doi:10.25143/prom-rsu_2011-02_dts

RIGA STRADINS UNIVERSITY

Agrita Puzuka

EVALUATION OF GENETIC RISK FACTORS IN MALE REPRODUCTIVE PATHOLOGY: LATVIAN POPULATION STUDY

Summary of Doctorate thesis

(speciality – Medical Genetics)

Supervisors: RSU prof., Dr.h.biol. Astrīda Krūmiņa

LU prof., Dr.biol. Viesturs Baumanis

ESF EIROPAS SOCIĀLAIS FONDS

The work was supported by ESF national programme „Support for doctoral and postdoctoral research in medical sciences"

Riga, 2011

RIGA STRADINS UNIVERSITY

Agrita Puzuka

EVALUATION OF GENETIC RISK FACTORS IN MALE REPRODUCTIVE PATHOLOGY: LATVIAN POPULATION STUDY

Summary of Doctorate thesis

(speciality – Medical Genetics)

Supervisors: RSU prof., Dr.h.biol. Astrīda Krūmiņa

LU prof., Dr.biol. Viesturs Baumanis

ESF
EIROPAS SOCIĀLAIS FONDS

The work was supported by ESF national programme „Support for doctoral and postdoctoral research in medical sciences"

Riga, 2011

The study has been worked out in Molecular Genetics Scientific Laboratory of Riga Stradins University and Latvian Biomedical Research and Study Centre

Scientific supervisor:

RSU prof., Dr.h.biol. Astrīda Krūmiņa

LU prof., Dr.biol. Viesturs Baumanis

Approved reviewers:

RSU asoc.prof., Dr.biol. Edvīns Miklaševics

Dr.biol. Jānis Kloviņš

LU prof., Dr.h.biol. Īzaks Rašals

Presentation of doctoral study will be held on the 24th January, 2011 in the Hippocrates Lecture Room of the Riga Stradins University, Dzirciema Street 16, Riga.

The Doctoral Thesis is available for reading in the library of RSU

Secretary of Promotion Council:
Dr.habil.med., professor Līga Aberberga - Augškalne

Annotation

Infertility is a topical problem nowadays. This problem affects approximately 10-15% of couples and about half of the cases refer to male infertility. Genetic reasons for male infertility should be looked in genes that are involved in normal male development and spermatogenesis processes. Those genes are located on human Y chromosome and on autosomes.

Fertility markers-genes localised on human Y-chromosome enable scientists to study Y-chromosomal AZF (azoospermia) region microdeletions and their correlation with male infertility. The method of molecular genetic analysis of spermatogenesis disturbances has been introduced; and it has been found that frequency of Y chromosome microdeletions among infertile males is 5%, and AZF microdeletions were observed in cases of azoospermia or severe oligozoospermia. An algorithm has been developed, which can help select those infertility cases, when detection of Y chromosome microdeletions is purposeful.

Polymorphisms, characteristic to human Y chromosome, - biallelic markers and microsatellites – can be successfully applied for detection of existence of "infertility haplogroups". Studying incidence of Y haplogroups in the infertile males and control group, statistically significant differences have been found, which suggests possibility of existence of certain Y chromosome variants – "infertility" haplogroups.

Positive correlation exists between male infertility and mutations or polymorphisms found in *CFTR* gene (localised on human chromosome 7). Analyzing *CFTR* gene mutations delF508, R117H, as well as 8^{th} intron poly-T and poly-TG polymorphisms in males with idiopathic infertility and control group, statistically significant relation of these mutations and polymorphisms with male infertility was not found, which indicates that *CFTR* gene does not affect spermatogenesis process directly.

Gathering information about the cause of genetic risk of male infertility, the study deals with molecular genetic aspect that has not been studied in Latvia up to now, and is significant in genetic counselling of these patients.

Topicality of the study

Infertility is a serious health problem affecting 10–15% of couples trying to achieve pregnancy; and male factor is estimated as a contributing factor in about half of these cases. More than in 60% the cause of male reproductive tract dysfunction is not known. Of the frequently observed known causes of infertility, major role is played by hormonal disorders and cytogenetic abnormalities. Genetic background of male infertility could be explained analysing Y chromosomal and autosomal genes that are involved in normal male development and spermatogenesis process.

