

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**Studies and clinical significances of genetic aberrations  
in background of infertility**

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Supervisor: Prof. Dr. Eva Olah



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## 1. INTRODUCTION

In the developed countries (Europa and the USA) there is a tendency of decline in population due to different social and economic factors: the pleasure of baby project decreases along with an increase in mortality rate. One of the most important causes leading to fewer births is – among others – the high frequency of infertility, while cardiovascular diseases and cancers play a central role in determining the high mortality rate. The Hungarian population started to decrease at the beginning of 80s years, after the millenary it became 40 000 fewer every year. Today the number of Hungarian population is fewer than 10 million. In the background of this decline some important factors can be hypothesized: changed social situation of women (women's employment, high number of single mothers and the divorce etc.), deteriorated social status of families. Further factors contributing to reduced number of births are the high number of infertile couples, the repeated failures of *in vitro* fertilization (IVF) and/or early abortions of implantation in artificial reproduction technics, decreasing significantly the couple's success to produce offspring.

In developed countries 10-15% of couples are affected by infertility, in half of them genetic factors can be identified. Nowadays genetic studies in infertility are more and more important, most of them have integrated into the standard diagnostic procedures. The aim of genetic studies are the following: with clarification of genetic aberrations we can obtain information about the genetic risks of offspring, anticipate the succes of assisted reproduction technics and get help in choosing the most effective way of assisted reproduction, in deciding whether to use or not to use donor sperm/oocyta. It would be very important to perform genetic tests in every case preceeding the IVF, not after several unsuccessful attempts. We should not forget that the risk of the birth of genetically affected offspring is higher after successful IVF than in those born in natural way. With knowledge of genetic causes we can use preimplantation and prenatal tests in order to prevent the birth of an affected child.

## **1.1. Definition and incidence of infertility**

Infertility includes the different forms of male and female infertility, as well as recurrent spontaneous abortions insofar as in these backgrounds there are undetectable gynecological and andrological factors. It is generally accepted that the diagnostic workup of infertile couple should be initiated after one year of regular unprotected intercourses. Recurrent spontaneous abortions means two or more abortions/necrosis of foetus. According to the results of researches the background of infertility consist of 40% gynecological, 40% andrological factors while the causes of infertility should be searched in either gender in 20% of cases.

In developed countries 10-15% of couples are affected by infertility, about one out of seven European couples suffer from reproductive health disorders. The frequency of recurrent spontaneous abortions vary between 0.5-3% of the couples of reproductive age.

## **1.2. Genetic causes of male infertility**

The examination of infertile males begins with andrological investigation (semen analysis). In the lack of anatomic and physiologic causes the possibility of genetic origin can be supposed. It is speculated that in about 50% of cases genetic abnormalities could be present, but we managed to find them only about 15% of cases. Several factors are unknown and rare, epigenetic factors can also occur besides genetic aberrations, including chromosome aberrations, Mendelian inherited (monogenic) diseases and multifactorial problems.

### **Chromosome aberrations**

The incidence of chromosome abnormalities is about 1:100, half of them numerical, half of them structural aberrations, but these two anomalies can occur together as well. Numerical and structural chromosome aberrations are more common in males with infertility, compared to the general population. It could be estimated that the overall incidence of a chromosomal factor in infertile males is 5%, this value increases to about 15% in azoospermic males.

Sex chromosome abnormalities display 80% of chromosome aberrations in infertility with a wide range of numerical chromosome aberrations. Structural abnormalities are fewer, most of them are macroscopic structural aberrations of chromosome Y. It can occur that the short arm of chromosome Y translocates to chromosome X or any autosomes. In cases of Y to X translocation male phenotype develops with 46,XX karyotype. This can explain by the presence of *SRY* (sex determination region) in 90% of cases. Klinefelter syndrome or the other numerical and structural aberrations of sex chromosomes constitute the majority of male infertility (3.7%).

Balanced reciprocal and Robertsonian translocations of autosomes can cause infertility. The incidence of reciprocal translocation in infertile population is 0.7-1.2% compared to the general population (0.09%). These translocations involve the exchange of chromosome material between the arms of two heterologous chromosomes. This balanced translocation does not cause phenotypic aberrations in general but it can cause recurrent spontaneous abortions because of the higher frequency of sperm with unbalanced chromosome translocation (20-80%). Robertsonian translocations result in the formation of a chromosome composed of the long arms of two acrocentric chromosomes and the subsequent loss of their short arms. The translocated dicentric chromosome consists of two long arms. These aberrations can impair the spermatogenesis by disturbing the meiosis. The incidence of Robertsonian translocations is 0.1% in the general population compared to the 0.9% incidence in the infertile males.

### **Y microdeletions of chromosome Y long arm**

Microdeletions of the Y chromosome long arm are the second most frequent genetic causes of spermatogenic failure in infertile men after Klinefelter syndrome. Azoospermic men have a higher incidence of chromosome Y microdeletions than oligozoospermic men, this frequency can reach 15%. The AZF (azoospermia factor) of chromosome Y is responsible

for spermatogenesis, which has been classically subdivided into three regions called AZFa, AZFb and AZFc, respectively. Frequencies of AZF microdeletions are the following: deletions of the AZFc region are the leading group (79% of all deletions), followed by AZFb (9%), AZFb+c (6%), AZFa (3%) and AZFa+b+c (46,XX male, 3%).

Deletions of the AZFa region result in complete Sertoli cell only (SCO) syndrome and azoospermia. The diagnosis of a complete deletion of the AZFa region implies the virtual impossibility to retrieve testicular sperm for intracytoplasmic sperm injection (ICSI). Complete deletions of AZFb and AZFb+c are characterized by SCO syndrome or spermatogenetic arrest resulting in azoospermia. Several reports demonstrated that no sperm is found upon attempts of testicular sperm extraction (TESE) in these patients. Like in the case of complete deletion of AZFa, AZFb and AZFb+c it is impossible to retrieve sperm and ICSI should not be recommended to these patients. Deletions of the AZFc region are associated with a variable clinical and histological phenotype. AZFc deletions can be found in men with azoospermia or oligozoospermia. In men with AZFc deletion there is a fairly good chance of retrieving sperm from TESE and children can be conceived by ICSI. The sons of these patients will be AZFc-deleted.

Taking into consideration the Y chromosome structure and the deletion mechanism, a number of other possible partial deletions have been proposed in both the AZFb and AZFc regions. The frequency and the pathological significance of these partial deletions is not yet clear. Gr/gr deletions correlate with spermatogenetic failure. Gr/gr deletion removes half of the AZFc genes, including two copies of the most important AZFc gene *DAZ* (deleted in azoospermia). Other most frequent deletions, named b2/b3 and b1/b3, seems to have no effect on fertility status.

## ***SRY* gene**

Besides the deletions of chromosome Y AZF region, the *SRY* gene is very important, which is located on the proximal end of the short arm of chromosome Y. The *SRY* gene encodes a transcription factor, which participates in the regulation of several genes. The *SRY* gene can translocate from chromosome Y to chromosome X, causing 46,XX male syndrome. The prevalence of this syndrome is 1:20000. These males are phenotypically normal, he has smaller testes, abnormal vas deferens, azoospermia, higher FSH level and decreased testosterone level.

## **Gene mutations**

*Kallmann-syndrome* is hypogonadotrophic hypogonadism with anosmia or hyposmia with an incidence of 1:60000. The underlying cause of the syndrome is *KAL1* gene mutations located on chromosome X, causing GnRH secretion deficiency of the hypothalamus. The mutant gene encodes pathological anosmin, causing diseased synapsis complexes of GnRH neurons. This syndrome causes late puberty, tall figure, hypoplastic testes and penis, cryptorchidism and insufficient olfactory sense.

There are several forms of *androgen insensitivity syndrome*, whose mild form (MAIS) is associated with infertility. In the background of the disease is decreased androgen, testosterone, dihydrotestosterone sensitivity of the target tissues, which causes pathological functioning of the androgen receptor (AR) protein. The *AR* gene is located on the long arm of chromosome X and has transactivator, DNA- and steroid-binding domains. This gene consists of two polymorphic trinucleotide microsatellites in the 1. exon of the transactivator domain. The first microsatellite contains of 8-60 repeat polyglutamin phase (CAG), the second polyglycine (GGC) repeat alters about 4-31 repetition. Several diseases can correlate with the length of polyglutamin repeat. The extreme shortness of the repeat is characteristic of prostate cancer, hepatocellular carcinoma and mental retardation, while the repeat above 40 is characteristic of

spinal and bulbular muscular atrophy (SBMA). The expansion of poliglutamin repeat relates to male infertility and insufficient masculinisation (MAIS).

