZF region (AZFa, AZFb, AZFc) according to the European Academy of Andrology and the European Molecular Genetics Quality Network (EAA/EMQN) 2014 guidelines⁽¹¹⁾. These STSs were: sY84 and sY86 for AZFa; sY127 and sY134 AZFb; and sY254 and sY255 for AZFc.

Second step amplification was performed to screen for partial deletions of AZFc region using STSs: sY1191 (specific for the gr/gr-

g1/g2 and b1/b3 deletions) and sY1291 (specific for the b2/b3 and gr/gr-g1/g3 deletions)⁽¹²⁾. The diagnosis of gr/gr deletion was based on the absence of marker sY1291 and presence of sY1191; while the absence of sY1191 and presence of sY1291 represented b2/b3 deletion⁽¹¹⁾.

Single PCR technique was performed. In the case of the absence of any marker the PCR was repeated two times at the same annealing temperature and two times at lower annealing temperature and an internal control (SRY marker) to confirm the deletion and to rule out false negative PCR result. A 2.0 µl (50 ng) aliquot of the genomic DNA was amplified by PCR, in a total volume of 12 µl using PCR master mix from Promega, USA. The PCR was carried out according to the following protocol: 36 cycles at 94°C for 30 s, 60°C for 50 s and 72°C for 60 s. The program was preceded by a 4 min denaturation step at 95°C and followed by a final extension step at 72°C for 5 min. In situations of absent PCR product, the reaction was repeated at 55°C annealing temperature with internal control (SRY) to rule out false results.

3.3- Visualization of PCR products:

Visualization of PCR products was performed by running the samples against a 1 kb DNA ladder (Promega; USA) as a marker on a 2% agarose gel stained with ethidium bromide 0.5mg/ml. In all amplification reactions, a female DNA and a water sample (with no template) were included as negative controls. As a positive control, a male DNA previously proved positive for all the markers was included in the amplification reaction. The PCR data was compared to the latest Y chromosome map reported^(6, 12).

3.4- Reagents preparation:

3.4.1- PCR buffer: 10X PCR buffer contains: 10 mM Tris-HCL pH8.3; 1.5 mM MgCl₂; 50 mM KCl.

3.4.2- Tris-acetate-EDTA (TAE) Electrophoresis buffer: 1X = 40 mM Tris base, 40 mM acetic acid, 1 mM EDTA (ethylenediaminetetraacetate).

3.4.3- Loading buffer: 0.5 gmbromophenol blue, 40% sucrose, up to 100 ml water.

3.4.4- Ethidium Bromide stain: Stock solution of 0.5 gm/ml is prepared. Final concentration included in agarose gel is 0.1 to 0.5 ug/ml.

Statistical analysis:

Data were analyzed using Statistical Package for Social Sciences soft ware

program (SPSS, version 24). Qualitative variables were recorded as frequencies and percentages and were compared by chi-square test. Quantitative variables were presented as means \pm standard deviation (SD) and were compared by independent *t*-test. *P* value < 0.05 was considered statistically significant.

RESULTS

The study populations were classified according to sperm concentration into 3 groups: azoospermia (n=146), oligozoospermia (n=64) and fertile normozoospermic men (n=30).

Demographic data:

The mean age \pm SD of the infertile patients was 34.12 \pm 5.95 years, with 105 (50%) of them from urban areas and 76 (36.2%) of them were smokers. The mean duration of marriage \pm SD of the infertile patients was 5.40 \pm 3.96 years. Family history of infertility was positive in 14 (6.7%) of the infertile patients. The demographic data of the infertile men groups as well as control group are shown in table 1.

Table 1: Demographic data in the study population.

Item	Azoospermia positive microdeletion (n= 14)	Azoospermia negative microdeletion (n= 132)	Oligozoospermia negative microdeletion (n= 63)	Normozoospermia negative microdeletion (n= 30)	Overall <i>P</i> value*	<i>P</i> 1	<i>P</i> 2	<i>P</i> 3	
Age (years)	36.6 \pm 5.9	34.2 \pm 6.5	33.6 \pm 5.4	33.4 \pm 3.2	0.11	0.17	0.10	0.09	
Residence	Urban	6 (43%)	69 (52.3%)	30 (47.6%)	0.56	0.51	0.75	0.29	
	Rural	8 (57%)	63 (47.7%)	33 (52.4%)					12 (40%)
Occupation	Employe	2 (14.3%)	30 (22.7%)	23 (36.5%)	0.51	0.96	0.49	0.57	
	Farmer	4 (28.6%)	47 (35.6%)	20 (31.7%)					8 (26.7%)
	Worker	7 (50%)	51 (38.6%)	20 (31.7%)					11 (36.7%)
	Others	1 (7.1%)	4 (3%)	0					1 (3.3%)
Smoking	4 (26.7%)	48 (36.4%)	24 (38.1%)	7 (23.3%)	0.45	0.78	0.50	0.99	
Duration of current marriage (years)	5.43 \pm 2.34	5.65 \pm 4.46	5.59 \pm 3.74	4.03 \pm 2.09	0.23	0.77	0.84	0.07	
Family history of infertility	1 (7.1%)	10 (7.6%)	3 (4.8%)	2 (6.7%)	0.91	0.64	0.86	0.94	