Early cytogenetic studies showed that deletions in the long arm of Y chromosome are responsible for azoospermia. With the advancement of molecular biology, three regions named "azoospermia factors" (AZFa, AZFb and AZFc regions; Yq11 locus) have been defined as spermatogenesis loci. It is now widely accepted that deletions within those regions severely diminish the sperm production. Interestingly, these microdeletions interrupted spermatogenesis at three different phases. A complete Sertoli-cell-only syndrome (SCOS) is observed in patients with deletion of AZFa, i.e. only Sertoli cells, but no germ cells are visible in the tubules of their testis tissue sections. Arrest at the spermatocyte stage is observed in the testis tissue of all patients with deletion of the complete AZFb locus. A variable testicular pathology has been found in patients with AZFc deletions; in most tubules, only Sertoli cells are identified but in some tubules germ cells of different developmental stages are clearly visible. Y chromosome microdeletion analysis in Latvia was absent.

Male infertility-causing genetic factors are thought to be associated with certain Y chromosome-specific polymorphisms – Y hromosome haplogroups and haplotypes. On the basis of Y chromosome haplogroup and haplotype data characteristic to the Latvian population, it is possible to identify haplogroups or lineages of the Y chromosome that may be at increased risk of developing male infertility.

Since it is known that frequency of Y chromosome microdeletions in men with idiopathic infertility is ~ 10%, it is necessary to look for other genetic mechanisms that are involved in development of male infertility. One of these factors is mutations and polymorphisms in cystic fibrosis (*CFTR*) gene. In males severe deficiency of protein encoded by *CFTR* gene results in congenital bilateral absence or atrophy of

the vas deferens (CBAVD) and obstructive azoospermia. The most common mutations in the *CFTR* gene that correlate with male infertility are delF508 and R117H, as well as poly-T and poly-TG tract 5T/12TG allele localised in the 8th intron of *CFTR* gene.

The aim

To study genetic aspects of idiopathic male infertility in Latvian population.

Study objectives

1. To detect Y chromosome AZF region microdeletions in selected group of patients with idiopathic impairment of spermatogenesis.
2. To compare existing Y-chromosome microdeletion analysis methods in order to assess their suitability for rapid and accurate detection of the Y chromosome microdeletions in laboratory of molecular genetics.
3. To develop an algorithm that would enable doctors-andrologists to select patients to who identification of the Y chromosome microdeletions is significant.
4. To approve and introduce in Latvia a convenient and effective Y chromosome microdeletion detection method as a molecular diagnostic tool of male infertility.
5. To analyse Y chromosome neutral polymorphism variants – Y haplogroups and haplotypes in men with idiopathic infertility, and assess their correlation with disturbances of spermatogenesis.
6. To study Y chromosome microdeletions in males affected with cystic fibrosis.
7. To analyse the *CFTR* gene mutation delF508 and mutation R117H, as well as *CFTR* gene poly-T and poly-GT polymorphisms in infertile men.
8. To compare *CFTR* gene poly-T and poly-GT polymorphisms in infertile and control group men.

Novelty of study

Molecular genetic studies of idiopathic male infertility in Latvia were absent, and it makes this research a novelty of this study. It was found that in men with azoospermia and severe oligozoospermiju frequency of Y chromosome microdeletions is 5%, which corresponds to the incidence of Y microdeletions in other European countries. Y chromosome haplogroup analysis showed that there are Y chromosome lineages that may be at increased risk of developing infertility. *CFTR* gene mutations (delF508 and R117H) and poly-T/poly-TG polymorphisms have been identified in men with idiopathic infertility. For the first time in Latvia the importance of molecular genetic studies is emphasized in clarification of diagnosis, in genetic association studies and in genetic counselling of male infertility.

Implications to practice

Approbated and introduced a convenient and efficient molecular method for detection of spermatogenesis disorder by which, in Latvia it will be possible to diagnose the Y chromosome microdelerions. An algorithm was introduced that could help doctors - andrologists to select infertile males, to whom it is important to identify the Y chromosome microdeletions. It is recommended to analyse all cases of idiopathic infertility, when hormone levels and the karyotype is normal, but sperm count is $<5x10^6$/mL. Analysis of Y chromosome microdeletions in men with less serious forms of infertility is not advised, because the incidence of Y chromosome microdeletions in this group is very low. Y-chromosome microdeletion analysis specifies the diagnosis of infertility, detects aetiology of disease, and is informative in case of genetic counselling before in vitro fertilisation.