*Cystic fibrosis* is one of the most common autosomal recessive disease, affecting one in 2500 live births. Insufficient functioning of the CFTR protein, which regulates the clorid chanel of plasma membrane, lies in the background of the disease. Profused secretions obstruct the outlets of the exocrin glandulae. Two vas deferens lack in males with *congenital bilateral absence of vas deferens* (CBAVD) causing azoospermia, while if one vas deferens is defective in males with *congenital unliateral absence of vas deferens* (CUAVD) causes severe oligozoospermia (sperm number  $<5 \times 10^6/\text{ml}$ ). The most frequent *CFTR* mutation in Hungary is the  $\Delta$ -F508 with the incidence about 60%. Further frequent mutations are the following: R117H, 218delA and 1148T, but compaund heterozygocity can also occur.

The mutations of the *GnRH receptor* cause hypogonadotrophic hypogonadism, while mutations of *gonadotropin (LH, FSH) genes* can be responsible for low estradiol level, hypogonadism, spermatogenetic failure. The mutations of *LH* gene effect more severe phenotipical consequence, than *FSH* mutations, because the LH regulates the testosteron production of the testis, while the FSH controls spermatogenesis.

### **Genetic aberrations of sperm**

Sperm cells carry on genetic abnormalities (chromosome aberrations, gene mutations) more frequently than somatic cells. On the one hand the studies of sperm is more dificult, on the other hand by studying sperm cells we can get information only about genetic aberrations of the present sample. The proportion of immature sperm and the frequency of chromosomal disomies are closely related to each other, suggesting that disomies originate primarily in immature sperm. The proportion of sperm with arrested development declines as the sperm concentration rises. Infertile men with normal karyotypes and low sperm concentrations or higher levels of morphologically abnormal sperm have significantly increased risks of

producing aneuploid spermatozoa, particularly for sex chromosomes. The fluorescence in situ hybridisation (FISH) is capable of determining numerous sperm cells numerical chromosome aberration at the same time. The simplest method for the determination of sperm sample estimated numerical chromosome aberrations is the so-called conservative method by which the frequency of one autosome and the two sex chromosomes numerical aberrations is determined by fluorescence signal of this chromosomes.

Simultaneously with cytoplasmic extrusion in spermiogenesis, there is a remodeling of the plasma membrane that facilitates the formation of the zona pellucida- and hyaluronic acid (HA)-binding sites. HA-binding associated with the presence of the HA receptors on the sperm surface is related to sperm development. Sperms with HA-binding ability are viable, having either intact or slightly capacitated acrosomal status HA-selected sperm are devoid of DNA degradation. Some authors suggested the relationship between diminished sperm development, low levels of HspA2 expression, increased frequency of chromosomal aneuploidies, presence of apoptotic process and fragmented DNA. The HA sperm selection methods are likely to reduce the frequency of chromosomal aberrations. This issue has a major impact on sperm selection for ICSI, because the sperm of infertile men has an increased proportion of immature sperm, showing a higher incidence of fragmented DNA and chromosomal aneuploidy.

Males with balanced reciproc translocation do not usually show any symptoms, but several sperm cells carry on unbalanced chromosome assortment because of non-disjunction of spermatogenesis. This aberration causes recurrent spontaneous abortions leading to infertility.

During meiosis I, quadrivalent is formed between the translocated chromosomes and their normal homologues in reciprocal translocation carriers in spermatocytes I at pachytene stage, leading to five different segregation modes. Only the alternate segregation mode

produces chromosomally normal and balanced gametes. The other four segregation modes (adjacent I, adjacent II, 3:1 and 4:0) produce chromosomally unbalanced sperm cells. These structural reorganisations through interchromosomal effect (ICE) cause pathological bivalents not affected in translocation, leading to non-disjunction and aneuploid spermatozoa.

Until the late 80s, the study of meiotic segregation was possible owing to the heterospecific fecundation of golden hamster oocytes with human spermatozoa. The introduction of FISH has allowed to study the chromosomal equipment of spermatozoa.

### **1.3. The most important genetic causes of female infertility**

Following the progress of molecular biology, many studies have been undertaken in recent years to clarify the genetic mechanism underlying female reproductive disorders. Apart from chromosomal abnormalities, alterations in female sexual maturation or reproductive function may be caused by gene defects at various levels of the hypothalamic-pituitary-ovarian axis or in gonadal and adrenal steroid biosynthesis or reception. The roles of genetic factors emerge in poor responders during assisted reproduction techniques. Females with normal karyotype produce chromosomally abnormal oocytes at a very high rate. Genetic aberrations of oocytes is increasing with advanced maternal age. Therefore genetic counselling is especially recommended when assisted reproduction techniques are performed in older women.

#### **Chromosome aberrations**

Turner syndrome is the most common chromosome abnormality in infertile women, but also a variety of structural autosomal aberrations may be found with relatively high frequency. The frequency of chromosomal abnormalities in female infertility can be estimated about 5%, 2.8% has numerical sex chromosome abnormalities and 2.1% structural autosomal abnormalities. The phenotype of women affected by sex chromosomal aberrations is highly variable, a feature shared by all these chromosome imbalances is primary ovarian dysfunction

with primary or secondary amenorrhoea or oligomenorrhoea. About 30% of primary amenorrhoeas are caused by Turner syndrome. Autosomal structural abnormalities may cause recurrent foetal loss.

### **Gene mutations**

Fragile X syndrome is the most common cause of mental retardation in males and it is caused by the expansion of the CGG trinucleotide repeat in exon 1 of the *FMRI* gene. The normal number of CGG repeat of the *FMRI* gene is defined as 6-45 repeats. Premutation is determined when the repeat number expanded between 54 and 200 and the allele is hypomethylated, while more than 200 repeats cause mental retardation. In fact, *FMRI* gene premutation causes sporadic occurrence of premature ovarian failure (POF) in 1.6% of cases, while familial POF in 16% of affected females. 15-25% of premutated women are affected by POF. Patients who develop POF frequently show a period of oligomenorrhoea with a progressive increase in gonadotropins. Premutation has been shown to be associated with low response to ovarian stimulation during *in vitro* assisted reproduction.

*Kallmann syndrome* due to mutation in the *KALI* gene is exceedingly rare in women, resulting in primary amenorrhoea with hypergonadotropinism and anosmia. A higher incidence of uterine malformations has also been reported.

Androgen receptor gene mutations with 46,XY karyotype can cause complete androgen insensitivity syndrome, syndromes with normal female genitalia in which infertility is a minor manifestation of symptoms. There are syndromes in which infertility represents the major phenotype: mutations in *FSH* and *LH* receptors, *FSH* gene, *GnRH* receptor.

## 2. AIMS

The aims of my research were to study the genetic aberrations background of infertility and to compare the results in the Hungarian population with the international literary data. On the bases of my research I have studied the following issues:

- Study and comparison of the balanced chromosome aberrations frequencies with the literary data of infertile males and females.
- Study of microdeletions and copy number variations (partial microdeletions) of chromosome Y AZF region in males with low sperm number. Comparison of the frequencies of the established deletions with data of control groups of normozoospermic and proven fertile (males with minimum one child) males and literary data.
- Study of sperm cells of infertile men with decreased sperm number and/or sperm motility with a typical biochemical marker: the determination of sperm hyaluronic binding capacity. Investigation of sperm chromosome aberration frequencies of same males with FISH method. Comparison of the established aberrations in males with different pathological and normal sperm parameters. Study of the correlation between sperm concentration, sperm motility, HA-binding capacity and the frequencies of estimated numerical chromosome aberrations.
- Determination of segregation patterns of balanced reciprocal translocation carrier infertile male with the study of sperm meiotic segregation and the comparison of the results with literary data. Furthermore, study of interchromosomal effect of chromosomes affected in translocation and comparison of the results with control groups.
- Investigation of CGG triplet expansion of *FMRI* gene in females affected by premature ovarian dysfunction. Establishing anomalies frequency and comparison of international literary data.

- Finally, my aim was to familiarize Hungarian specialists interested in the checkup of infertility with the results of the genetic studies, as well as drawing the attention of obstetricians/ gynecologists, andrologists and specialists in IVF to the necessity of genetic studies before IVF.

### **3. PATIENTS AND METHODS**

#### **3.1. Patients**

##### *Males and females with infertility*

We studied cytogenetic aberrations in 500 cases (305 male and 195 females) between 2007 January 1. and 2012 March 31. after excluding organic and endocrinological causes. We carried out chromosome Y microdeletion and partial microdeletion analysis in 150 idiopathic – without clinical alterations and with normal karyotype – infertile males. We classified the infertile males by their sperm samples parameters into azoospermic (no sperm in the ejaculum, 64 persons) and oligozoospermic (average sperm concentration:  $4.8 \times 10^6/\text{mL}$ ;  $0,1-14 \times 10^6/\text{mL}$ , 86 persons) groups according to WHO criteria.