* P value < 0.05 was considered significant.

P1: between azoospermia positive deletion and azoospermia negative deletion.

P2: between azoospermia positive deletion and oligozoospermia negative deletion.

P3: between azoospermia positive deletion and normozoospermia negative deletion.

Clinical and laboratory data:

In the infertile men; the mean volume \pm SD of the right testis was 11.79 ± 2.85 ml and that of the left testis was 10.87 ± 2.87 ml. The mean serum level \pm SD of hormones were FSH (21.89 ± 10.23 mIU/ml), LH (14.12 ± 6.19 mIU/ml), testosterone (5.60 ± 1.37 ng/ml), and prolactin (6.88 ± 1.60 ng/ml). The clinical and laboratory data in the infertile men groups as well as control group are shown in table 2.

Table 2: Clinical and laboratory data in the study population.

Item		Azoospermia positive microdeletion (n=15)	Azoospermia negative microdeletion (n=132)	Oligozoospermia negative microdeletion (n=63)	Normozoospermia negative microdeletion (n=30)	Overall P value*	P1	P2	P3
		with	with	with	with				
Testicular volume (ml)	Right testis	11.71 \pm 1.44	10.80 \pm 3.13	12.46 \pm 1.80	14.70 \pm 0.66	<0.001	0.62	0.11	<0.001
	Left testis	10.43 \pm 1.70	9.92 \pm 3.10	11.63 \pm 2.07	13.60 \pm 0.81	<0.001	0.35	0.31	<0.001
Hormonal profile	FSH (mIU/ml)	21.29 \pm 7.27	22.5 \pm 11.04	12.98 \pm 4.18	8.45 \pm 1.55	<0.001	0.69	0.002	<0.001
	LH (mIU/ml)	14.26 \pm 4.83	14.08 \pm 6.57	8.61 \pm 3.19	5.75 \pm 1.71	<0.001	0.92	0.001	<0.001
	Testosterone (ng/ml)	5.38 \pm 1.39	5.39 \pm 1.41	5.96 \pm 1.41	5.82 \pm 0.88	0.44	0.98	0.18	0.29
	Prolactin (ng/ml)	7.07 \pm 1.62	6.77 \pm 1.90	6.59 \pm 1.02	6.38 \pm 0.83	0.12	0.52	0.29	0.09

FSH= follicle stimulating hormone;; LH= luteinizing hormone

* P value < 0.05 was considered significant.

P1: between azoospermia positive deletion and azoospermia negative deletion.

P2: between azoospermia positive deletion and oligozoospermia negative deletion.

P3: between azoospermia positive deletion and normozoospermia negative deletion.

Results of PCR:

Regarding the examination of Y chromosome microdeletions; deletions were found in 15 of the 210 infertile patients (7.14%). AZFc deletion was found in 3 of the 210 infertile patients (1.4%), b2/b3 deletion in 1 (0.48%) and gr/gr deletion in 11 (5.24%) of infertile

patients. No deletion was found in the fertile normozoospermic men. Only one case of gr/gr deletion was found in oligozoospermic men (1.56%). Out of the 146 azoospermic men; 3 (2.05%) had complete AZFc deletion, 1 (0.69%) had b2/b3 deletion, and 10 (6.85%) had gr/gr deletion.

The age of oligozoospermic man with gr/gr deletion was 24 years, testicular volume was 13 ml bilaterally, sperm concentration was 2 million/ml, and hormonal levels were FSH (13.6 mIU/ml), LH (8.6 mIU/ml), total testosterone (6.4 ng/ml), and prolactin (7.2 ng/ml).

DISCUSSION

Microdeletions of Y chromosome are the second most frequent genetic cause of male infertility after Klinefelter syndrome⁽⁵⁾. A correlation between Y chromosome deletions in AZF regions and male infertility was first documented in 1976⁽¹⁹⁾. Partial AZFc deletions were identified, with gr/gr deletion as the most common⁽¹²⁾. The effect of the deletion is largely dependent on the ethnic and geographic origin of the study population⁽¹¹⁾.