Material and methods

Objects

Subjects analysed in this study were 100 men from infertile couples (failure to achieve pregnancy during at least one year) (Table1). Inclusion criteria were azoospermia (lack of sperm in ejaculate), oligozoospermia (defined as sperm concentration $<5x10^6$/mL) or oligo- astheno- teratozoospermia (sperm structural defects). All cases of spermatogenic failure resulting from endocrine or proved cytogenetic abnormality (except two cases) were excluded from our study. The average age of infertile men was 35 ± 11 years (ranged from 24 to 46 years). The average duration of infertility was 4,5 years (ranged from 1 to 20 years). Each patient was asked and examined for potential gonadal anomalies, testis volumes, varicoceles, potential epididymal or prostata abnormalities, levels of hormones determined: follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone. Sperm and its ejaculates were analysed for their mobility, number and morphology according to the guidelines of World Health Organization (WHO). Our study was approved by the Latvian Central Committee of Medical Ethics, and informed consent was obtained from each patient.

Overview of male reproductive pathology molecular genetics study (see Table 1)

Objects for Y chromosome microdeletion analysis were:

- 100 infertile males
- 15 males affected by cystic fibrosis (CF patients)

Objects for Y haplogroup analysis were:

- 79 infertile males
- Control group 153 males (Latvians in three generations)

Objects for Y haplotype (Y-STR microsatellite) analysis were:

- 5 infertile males with AZF region deletions

Objects for CFTR gene analysis were:

- 82 infertile males
- Control group 60 males (Latvians in three generations)
- 15 males affected by cystic fibrosis (CF patients)

Table 1. **Overview of analysed objects/DNA samples**

Objects (DNA samples)	Type of analysis	Used methods
100 infertile males	Y chromosome microdeletion analysis	Multiplex polymerase chain reaction (Multiplex PCR)
	Y chromosome haplogroup analysis	Polymerase chain reaction (PCR) followed by analysis of restriction fragment length polymorphism (RFLP) and DNA sequencing
	Y chromosome haplotype analysis	Genotyping of Y chromosome microsatellite (Y-STR or DYS)
	Analysis of *CFTR* gene mutations delF508	PCR followed by heteroduplex analysis
	Analysis of *CFTR* gene mutations R117H	PCR followed by RFLP
	Analysis of *CFTR* gene poly-T un poly-TG polimorphisms	DNA sequencing
153 males (Latvians in three generations) – control group	Y chromosome haplogroup analysis	PCR followed by RFLP and DNA sequencing
	Analysis of *CFTR* gene poly-T un poly-TG polimorphisms	DNA sequencing
15 males affected by cystic fibrosis (CF patients)	Y chromosome microdeletion analysis	Multiplex PCR
	Analysis of *CFTR* gene poly-T un poly-TG polimorphisms	DNA sequencing

Isolation of DNA

Peripheral blood samples from infertile males were collected, and DNA was isolated using Nucleon BACC1 kit (Amersham, USA). DNA samples that represent control group and cystic fibrosis patients were used from RSU Molecular genetics laboratory DNA sample collection and previously were isolated using phenol-chloroform method.

Y chromosome microdeletion analysis

Microdeletions of AZF regions were detected by two multiplex PCR reactions – multiplex A and multiplex B. The following primer sets were used - sY14, ZFX/ZFY as male and both gender controls, but sY84, sY86 for AZFa, sY127, sY134 for AZFb and sY254, sY255 for AZFc deletions which are recommended to be used in multiplex PCR as the best choice for the diagnosis of Y chromosome microdeletions of the AZF region. Those primer sets were initially used by Simoni et al (2004) and are recommended by European Academy of Andrology. A deletion was confirmed when two consecutive PCR amplifications yielded positive results. To compare molecular Y chromosome microdeletion detection methods DNA samples with Y chromosome microdeletions were analysed with commercial kit – Y Chromosome Deletion Detection System, Version 2.0 (Promega, USA).