We analysed the sperm chromosome aberrations frequency and hialuronacid-binding capacity of 28 infertile males with normal karyotype. According to the sperm concentration and motility patients were divided into three groups: oligozoospermic, asthenozoospermic and oligoasthenozoospermic groups. The oligozoospermic group consisted of ten men with infertility (age range: 31-42). The sperm concentration of these patients was up to 15 million/ml (range: 1-15) along with above 32% progressive motility (range: 35-80). The asthenozoospermic group consisted of nine men with subfertility (age range: 31-39). Their sperm concentration was above 15 million/mL (range: 18-58) with under 32% progressive motility (range: 5-30). In the oligoasthenozoospermic group (n=9; age range: 27-37) the sperm concentration and the progressive motility was under 15 million/mL (range: 4-12) and up to 32% (range: 0-32), respectively.

In the segregation study we carried out sperm FISH studies of a male with balanced t(3;6)(q21;q23) translocation. The patient's wife had four spontaneous abortions during the four previous years before checkup. The patient showed sperm concentration of  $81 \times 10^6/\text{mL}$  with 60% normal motility.

We carried out *FMRI* gene study in 17 females with premature ovarian dysfunction and normal karyotype. The necessity of the *FMRI* gene triplet expansion study was revealed after endocrinological aberrations (oligomenorrhoea and high gonadotropin level) and poor responders of ovarian stimulation in assisted reproduction.

Patients were referred to our laboratory from the infertility/endocrinological outpatient hours of the Obstetrical and Gynecological Departments of the University of Debrecen, Medical and Health Science Center (UD MHSC), the andrological outpatient department of the Department of Urology of the UD MHSC, the outpatient department of the Clinical Genetics Center of the Pediatrics Institute of the UD MHSC, as well as from the Kaáli Institute.

#### *Control groups*

There were 110 normozoospermic males (mean sperm concentration:  $46.2 \times 10^6/\text{mL}$ , range: 18-114) and 22 proven fertile males (with at least one health child) in the partial microdeletion study as the members of control groups.

There were 17 normozoospermic sperm donors (mean sperm concentration:  $85.8 \times 10^6/\text{mL}$ , range: 40-160) in the study of the determination of sperm chromosome aberrations and hialuronacid binding capacity as a control group.

There were five normozoospermic males (1. group: mean sperm concentration:  $95,3 \times 10^6/\text{mL}$ , range: 41-160; with above 60% normal motile sperm, range: 60-95) and five oligozoospermic males with normal karyotype (2. group: mean sperm concentration:  $6,4 \times 10^6/\text{mL}$ , range: 3-15; and above 60% normal motile sperm, range: 60-85) involved in the

segregation study for the examination of the interchromosomal effect as controls. The proportion of the sperm with normal morphology was above 30% in both control groups.

Sperm analyses of all control groups were carried out according to WHO criteria. The samples of the control groups were collected from the andrological outpatient hours of the Department of Urology of the UD MHSC, the consulting hours of the Clinical Genetics Center of the Pediatrics Institute of the UD MHSC, as well as in the Kaáli Institute.

### **3.2. Methods**

#### **Cytogenetics and FISH**

Constitutional karyotype of the patients were determined with conventional cytogenetic analysis. 3-5 mL Na-heparin anticoagulated blood was applied for the investigation. 5 mL culture solution (lymphochrome with phytohemagglutinin (PHA)) and 0.5 mL anticoagulated blood was pipetted in every flask. The flasks were put in the CO<sub>2</sub> (5%) thermostat for 72 hours, 37 °C. Blocking of the cell culture in methaphase happened with colchicine, the recovery of nucleus in interphase and chromosomes in methaphases were carried out with standard method (hypotonisation: 0.075 M KCL and fixation: mixture of the methanol:acetic acid in 3:1 rate, Carnoy fixative).

Giemsa staining (G-banding) is the most common process in rutin cytogenetic analysis. The slides were put in 0.005% trypsin solution for some seconds, then they were stained with Giemsa paint for 5 minutes. Leica, Leitz Diaplan, Nikon microscopes and Lucia Cytogenetics System software were used for analysis of methaphases. Determination of the patients karyotype was performed according to International System for Human Cytogenetic Nomenclature (ISCN) 2009.

Cell suspension gained from chromosome preparations were used for FISH investigations. FISH investigations on chromosome preparations from the blood were applied to reveale numerical mosaicism of sex chromosomes, determination of mosaicism degree,

certification of the presence of *SRY* gene, determination of its localisation and demonstrations of translocations.

Blocked mitosis, hypotonised, fixed cells were dropped on slides during FISH investigations. Slides were put in 37°C 2 X SSC/0.5% NP-40 (Nonidet P-40 Substitute, Sigma-Aldrich, St. Louis, MO, USA) solution after drying for 15 minutes, then extracellular proteins were removed with some minute pepsin digestion (Pepsin lyophilized powder, Sigma-Aldrich, St. Louis, MO, USA). After washing dehydration happened in ascended alcohol series. After drying probes were put on the advanced part of the slides. The FISH was performed using alpha-satellite sequence specific centromeric probes for chromosome X (DXZ1, SpectrumOrange), Y (DYZ1, SpectrumGreen), *SRY* specific probes (SpectrumOrange) (Abbott/Vysis, Des Plaines, IL, USA) and in the segregation studies alpha-satellite DNA probes were used for chromosome 3 (CEP3, Red; Cytocell, Cambridge, UK), for chromosome 6 (CEP6, Spectrum Aqua; Kreatech Diagnostics, Durham, UK) and a subtelomeric DNA probe for the distal portion of the long arm of chromosome 3 (3qter, Spectrum Green; Cytocell, Cambridge, UK). Slide and probe codenaturation was carried out at 76 °C for 3 min, hybridization was provided at 37 °C in a moist chamber for 16 - 18 h (Hybrite, Abbott/Vysis, Des Plaines, IL, USA). Posthybridization washes were performed with 50% formamide/ 2X SSC at 42 °C for 15 min. The slides were then washed with 2X SSC at room temperature for 10 min and 2X SSC/ 0,1 % NP-40 for 5 min. After washing, the nuclei were counterstained with 4'-6' diamidino-2 - phenylindole (DAPI, Abbott/Vysis, Des Plaines, IL, USA).

Scoring was performed using Zeiss Axioplan2 (Carl Zeiss, Jena, Germany) fluorescence microscope, the images were captured and analysed by ISIS software (Metasystems, Althusheim, Germany).

## **DNA isolation**

DNA isolations for chromosome Y molecular genetic and *FMR1* gene fragment analysis were performed from EDTA anticoagulated periferic blood. Genomic DNA was isolated using QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols.

## **Studies of chromosome Y AZF region**

Exactly determined sequences of AZF region - sequence tagged site (STS) – were amplified. These assay was performed by STS primers specific for the three AZF regions, two primer pairs were used in single PCR for every region: in AZFa region: sY84, sY86, in AZFb rregion: sY127, sY134, in AZFc region: sY254, sY255 (both in *DAZ* gene). Lack of amplification was implied for deletion. Five STS regions were studied for determination of partial deletions: gr/gr type of deletion was defined, when sY1291 was deleted, sY1161, sY1191, sY1201 and sY1206 were presented; b2/b3 deletion means deletion of sY1191 and amplification of sY1161, sY1201, sY1206 and sY1291, in case of b1/b3 deletion sY1161, sY1191 and sY1291 were deleted and sY1201 and sY1206 were present, respectively.

Control PCRs were used for determinations of chromosome Y microdeletions: DNA samples of males with normal spermatogenesis were applied for positive control, while DNA samples of females for negative control. Reagent contaminations were verified with water control. *ZFX/ZFY* genes were used for internal control because it was present in females and males as well. The presence of chromosome Y was proved by the study of *SRY* gene on the short arm of chromosome Y.

Parameters of STS PCRs were the following: 100ng DNA, 0.2 mM dNTP, 0.1  $\mu$ M each primer and 2 U DreamTaq DNS polimerase in 10X PCR buffer. PCR products were amplified in Veriti thermal cycler (Applied Biosystem, Foster City, CA, USA) PCR appliance. PCR products were separated on 2% agarose gel.

Significance levels among results of males with different sperm concentrations were represented with  $\chi^2$ -probe (SigmaStat software, San Jose, CA, USA),  $P=0.05$  levels were considered as significant difference.