In the current study; microdeletions were found in 7.14% of infertile men, which is less than that previously reported in Egyptian studies; 12%⁽¹³⁾, 37%⁽¹⁴⁾, 20.4%⁽¹⁵⁾, and 10.3%⁽¹⁶⁾. These differences may be related to different inclusion criteria, sample size and the used technique.

Complete AZFc deletion was found in 1.4% of the infertile population. This was near that previously reported in Germany (1%)⁽²⁰⁾, South Iran (1.25%)⁽²¹⁾, and India (0.97%)⁽²²⁾. This rate was less than previously reported in Egyptian studies; 6%⁽¹³⁾, and 9.2%⁽¹⁵⁾. Higher prevalence was also reported from other studies; 7.4 in a Han-Chinese population⁽²³⁾, and 9.17% in Dravidian-Indian⁽²⁴⁾.

In the present study; partial AZFc deletions were found in 5.7% of infertile men, with gr/gr deletion in 5.24% and b2/b3 deletion in 0.48%. This in accordance with a previous study from

Northern Italy that reported partial deletions in 5.2% of infertile men with gr/gr deletions in 5% and b2/b3 deletions in 0.2%⁽²⁵⁾. Another study reported partial

deletions in 6.2% of infertile men in China with gr/gr deletions in 5.84% and b2/b3 deletions in 0.24%⁽²²⁾. Similar prevalences of gr/gr deletions were previously reported: 4.8% in Gaza Strip-Palestine area⁽²⁶⁾, 4.2% in Spain⁽²⁷⁾, and 5% of infertile men in South Iran⁽²¹⁾.

Higher prevalence were previously reported: gr/gr deletions in 9.2% of infertile Egyptian men⁽¹⁵⁾, gr/gr deletions in 12.5% and b2/b3 deletion in 9.3% in China⁽²³⁾, gr/gr deletions in 7.2% and b2/b3 deletions in 1.4% of infertile Indian men⁽²⁸⁾, gr/gr deletions in 8.5% and b2/b3 deletions in 5.8% of infertile Korean men⁽²⁹⁾, gr/gr deletions in 10% and b2/b3 deletions in 5% of infertile men from Iran⁽³⁰⁾, gr/gr deletions in 6.25% and b2/b3 deletions in 7.21% in Dravidian-Indians⁽²⁴⁾, and gr/gr deletions in 12.4% and b2/b3 deletions in 4.96% of infertile Chinese men⁽³¹⁾.

Rozen and colleagues reported gr/gr deletions in 2.4%, b2/b3 deletion in 1.1%, and b1/b3 in 0.1% of the studied populations from five different locations (India, Poland, Tunisia, United States and Vietnam)⁽³²⁾. A previous study documented gr/gr deletion in 3.2% and b2/b3 deletion in 0.5% of infertile men in Italy⁽³³⁾. Another study documented gr/gr deletion in 3.9% and b2/b3 deletion in 1.3% of infertile men in Spain⁽³⁴⁾.

In the current study; no deletions were recorded in the normozoospermic control group. This is in accordance with previous studies^(12, 15, 27, 35). Other studies reported partial AZFc subdeletions in the control groups^(21, 24, 28-31, 36). The variation in the frequency may be related to ethnic variation, genetic background and Y

haplotypes. The discovery of specific haplogroups with an increased propensity for the occurrence of partial AZFc deletions may explain differences in the frequencies of partial AZFc deletions between populations⁽⁷⁾.

In the current study; there was significant difference in testicular volume between patients with AZF deletions and normozoospermic men. However; there was no significant difference in testicular volume between azoospermic patients with AZF deletions and azoospermic men without deletions. This is in accordance with previous report⁽¹⁶⁾. This finding implied that AZF microdeletions in infertile patients are not related to the testicular volume.

In the current study; in spite of significant difference between men with AZF deletions and normozoospermic men in the levels of FSH and LH; there was no significant difference between the levels of reproductive hormones (FSH, LH, testosterone and prolactin) in azoospermic men with and without AZF deletions. These findings are in accordance with previous studies^(16, 20, 24, 37, 38). These results implied that AZF microdeletions in infertile patients were not be related to the levels of FSH, LH and testosterone.

To the contrary; in a previous study; the concentrations of FSH and testosterone in patients with microdeletion were significantly lower than those without microdeletions, while concentration of LH was significantly higher in patients with microdeletions⁽³⁹⁾. This may be related to inclusion of patients with hypogonadotrophic hypogonadism and chromosomal abnormalities who were excluded from the current study.