### **Studies of sperm hyaluronic acid binding**

Hyaluronic acid binding capacity of 45 sperm samples for FISH studies were examined: in cases of ten oligozoospermic, nine asthenozoospermic, nine oligoasthenozoospermic and 17 normozoospermic control males. The sperm sample was maintained at room temperature (18-28°C) for 30 to 60 minutes to allow it to liquefy. HBA-test (hyaluronic acid binding assay) (MidAtlantic Diagnostics, Marlton, NJ, USA) was carried out at room temperature: the sample was mixed and pipetted 7-10  $\mu$ L of it near the center of the chamber. The CELL-VU gridded cover slip was located over the chamber. The chamber was incubated at room temperature for at least 10 minutes, this period proved to be necessary for sperm to bind to HA.

### **Sperm FISH**

Smears of semen samples (10  $\mu$ L) were fixed with methanol: acetic acid (3: 1) for 10 min, air dried, dehydrated in a series of 70, 85 and 100 % ethanol for 2 min each. For decondensation, the sperm slides were warmed to room temperature, and in order to render the sperm chromatin accessible to DNA probes they were treated first with 10 mmol/L dithiothreitol (DTT; Sigma, St. Louis, MO, USA) in 0.1 mol/L Tris-HCl, pH 8.0, for 30 min, then with 10 mmol/L lithium diiodosalicylate (LIS; Sigma, St. Louis, MO, USA) in Tris-HCl for 1-3 h. Numerical chromosome aberrations of three chromosomes were examined according to the literary conventional protocol for determination of the aneuploidy with the help of alpha-satellite sequence specific centromeric probes for chromosome 17 (D17Z1, SpectrumAqua), X (DXZ1, SpectrumOrange) and Y (DYZ1, SpectrumGreen) (Abbott/Vysis, Des Plaines, IL, USA). FISH probes specific for balanced translocation revealed in peripheric

blood were used in the segregation study, centromeric DNA probes for chromosome 3 (CEP3, Red; Cytocell, Cambridge, UK), for chromosome 6 (CEP6, Spectrum Aqua; Kreatech Diagnostics, Durham, UK) and a subtelomeric DNA probe for the distal part of the long arm of chromosome 3 (3qter, Spectrum Green; Cytocell, Cambridge, UK) were used in the case of males with t(3;6)(q21;q23) translocation. FISH studies were performed according to the manufacturer's protocols of the probes.

5000 cells per smears were examined for the determination of sperm chromosome aberrations and for the study of interchromosomal effect. The overall hybridization efficiency was >98%. Nuclei that were overlapped or displayed no signal due to hybridization failure were omitted from the scoring. Scoring was performed using Zeiss Axioplan2 (Carl Zeiss, Jena, Germany) fluorescence microscope, the images were captured and analysed by ISIS software (Metasystems, Althussheim, Germany).

An accepted method to estimate cumulative aneuploidy is the knowledge of the disomy frequency of sex chromosomes and at least one autosomal chromosome, because simultaneous examination of all the chromosomes on numerous sperm is extremely time- and work-consuming.

2000 sperm cells were examined in the segregation study, where the signal patterns revealed individual variability according to meiotic segregation.

We analysed the samples normality using Shapiro-Wilk test, the samples homogeneity using Barlett test. Differences in the sperm concentration, HA-binding ability, disomy frequencies, diploidy frequencies and estimated numerical chromosome aberrations in the different groups of patients were analyzed using Mann-Whitney/Wilcoxon Two-Sample Test, Kruskal-Wallis test (when normality does not exist) and Two-sample t-probe (when normality is exist) (SigmaStat and SPSS softver, San Jose, CA, USA). Correlation analysis between the sperm concentration, HA-binding capacity and sperm estimated numerical chromosome

aberrations using all samples in the four groups were examined with Pearson correlation test. The strength of the linear relationship between each pair of variables, having been corrected for other variables, were studied with partial correlation analysis. In the segregation study for the examination of interchromosomal effect significance levels of differences were represented with  $\chi^2$ -probe (Statistica software, StatSoft, Tulsa, OK, USA). A value of  $P < 0.05$  was considered as significant difference.

### **Studies of *FMR1* gene**

The region of the 5' end of the *FMR1* gene with CGG trinucleotide repeats were amplified with polymerase chain reaction (PCR) with the help of Amplidex<sup>TM</sup> *FMR1* PCR Kit (Asuragen, Austin, TX, USA). The parameters of PCRs, according to the kit protocol, were the following: 11.45  $\mu$ L GC-Rich Amp Puffer, 0.5  $\mu$ L *FMR1* F,R FAM-Primers, 0.5  $\mu$ L *FMR1* CGG Primer, 0.5  $\mu$ L Diluent, 0.05  $\mu$ L GC-Rich Polymerase Mix and 2  $\mu$ L DNA (in the reaction solution 20-40 ng). PCR products were amplified in Veriti thermal cycler (Applied Biosystem, Foster City, CA, USA) PCR appliance. Reactions to capillary electrophoresis were pipetted as follows: 11  $\mu$ L Hi-Di formamid, 2  $\mu$ L ROX 1000 Size Ladder and 2  $\mu$ L denaturated PCR product (95°C, 2 min). The separation of the samples took place on 3500xL-Avant Genetic Analyzer apparatus with POP-7 polymer (Applied Biosystems, Foster City, CA, USA). The measurement receiving fragment analysis was evaluated with the help of GeneMapper 4.0/4.1 software.

## **4. RESULTS**

### **4.1. Cytogenetics and molecular cytogenetics**

Cytogenetic aberrations were revealed in 20 patients out of 305 infertile males (6.6%), sex chromosome aberrations were proved in 12 cases (3.9%). Klinefelter-syndrome was identified in ten males (47,XXY, 3.3%), as well as mosaic ring chromosome Y was detected in one case (0.33%). *SRY* gene was revealed on the short arm of chromosome X in a male

with 46,XX karyotype according to FISH analysis. Balanced translocation performed by autosomes was revealed in six cases (2%); reciprocal translocations in three cases - 46,XY,t(3;6)(q21;q23), 46,XY,t(2;5)(p13;q21), 46,XY,t(7;20)(p15;p13) – as well as Robertsonian-translocations in three patients (45,XY,rob(13;14)mat). Pericentric inversion of chromosome 9 was revealed in two patients (0.66%).

Cytogenetic aberrations were identified in 15 patients out of 195 infertile females (7.7%). Mosaic form of sex chromosomes numerical aberrations proved one of the most frequent chromosome aberrations: 45,X/46,XX/47,XXX karyotype was detected in seven cases out of 195 patients (3.6%). The degree of mosaicism was refined with the help of FISH: two chromosome X were found in 93% of cells, one chromosome X in 4.5% of cells and three chromosome X in 2.5% of cells. Robertsonian-translocation was detected in one patient (0.5%) (45,XX,rob(14;21)mat) and pericentric inversion of chromosome 9 in seven cases (3.6%).

#### **4.2. Studies of chromosome Y AZF region**

Chromosome Y microdeletions were found in eight cases out of the 64 *azoospermic males*, AZFb+c in three (4.69%) and AZFc deletion in five (7.81%) patients. Two types of partial microdeletions were detected in the AZF region: b2/b3 deletion in two cases (3.13%) as well as b1/b3 deletion in one patient (1.56%). Among the 86 *oligozoospermic males* one full AZFc microdeletion was detected (1.16%) and five partial microdeletions inside the AZFc region were revealed: three gr/gr (3.49%) and two b2/b3 deletion (2.33%). Seven partial microdeletions were detected in the *normozoospermic group* (n=110): four gr/gr (3.64%) and three b2/b3 deletions (2.73%). Neither chromosome Y microdeletions, nor partial microdeletions did occur among the proven fertile cases.

AZFb+c deletion and b1/b3 partial microdeletion were detected only in the azoospermic cases; AZFc deletion occurred significantly more frequently among azoospermic patients, than

among oligozoospermic males ( $P<0.05$ ), while AZFc deletion was not detected in normozoospermic males. Gr/gr deletion did not occur in azoospermia and no significant differences in the frequencies of these deletions between oligozoospermic and normozoospermic groups were observed. B2/b3 partial deletion was detected in each of the three groups, but significant differences were not established among them.

#### **4.3. Studies of sperm hilauronic acid binding capacity**

The average sperm HA-binding capacity of the motile sperm was 81% (range: 58-95) in the normozoospermic group, 53% (range: 10-68) in the oligozoospermic group, 37% (range: 0-83) in the asthenozoospermic group and 30% (range: 2-55) in the oligoasthenozoospermic group. The HA-binding capacity of the normozoospermic men proved to be significantly higher than the oligozoospermic ( $P<0.001$ ), the asthenozoospermic ( $P<0.001$ ) and the oligoasthenozoospermic ( $P<0.001$ ) men.

#### **4.4. Studies of sperm chromosome aberrations**

Sex chromosome disomy frequencies were significantly higher in the oligoasthenozoospermic group compared to the three other groups ( $P=0.001$  in the normozoospermic,  $P=0.014$  in the oligozoospermic and  $P=0.004$  in the asthenozoospermic group). We have also found a significantly higher frequency of disomy 17 in the oligoasthenozoospermic group compared to normozoospermic ( $P=0.0001$ ), oligozoospermic ( $P=0.0019$ ) and asthenozoospermic ( $P=0.0011$ ) patients. The total diploidy frequency of oligoasthenozoospermic patients was significantly higher compared to the three other groups (normozoospermic controls:  $P<0.0001$ ; oligozoospermic men:  $P=0.03$ ; asthenozoospermic patients:  $P=0.001$ ). A significant difference was observed in the frequency of estimated numerical chromosome aberrations between oligoasthenozoospermic and normozoospermic ( $P<0.001$ ), oligoasthenozoospermic and oligozoospermic ( $P=0.004$ ) as well as oligoasthenozoospermic and asthenozoospermic ( $P=0.001$ ) groups. There were significantly

higher disomy 17 ( $P=0.0019$ ), diploidy ( $P=0.001$ ) and estimated numerical chromosome aberrations ( $P=0.002$ ) found in the asthenozoospermic group compared to the normozoospermic one. We have detected a significantly higher disomy 17 frequency in oligozoospermic men compared to the normozoospermic patients ( $P=0.0387$ ).

#### **4.5. Correlation between sperm concentration, HA-binding capacity and estimated numerical chromosome aberrations**

We studied Pearson correlation ( $r$ ) between the sperm concentration, HA-binding capacity and estimated numerical chromosome aberrations comparing 28 infertile and 17 normozoospermic sperm samples. A statistically significant positive correlation was found between the sperm concentration and the HA-binding capacity ( $r=0.658$ ), and statistically significant negative correlations were observed between the sperm concentration and the estimated numerical chromosomes aberrations ( $r= - 0.668$ ) and the HA-binding ability and the estimated numerical chromosome aberrations ( $r= - 0.682$ ).

#### **4.6. Segregation analysis**

The cytogenetic analysis of a male revealed a balanced translocation between the long arms of chromosome 3 and chromosome 6. The karyotype of the patient was  $46,XY,t(3;6)(q21;q23)$ , the translocation was confirmed by FISH.

From the sperm FISH signal pattern we concluded to the method of chromosome segregation in cells. 46.8% of spermatozoa showed normal/balanced (alternate) segregation mode. 53.2% of sperm showed abnormal signal pattern caused by the following unbalanced segregation: adjacent 1 segregation was observed in 26.07% of spermatozoa, adjacent 2 in 13.63% and 3:1 in 8.49% of sperm. Diploidy and 4:0 segregation mode occurred in 0.45% of sperm – their individual frequencies could not be distinguished due to the similar signal patterns of the FISH probes. Other signal patterns were observed in 4.54% of sperm heads.

The existence of interchromosomal effect was tested by comparing the patient's estimated numerical chromosome aberration frequencies with the results in the two control groups: a significant difference in the prevalence of the estimated numerical chromosome anomalies was seen between the translocation carrier and the two groups ( $P < 0.0001$ ). We found a significantly higher frequency of sex chromosome disomy in the translocation carrier compared to the normozoospermic ( $P < 0.05$ ) and the oligozoospermic groups ( $P = 0.05$ ). Though higher frequency of disomy 17 was observed in the translocation carrier compared to the normozoospermic controls and the oligozoospermic men, these differences were not significant ( $P = 0.11$  and  $P = 0.30$ ). The total diploidy frequency of the translocation carrier was significantly higher compared to that of the two control groups ( $P < 0.0001$ , normozoospermic controls;  $P = 0.006$ , oligozoospermic men).

#### **4.7. Studies of *FMRI* gene**

*FMRI* gene premutation was detected in two cases out of 17 females with premature ovarian dysfunction (11.8%). Premutant allele was present in mosaic form in one case (repeat number=59), as well as triplet expansion was present in all somatic cells of the other patient (repeat number=54). The average CGG repeat number of the 15 normal females without triplet expansion was 23 on the both alleles.

### **5. DISCUSSION**

#### **5.1. Chromosome aberrations in male infertility**

In the background of male infertility chromosomal abnormalities can be detected in about 5% of cases, which can increase to 15% in azoospermic men. In our study, Klinefelter-syndrome (3.3%) and the other numerical or structural aberrations of sex chromosomes (0.66%) proved to be the most frequent abnormalities in the background of male infertility, as well as cytogenetic aberrations of autosomes with frequency of 2%. The frequencies of

pericentric inversion of chromosome 9 were more common in infertile males (0.66%) than known in the general population (1.65‰).

Klinefelter-syndrome is the most frequent genetic cause of male infertility. Its frequency is 0.1-0.16% in the population and 3-11% in infertile males. 80-90% of Klinefelter-syndrome include the classic 47,XXY form and a higher degree of aneuploidy and mosaicism can be observed in 10–20% of the cases. In our study we detected ten males with 47, XXY karyotypes. Non-disjunction during the paternal gametes development in meiosis or preembryonic cell division in mitosis is in the background of the syndrome. According to latest studies Klinefelter-syndrome proved to be related to roles of androgenes and CAG repeat polymorphism of androgen receptor gene (*AR*) located on chromosome X. It was detected that the shorter allele of the two *AR* alleles should get inactivated in Klinefelter-syndrome, while the length of the active allele is directly proportional to the height and the presence of gynecomastia.

47,XYY karyotype affects one boy in 1000 live births (0.1%). This ratio is smaller in infertile males (0.084%). The majority of affected males are phenotypically normal, with variable clinical manifestation, the ability of sperm production varies between severe oligozoospermia and normozoospermia, they can often father healthy children. The risks of chromosome aberrations and spontaneous abortions increase in the offspring of males with 47,XYY karyotype, because the sex chromosome disomy frequency in the sperm of these males (0.2 - 1%) is significantly higher compared to that of the general population (0.1%). In our study, 47,XYY karyotype was not detected among infertile males.

Among structural chromosome Y aberrations in infertility the ring chromosome Y is the most frequent aberration and it should cause phenotypic consequences (incidence under 0.5%). This chromosome aberration causes azoospermia or severe oligozoospermia by disturbance of genes responsible for spermatogenesis. The ring chromosome Y can often

disappear under spermatogenesis causing Turner-syndrome in the offspring. The incidence of boys born with constitutional, non-mosaic ring chromosome Y is very low. Mosaic cases are more frequent, where the aberrant chromosome Y can get lost in some cell lines, or it can appear with cells with normal chromosome Y in mosaic form. We detected a mosaic form of this aberration, the karyotype of the patient was 46,XY/46,X,r(Y). Chromosome Y with the presence of aberrant AZF region in some cell lines can cause the severe oligozoospermia of the patient.

In the background of the phenotype of males with 46,XX karyotype there are several genetic factors, causing varied clinical phenotype. This abnormality is very rare, one male is affected out of 20 000. The translocation of the *SRY* gene of chromosome Y to the autosomes or chromosome X constitutes largest part of the background of the cases (80%). Male phenotype was determined by the translocated gene, but the patient is azoospermic because of the lack of chromosome Y region responsible for spermatogenesis (AZF region), the testes are hypoplastic, in addition, gynecomastia and cryptorchismus can sometimes be observed. In our laboratory we diagnosed one case with 46,XX male syndrome, in this case the *SRY* gene translocated to the chromosome X. It is necessary to apply donor sperm for IVF in these cases because of the lack of spermatogenesis.

Balanced reciprocal and Robertsonian-translocations of autosomes can play a role in the emergence of infertility. The frequency of autosome translocations was detected 5.63% among infertile patients compared to the frequency of the control group (1.28%). The frequency of Robertsonian-translocation carriers proved higher among oligozoospermic/azoospermic males than in the average population. The reciprocal translocation is rarer than the Robertsonian-translocation, its frequency is about 1.5% among infertile males. Reciprocal translocation usually affects azoospermic males (0.9%), while oligozoospermic men are affected rarely (0.6%). Infertility due to autosomal abnormalities is

caused by mutations, deletions or inactivation of other genes playing role in sex determination. On the other hand the crossing over of abnormal homologue chromosome pairs during the meiosis can cause aneuploidy of other chromosomes by interchromosomal effect. In our laboratory we detected three reciprocal and three Robertsonian-translocations. We found a very rare aberration among the reciprocal translocations: 46,XY,t(3;6)(q21;q23), which has not been described yet in infertility. The other two balanced reciprocal translocations - 46,XY,t(2;5)(p13;q21), 46,XY,t(7;20)(p15;p13) – are already known, but these found in the literature, show different chromosome breakpoints. Robertsonian-translocations between chromosome 13 and 14 were mentioned as the most frequent cause of infertility among the Robertsonian-translocations. We also detected three Robertsonian-translocations with 45,XY,rob(13;14)mat karyotype. All three infertile men inherited the chromosome aberration from the mother.