This study provides further evidence that partial deletions of the AZFc region are a risk factor for decreased sperm quality. Several partial deletions of AZFc were found to be associated with impaired spermatogenesis, suggesting multiple genes related to this process are

located in this region. These findings reinforce the necessity of AZF microdeletion testing among infertile males prior to employment of assisted reproduction techniques.

There were some limitations to this study. Small number of normozoospermic men was examined due to difficulties in enrollment. The Y haplogroups in the study population and gene dosage in AZF regions were out of the scope of this study. No germ-line analyses were performed to determine if the deletions were inherited, somatic, or mosaic mutations. Further studies are warranted to overcome these limitations.

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الملخص العربي

تحليل الفقد المجهري للجسيم الصبغي "واي" عند الرجال غير المخصبين في

صعيد مصر

أ.د / كمال زكى شعير*، أ.د / عصام الدين عبد العزيز ندا**، د / رمضان صالح**، د / منال أسامة الهمشري***، ط /
أشرف أحمد عبد اللطيف**

* قسم أمراض الذكورة والتناسل- كلية الطب- جامعة القاهرة، ** قسم الأمراض الجلدية والتناسلية وطب الذكورة- كلية الطب- جامعة سوهاج، *** قسم المشخصات الجزيئية والعلاجات- معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية- جامعة مدينة السادات

الهدف من البحث:

- 1- تقييم الفقد الدقيق للجسيم الصبغي "واي" عند الرجال غير المخصبين في صعيد مصر.
- 2- تحليل علاقة الفقد الدقيق للجسيم الصبغي "واي" بالخصائص الإكلينيكية والمعملية.

المرضى وطرق البحث:

ضم هذا البحث مجموعة من المرضى الغير المخصبين بعيادة الذكورة بمستشفيات سوهاج الجامعية (العدد = 210) وكذلك عدد 30 من الرجال المخصبين وتحليل السائل المنوي طبيعى وذلك بعد موافقة لجنة أخلاقيات البحث العلمى بالكلية. تم أخذ موافقة مبنية على المعرفة من جميع المشاركين فى البحث. تم أخذ التاريخ المرضى للمرضى مع فحصهم إكلينكياً. تم عمل تحليل سائل منوى لجميع المشاركين فى البحث وبناءً على عدد الحيوانات المنوية تم تقسيم المرضى إلى ثلاثة مجموعات: عديمى الحيوانات المنوية (العدد = 146)، قليلى الحيوانات المنوية (> 15 مليون/مل) (العدد = 64)، و طبيعى عدد الحيوانات المنوية (< 15 مليون/مل) (العدد = 30). تم عمل تحليل لمستوى الهرمونات بالدم (الهرمون المحفز للحويصلات و الهرمون المنشط للتبويض و هرمون التستوستيرون و هرمون البرولاكتين). تم أخذ عينة من الدم و فحصها لتحديد الفقد المجهري للجسيم الصبغي "واي" عن طريق تفاعل البوليميراز المتسلسل. تم استبعاد المرضى الذين يعانون من إنسداد أو التهابات فى الأعضاء التناسلية، وجود شذوذ فى الكروموسومات، وجود دوالى الخصيتين وكذلك وجود تاريخ مرضى للعلاج الكيماوى أو الإشعاعى.

نتائج البحث: تتمثل نتائج البحث فى الملاحظات الآتية:

- 7,14% من الرجال غير المخصبين يعانون من فقد فى منطقة عامل النطاف (9,09% من عديمى الحيوانات المنوية و 1,06% من قليلى الحيوانات المنوية).
- الفقد الكامل لجزء من منطقة عامل النطاف كان موجود فقط فى المنطقة سى (فى 2,05% من عديمى الحيوانات المنوية).
- الفقد الجزئى فى منطقة عامل النطاف سى كان موجود فى 0,7% من الرجال غير المخصبين (0,24% فى مذقة جى ار/جى ار، و 0,48% فى منطقة بى 2/بى 3).
- لم يكن هناك فروق ذات دلالات احصائية بين المرضى عديمى الحيوانات المنوية سواء حاملى الفقد فى منطقة عامل النطاف أو غير حاملها فيما يتعلق بحجم الخصيتين أو مستوى الهرمونات.

الخلاصة:

- وجود فقد مجهري فى منطقة عامل النطاف قد يكون أحد العوامل المهمة لعدم الانجاب خاصة عند الرجال الذين يعانون من انعدام فى الحيوانات المنوية.
- لا يمكن توقع الفقد فى منطقة عامل النطاف عن طريق حجم الخصيتين أو مستوى هرمونات الخصوبة بالدم.