The incidence of chromosomal inversion is 0.8% in the general population. The pericentric inversion of centromeric heterochromatin on chromosome 9 can be classified as chromosome polymorphism, but its role should be considered in infertility. One study found that 20% of people with pericentric inversion have problem of infertility, while in the parents with normal karyotype this problem was significantly rarer (6%). Other authors detected more frequent heterochromatin inversion of chromosomes among azoospermic males than in the general population. The effect of inversion to spermatogenesis is manifested as modification of sperm morphology, motility and meiotic segregation. We found two chromosome 9 pericentric inversions among 305 infertile males, which is equal (0.65%) as that in the general population (0.66%).

## **5.2. Chromosome aberrations in female infertility**

Chromosome aberration frequency in female infertility is about 5%, which affects sex chromosomes in 2.8%, while autosomes in 2.1%. In our study the mosaic form of the

numerical aberration of chromosome X was one of the most frequent chromosome aberrations among the studied females (n=7, 3.6%), one Robertsonian-translocation (0.5%) and seven pericentric inversion of chromosome 9 (3.6%) were detected.

Turner-syndrome is the most common chromosome aberration in female infertility. 30 % of primer amenorrhoeas were caused by Turner-syndrome. The incidence of this syndrome among newborn girls is about 1:2000 and 1:2500. Prenatal prevalence of this syndrome is much higher than postnatal one because of intrauterin selection. Low height, gonad dysgenesis, ovarian dysfunction and infertility due to the insufficient production of female sex hormones are the specific symptoms of Turner-syndrome. The most common chromosome aberrations in the background of Turner-syndrome are the following: X-monosomy – 45,X; mosaicism (50-75%) – including 45,X/46,XX (10-15%), 45,X/46,XY (2-6%), 45X/46,X,i(Xq), 45,X/46,X,del(Xp), 45,X/46,XX/47,XXX karyotypes; structural chromosome X aberrations – complete or partial short arm deletion of chromosome X: 46,X,del(Xp), long arm isochromosome of chromosome X: 46,X,i(Xq), ring chromosome X: 46,X,r(X), marker chromosome: 46,X+m. In our study we detected seven mosaic Turner-syndromes, on average two chromosome X were confirmed in 93% of cells, one chromosome X in 4.5% of cells and three chromosome X in 2.5% of cells. Extra chromosome X was present in a small percentage of cells, which is common in such cases. The incidence of the triple X syndrome resulting in 47 chromosomes among women are about 0.1%.. Number of women affected triple X syndrome is higher than average, characteristic physical differences cannot be discovered. The female genital organs may be regular or poorly developed. Typical of this syndrome is cycle disorders or premature menopause. 75% of patients have childbearing potential.

Balanced reciprocal and Robertsonian-translocations of autosomes play a role in female infertility as well. The frequency of autosome translocation in female infertility was estimated

under 0.5%. Abnormalities of autosomes can lead to infertility, when the translocation-affected region plays a role in sexual development, as well as aneuploidy of the other chromosomes causing abnormal homologous chromosome pairing during crossing over by interchromosomal effect. We detected one Robertsonian-translocation between chromosome 14 and 21. Robertsonian-translocations of chromosome 13, 14 and 21 were mentioned as the most frequent cause of female infertility among the Robertsonian-translocations similarly to male infertility. Comparing the 45,XX,der(13;14) and 45,XX,der(14;21) translocation carrier females it was found that oocytes with normal karyotype in both types were significantly more frequent than cells with balanced chromosome equipment, as well as oocytes with unbalanced chromosome equipment proved to be increased in the cases with 45,XX,der(14;21) karyotype.

The prevalence of chromosome inversion is 0.8‰, while the frequency of the pericentric inversion of chromosome 9 heterochromatin is twice as high. Diploid oocyte can be produced by inversion during meiotic chromosome segregation. No difference in phenotype is caused, it was associated with decreased fertilization ability (subfertility) rather than with infertility. We found seven pericentric inversions of chromosome 9 (3.6%) during genetic investigations of female infertility, which is higher than inversion frequency in the general population.

### **5.3. Deletions of chromosome Y AZF region**

6.2-7.7 Mb size AZFb and AZFc deletions were identified only in azoospermic and severe oligozoospermic men, but not in normozoospermic and proven fertile males. AZFc deletion proved to be significantly more frequent in azoospermic males compared to other groups. B1/b3 partial deletion was detected only in one azoospermic male. We detected gr/gr deletion in oligozoospermic and normozoospermic men, there was no difference in its frequency, but it could not be found in azoospermic group. There was no difference in the

frequency of b2/b3 deletion among the three groups. Chromosome Y microdeletions were not detected in proven fertile males.

The world literature indicates that, as a rule, clinically relevant Y microdeletions are found in patients with azoospermia or sperm concentration  $<1 \times 10^6$ /ml (severe oligozoospermia). Very rarely, deletions can be found in infertile patients with sperm concentration between 1 and  $5 \times 10^6$ /ml. Gr/gr and b2/b3 partial deletions can occur in oligozoospermic and normozoospermic cases suggesting that these deletions are not related to spermatogenic failure, as well as gr/gr deletion is considered as a risk factor.

Gr/gr deletion removes half of the AZFc gene content (*DAZ*, *CDY1* and *BPY2* genes). Men with gr/gr deletion have seven times higher chance for oligozoospermia to emerge than males without deletions. Its frequency is about 4% among oligozoospermic males. In our study gr/gr microdeletions were found in three cases out of the 86 oligozoospermic males, as well as in four patients out of the control groups (110 normozoospermic and 22 proven fertile males) (3.03%). Significant difference between the two groups was not proven, but these deletions can refer to the emergence of spermatogenetic failure.

There is a chance for successful assisted reproduction in case with AZFc deletion, but genetic aberration with all phenotype consequences is transmitted to the male offspring. The chromosome Y with AZFc deletion can disappear during meiosis, therefore the majority of sperm cells of males with AZFc deletion are nullisomic with respect to the sex chromosomes. Concerns have been raised about the potential risk for the offspring to develop sex chromosomes aneuploidy, mosaicism and Turner-syndrome. In patients with azoospermia or severe oligozoospermia being candidates for ICSI or TESE/ICSI deletion screening should be indicated. TESE should not be recommended in cases of complete deletion of the AZFa region, or complete deletion of AZFb or deletion of the AZFb+c regions. Genetic counselling is mandatory for males with chromosome Y microdeletions. Preimplantation genetic

diagnosis is recommended for infertile males with chromosome Y microdeletions after ICSI for the prevention of the offspring to develop Turner-syndrome. In addition, genetic investigation is recommended for children born from fathers with chromosome Y microdeletion after ICSI for the detection of Turner syndrome girls and of genetic causes in the background of male infertility.

#### **5.4. Sperm hyaluronic binding capacity**

The HspA2 chaperone protein plays a central role in the last step of sperm cells maturation. The second wave of HspA2 expression occurs simultaneously with cytoplasmic extrusion, as well as with plasma membrane remodelling, related to maturity. During remodelling zona-binding sites and hyaluronic acid receptors appear on the surface of plasma membrane. Sperm with diminished maturity and cytoplasmic retention shows high creatine phosphokinase (CK) and low HspA2 level, so these cells are unable to bind to zona pellucida. Mature sperm is able to bind to hyaluronic acid according to zona pellucida selectively. Freshly ejaculated and cryopreserved mature sperm is capable of binding to hyaluronic acid in artificial circumstances, we can separate the mature and immature sperm accordingly.

We studied the hyaluronic acid binding capacity of the sperm of males with different sperm concentration and sperm movement with the help of HBA-assay. We found a significantly higher hyaluronic acid binding capacity between the normozoospermic, oligozoospermic, asthenozoospermic and oligoasthenozoospermic males. Males with normal sperm movement and sperm count produce mature sperm cells in contrast with oligozoospermic males with a high number of immature sperm. Our finding is supported by other studies, in which the normal, mature sperm distribution of the HBA-test is above 80 %.

#### **5.5. Sperm chromosome aberrations**

According to studies summarising sperm chromosome analysis, the aneuploidy frequency in the sperm of normozoospermic males is about 6.5%, some authors estimate this

ratio above 15%. The proportion of immature sperm and of sperm with numerical chromosome aberrations is very high in the majority of infertile males with low sperm concentration and impaired motility.

According to several authors, there is a close correlation between the proportion of immature sperm and chromosomal aneuploidy in sperm and an inverse correlation between sperm concentration and maturity. We verified this relation in our study, when sperm samples were analysed to evaluate aneuploidy frequencies of chromosomes X, Y and 17. In the present study we examined sperm samples of 45 different oligozoo-, asthenozoo-, oligoasthenozoo- and normozoospermic men to determine the frequency of chromosome aberration. We have found significantly higher disomy frequencies of sex chromosomes and chromosome 17 as well as total diploidy frequencies in oligoasthenozoospermic males compared to the other three groups. A significant difference was observed in the frequency of estimated numerical chromosome aberrations between oligoasthenozoospermic and normozoospermic, oligoasthenozoospermic and oligozoospermic and oligoasthenozoospermic and asthenozoospermic groups.

Chromosome aberrations, mostly sex chromosome aneuploidy is more frequent in children born after ICSI. Chromosome aberrations compatible with life (Klinefelter-syndrome) are associated with infertility in most cases, procreation of children can be successful with the help of ICSI. In this case the risk of transmission of chromosome aberrations is very high. Chromosome aberrations transmitted by father side are significantly responsible for the high rate of unsuccessful assisted reproduction processes and miscarriages.

Somatic chromosome aberrations cannot be observed in most of the patients with high frequency of aneuploid gametes. In a small ratio of cases fertilized with numerical chromosome aberrations carrier sperm appeared this aberration in the embryo, most of whom were aborted. According to FISH studies sperm aneuploidy frequency concerning any

chromosome is much higher than epidemiological frequency of trisomy in the foetus. 47,XXY and 47,XYY trisomies are the exception because this foetus is viable. The frequency of abnormal karyotype is four-fold higher among children conceived by ICSI (0.8%). Triploidy is a common cause of early pregnancy losses, it can occur in 12-13% of spontaneous abortions.

### **5.6. Correlation between sperm concentration, HA-binding capacity and estimated numerical chromosome aberrations**

Although the ratio of immature sperm is different among infertile males, cases with normozoospermia have a higher rate of mature sperm in the ejaculate. Males with decreased sperm concentration have a higher rate of aneuploidy in the sperm.

The relationship between sperm immaturity and aneuploidy can be explained with the function of the HspA2 protein. HspA2 is a chaperon, its expression happen in two waves during spermatogenesis and spermiogenesis. It appears first in the primary and secondary spermatocyte as a component of the synaptonemal complex between the homologous chromosomes during meiosis. The second wave of the expression occurs at the end of spermatogenesis parallel with the excrusion and the remodelling of plasma membrane, the emergence of the binding sites of zona pellucida and hialuronic receptors. Low expression of HspA2 protein leads to meiotic error, aneuploidy and chromosome aberrations because this protein is a component of the synaptonemal complex. HspA2 level of immature sperm with cytoplasmic retention is low.

Accordingly, there was a significant correlation ( $r=0.7$ ) between the proportion of immature sperm with cytoplasmic retention and frequency of aneuploidies, indicating that aneuploidies are primarily found in immature spermatozoa with cytoplasmic retention and diminished HspA2 levels. A correlation between zona pellucida binding sites and sperm immaturity was proved earlier: only the mature sperm is capable of binding to zona pellucida.

Mature sperm has hialuronic-binding receptors and bind to hialuronic acid like zona pellucida binding site selectively. Hialuronic acid receptors like zona pellucida binding sites can not be found on the surface of immature sperm with low HspA2 level. It was confirmed earlier that the frequency of chromosome aneuploidy in hialuronic acid binding sperm is low.

We have found significantly higher disomy and diploidy of sex chromosomes and chromosome 17 disomy and estimated numerical chromosome aberration frequencies in infertile patients with abnormal semen compared to normozoospermic patients. Our finding is supported by other studies, in which it has been well documented that the sperm of severely oligozoospermic men have higher rates of aneuploidies than the sperm of normozoospermic men. In our study the HA-binding capacity of normozoospermic men proved to be significantly higher than infertile males also. A significant correlation was found among the sperm concentration, the hialuronic acid binding and the chromosome aberrations.

During ICSI, when the selection barrier of the zona pellucida does not function, insemination could happen with immature and aneuploid sperm, too. The fertilization potential should improve significantly with hialuronic acid selected and mature sperm like in natural fertilization, so the occurrence of genetic complications can decrease with the help of this method.

### **5.7. Sperm segregation analysis of male carriers of balanced reciprocal translocation**

Balanced reciprocal translocations are the most common structural chromosomal abnormalities, with an incidence of one per 1175 newborns, the incidence of balanced Robertsonian translocation is one in 1085 births. Translocation carriers can produce a significant percentage of gametes with an unbalanced combination of the parental rearrangement, causing abortions and infertility.

During the spermatogenesis, in meiosis I in spermatocytes of reciprocal translocation carriers quadrivalent (trivalent during Robertsonian translocation) is formed between the

translocated chromosomes and their normal homologues. In alternate segregation, the translocated chromosomes segregate in a spermatocyte II and their normal homologues in another spermatocyte II, producing chromosomally balanced and normal spermatozoa, respectively. This segregation mode produces translocation carrier (like the father) or normal foetuses. The other segregation modes produce chromosomally unbalanced gametes. Adjacent segregation modes induce nullisomies or disomies in the spermatozoa and thus monosomies or trisomies in the embryo. When the segregation follows a 3:1 mode, a gamete with 22 chromosomes and another with 24 chromosomes are produced. During 4:0 segregation mode a gamete with 21 and 25 chromosomes are produced (the 3:0 segregation mode of Robertsonian translocation produce gametes with 21 and 24 chromosomes).

The frequencies of unbalanced spermatozoa of a man carrying a balanced reciprocal translocation vary from 19% to 80%. These variations confirm that the risk of meiotic imbalance varies according to the feature of the chromosomes involved in the rearrangements, according to the size of the translocated regions, centromere and breakpoint positions. The rate of unbalanced gametes during Robertsonian translocation varied from 2.7-26.5%, because the trivalent was always in cis configuration, suggesting that this cis configuration promoted an alternate segregation, which should produce an equal number of normal and balanced spermatocytes.

In our study the karyotype of the patient was 46,XY,t(3;6)(q21;q23), 53.2% of sperm showed abnormal signal pattern. This high ratio should determinate the chosen method of assisted reproduction and the necessity of using donor sperm. Our data are in accordance with the results of other studies where the translocation affected also the chromosomes 3 or 6, but at different breakpoints.

The existence of ICE with determination of chromosomes X, Y and 17 aneuploidy was proven: the disomy and diploidy of chromosome 17 was more frequent in translocation carrier

than in control groups. During ICE, chromosomes affected in translocation can influence the meiotic segregation of other chromosomes which are not involved in the reciprocal translocation. Aneuploid embryos can be produced during ICE of balanced reciprocal translocation carriers.

The meiotic segregation analysis of sperm together with aneuploidy study of X, Y and 17 chromosomes using FISH allows the determination of reproductive prognosis in male balanced translocation carriers and provide appropriate genetic counselling. During IVF pre-implantation genetic diagnosis has to be recommended to those couples in whom the male partner (as well as the female) carries a balanced translocation.

### **5.8. The *FMRI* gene triplet expansion in premature ovarian dysfunction**

Amenorrhoea before 40 years with high FSH and LH levels is characteristic of premature ovarian dysfunction (POF). Several genes can cause POF, especially those that participate in the regulation of hypothalamus-hypophysis-ovarium axis. The deletion and the translocation of the long arm of chromosome X (Xq) is related to the presence of POF because of deletions and loss of functions of genes influencing ovarium function. The 33% of POF cases show family accumulations, the other cases are sporadic.

The most frequent examined gene in POF is *FMRI* gene localised in the Xq27.3 region. The premutation of this gene is responsible for the emergence of symptoms: *FMRI* gene premutation should occur in 1.6% of sporadic cases, as well as in 16% of family accumulations. According to other studies, the aggregate incidence is 6%. 15-25% of premutated women are affected by POF.

In our study we proved *FMRI* gene premutation in two cases among 17 POF cases with sporadic amenorrhoea (11.8%). In one case premutant allele was present in mosaic form, while in the other it could be seen in all the somatic cells. The role of *FMRI* premutation in the emergence of amenorrhoea was verified in these two cases.

## 6. OBSERVATIONS

1. *In male infertility* the most frequent cytogenetic aberrations proved to be Klinefelter syndrome or other numerical and structural aberrations of sex chromosomes (3.9%), while the frequency of autosomal cytogenetic aberrations was 2%. The frequencies of pericentric inversion of chromosome 9 were more common in infertile males than in the general population.

2. The mosaic form of the numerical aberration of chromosome X was one of the most frequent chromosome aberration *among infertile females*; we also detected one Robertsonian translocation and seven pericentric inversion of chromosome 9.

3. AZFb and AZFc microdeletions were identified *only in azoospermic and severe oligozoospermic men*, but it was not identified in normozoospermic ones and proven fertile males. AZFc deletion proved to be significantly more frequent in azoospermic males compared to other groups.

4. We detected *gr/gr and b2/b3 partial microdeletion* in oligozoospermic and normozoospermic men, suggesting that *these deletions are not correlated with spermatogenic failure*, while *gr/gr deletion has to be considered a risk factor*.

5. *The HA-binding capacity of normozoospermic men proved to be significantly higher than oligozoospermic, asthenozoospermic and oligoasthenozoospermic men*, because males with sperm of normal motility and number have mostly mature sperm, unlike oligozoospermic males with mostly immature sperm.

6. We have found significantly higher *sperm disomy frequencies of sex chromosomes and chromosome 17* as well as *total diploidy frequencies* in males with spermatogenic failure compared to the normozoospermic controls.

7. *We detected correlation between sperm concentration, HA-binding capacity and sperm numerical chromosome aberrations.*

8. In our study the patient with 46,XY,t(3;6)(q21;q23) karyotype, showed in 53.2% of his sperm unbalanced chromosome assortment. This high ratio should determinate the chosen method of assisted reproduction and may indicate the necessity of using donor sperm.

9. The existence of *interchromosomal effect* (ICE) was proven with determination of chromosomes X, Y and 17 aneuploidy: the disomy and diploidy of chromosome 17 was more frequent in t(3;6) translocation carrier than in control groups.

10. We proved *FMRI gene premutation in two cases* among 17 premature ovarian dysfunction females with sporadic amenorrhoea. The role of *FMRI* premutation in the emergence of amenorrhoea was verified in these two cases.

## 7. SUMMARY

In the western countries infertility affects about 10-15% of couples, genetic factors can be identified in about half of the cases. The first step of the genetic diagnosis is the conventional chromosome analysis, followed by molecular genetic tests according to the specific symptoms. In our laboratory we carried out cytogenetic analysis in 500, FMR1 gene analysis in 17, Y chromosome microdeletion analysis in 150, determination of sperm numerical chromosome aberration in 28 cases and sperm meiotic segregation study in one balanced reciprocal translocation carrier. In female infertility the most common chromosome aberrations were the numerical aberration of chromosome X and inversion of chromosome 9 (3.6%). In two cases *FMR1* gene triplet expansion was found. In male infertility the most frequent cytogenetic aberration proved to be the Klinefelter syndrome (3.3%) and autosomal translocations (2%). Y chromosome microdeletions were identified only in azoospermic and severe oligozoospermic men, but partial microdeletions could also be detected in normozoospermic men. When sperm concentration and motility were damaged simultaneously, we found a higher chromosome aberration rate in sperm. In a man with 46,XY,t(3;6)(q21;q23) karyotype, about 53.2% of sperm carried out unbalanced chromosome assortment. To perform genetic test in infertility in the lack of any other organic and functional problems is reasonable in cases with low sperm count, recurrent foetal loss or premature ovarian dysfunction. In the knowledge of genetic alteration we can assess the genetic risk of the offspring and choose the appropriate mode of assisted reproduction. To determine the accurate genetic alteration of the embryo preimplantation and prenatal genetic diagnostics are needed. Unfortunately, the etiology in the background of infertility can not be clarified in all cases. The successfulness of the assisted reproduction techniques depends on the probability of genetic aberrations.

Register Number: DEENKÉTK/366/2012.

Item Number:

Subject: Ph.D. List of Publications

Candidate: Attila Mokánszki

Neptun ID: WT0X7Z

Doctoral School: Doctoral School of Clinical Medicine

### List of publications related to the dissertation

1. **Mokánszki A.**, Ujfalusi A., Balogh E., Molnár Z., Sápy T., Jakab A., Varga A., Oláh É.: Infertilitásban végzett citogenetikai és molekuláris genetikai vizsgálatok a kelet-magyarországi régióban.  
*Orv. Hetil.* 153 (2), 51-60, 2012.  
DOI: <http://dx.doi.org/10.1556/OH.2013.29510>
2. **Mokánszki, A.**, Ujfalusi, A., Balogh, E., Sümegi, A., Antal-Szalmás, P., Bazsáné Kassai, Z., Molnár, Z., Varga, A., Sápy, T., Jakab, A., Oláh, É.: Meiotic segregation study of a novel t(3;6)(q21;q23) in an infertile man using fluorescence in situ hybridization (FISH).  
*Syst. Biol. Reprod. Med.* 58 (3), 160-164, 2012.  
DOI: <http://dx.doi.org/10.3109/19396368.2012.670868>  
IF:1.524 (2011)
3. **Mokánszki, A.**, Molnár, Z., Ujfalusi, A., Balogh, E., Bazsáné Kassai, Z., Varga, A., Jakab, A., Oláh, É.: Correlation study between sperm concentration, hyaluronic acid-binding capacity and sperm aneuploidy in Hungarian patients.  
*Reprod. Biomed. Online.* 25 (6), 620-626, 2012.  
DOI: <http://dx.doi.org/10.1016/j.rbmo.2012.08.003>  
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List of other publications

5. **Mokánszki, A.**, Körhegyi, I., Szabó, N., Bereg, E., Gergev, G., Balogh, E., Bessenyei, B., Sümegi, A., Morris-Rosendahl, D.J., Sztriha, L., Oláh, É.: Lissencephaly and Band Heterotopia:LIS1, TUBA1A, and DCX Mutations in Hungary.  
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6. Molnár, Z., **Mokánszki, A.**, Benyó, M., Kassai, Z., Oláh, É., Jakab, A.: Sperm fertilization potential and chromosomal anomalies in testicular cancer before chemotherapy.  
*Hum. Reprod.* 26 (2011), i253, 2011.

**Total IF: 5.314**

**Total IF (publications related to the dissertation): 3.566**

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

07 December, 2012



## **List of presentations related to the dissertation**

**Mokánszki A.**, Balogh E., Ujfalusi A., Sümegi A., Varga A., Jakab A., Molnár Zs., Sáy T., Oláh É.: A spermium FISH vizsgálat jelentősége infertilitásban. *Magyar Humán-genetikai Társaság VIII. Kongresszusa*, Debrecen (2010)

**Mokánszki A.**, Molnár Zs., Bazsáné Kassai Zs., Balogh E., Ujfalusi A., Sümegi A., Jakab A., Oláh É.: Heredaganatos férfiak spermaképének, fertilizációs képességének és spermium kromoszóma anomáliáinak változása tumorelles kezelés előtt. *A Magyar Andrológiai Tudományos Társaság IV. Kongresszusa*, Bika (2010)

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**Mokánszki A.**, Sümegi A., Sáy T., Tóthné Varga E., Bodnár B., Varga A., Oláh É.: Az Y kromoszóma részleges mikrodélációinak vizsgálata férfi meddségben. *A Magyar Andrológiai Tudományos Társaság V. Kongresszusa*, Hévíz (2011)

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## **List of other presentations**

**Mokánszki A.**, Körhegyi I., Szabó N., Bereg E., Gyurgyinka G., Balogh E., Bessenyei B., Sümegi A., Sztrihá L., Oláh É.: Genetikai vizsgálatok neuronális migrációs zavarokban. *A Magyar Humán-genetikai Társaság VIII. Kongresszusa*, Debrecen (2010)

**Mokánszki A.**, Körhegyi I., Szabó N., Bereg E., Gyurgyinka G., Balogh E., Bessenyei B., Sümegi A., Sztriha L., Oláh É.: Genetikai vizsgálatok neuronális migrációs zavarokban. *A MTA Debreceni Akadémiai Bizottság, „Ritka genetikai betegségek korszerű diagnosztikája” tudományos ülése*, Debrecen (2011)

**Mokánszki A.**, Körhegyi I., Szabó N., Bereg E., Gyurgyinka G., Balogh E., Bessenyei B., Sümegi A., Sztriha L., Oláh É.: Genetikai vizsgálatok neuronális migrációs zavarokban. *A Magyar Gyermekorvosok Társaságának 2011. évi Nagygyűlése*, Pécs (2011)