Y chromosomal haplogroup analysis

Simultaneously, DNA samples from infertile patients were typed with 6 Y chromosomal binary markers to establish their haplogroup (Hg). Binary markers were chosen corresponding to those haplogroups that were found in ethnogenetic studies of Latvians (M9, SRY-1532, M17, Tat, P21, M170; unpublished data). Every sample was typed with all binary markers. Typing conditions were similar for all the markers, and were previously described by Zerjal et al (1997). Results are given according to the haplogroup nomenclature of the Y Chromosome Consortium (Y Chromosome Consortium, 2002).

Y chromosomal haplotype analysis

To detect Y chromosome haplotype or Y chromosome microsatellite (Y-STR or DYS) variants commercial kit – PowerPlex Y System (Promega, USA) was used. 12 Y-STRs: DYS392, DYS19, DYS385a, DYS385b, DYS389I, DYS389II, DYS390, DYS391, DYS393, DYS437, DYS438, and DYS439 were analysed. In Y chromosome microsatellite analysis, PCR primers were used that were labelled with fluorescent days that allows determining corresponding Y-STRs. PCR products were analysed with ABI PRISM 310 genetic analyser. Results were processed using Genotyper 3.7 and PowerTyper TM YMacro programs. Patients Y-STR data were

analysed also with computer programme (http://www.hprg.com/hapest5/) to obtain in some cases more precise information about Y Hg (Athey, 2005 un Athey, 2006).

Analysis of CFTR gene mutations delF508 and R117H

CFTR gene mutation delF508 was detected using designed primers and heteroduplex PCR analysis (Rommens et al, 1990). *CFTR* gene mutation R117H was detected appropriate PCR approach followed by RFLP analysis (Boucher et al, 1999).

Analysis of CFTR gene polyT and poly-TG polimorphisms

CFTR gene polymorphisms poly-T and poly-GT were detected using appropriate PCR (Chillon et al., 1995) followed by sequencing of corresponding PCR products (sequencing approach was introduced in frame work of our study)

Statistical analysis

Chi-square statistic. Differences in Y chromosome haplogroup frequencies and CFTR gene poly-T and poly-TG polymorphic variants frequencies between infertile males and control group were compared by the chi-square statistic. All p values were based on 2-sided comparisons and $p<0.05$ indicates statistical significance. Analysis was held using interactive calculator (http://www.quantpsy.org; Preacher, 2001). For multiple tests (Y chromosome haplogroup analysis; in case of Hg N3a1, Hg R1a1 and Hg I) *Bonferroni* correction was used (Bland and Altman, 1995). After *Bonferroni* correction in case of Hg N3a1 and Hg R1a1 $p<0.016$ and in case of Hg I $p<0.025$ indicates statistical significance.

Confidence Interval Analysis, CIA. Confidence Interval Analysis (Newcombe & Altman, 2000) was used to compare differences in frequencies of *CFTR* gene poly-T and poly-TG polimorphic variants between infertile males and control group. Analysis was held using computerprogramm CIA (*proportions and their diferences, 95% confidence interval for the difference, Newcombe method*). All p values were based on 2-sided comparisons (infertile males/control group; infertile male/CF patients; control group/CF patients) and $p<0.05$ indicates statistical significance.

Results

1. Y chromosome microdeletions and male infertility

Multiplex PCR analysis of Y chromosome microdeletions in the samples in all cases gave definite results – in both multiplex A and B polymerase chain reactions negative control contamination was not observed, and positive controls of both female DNA (PCR internal control) and fertile male DNA (positive control of male DNA) always gave positive signals.

Frequency of Y chromosome microdeletions in the analysed idiopathic infertile male population in Latvia was found to be 5% (5 samples of 100). In three cases of five microdeletions were observed in AZFc region, but in two cases they were found in all three AZF regions (AZFa+b+c deletion) Data on types of Y chromosome microdeletions are summarized in Figure 1.

In all cases microdeletions were found in infertile men having severe form of infertility – azoospermia or oligozoospermia. In infertile males with deletion in AZFc region (three cases) of Y chromosome long arm, number of spermatozoa in spermatograms is different – in one case spermatozoa were not found (azoospermia), but in two cases 1–5 immobile spermatozoa were found (severe oligozoospermia). In the individuals with full AZF region (AZFa+b+c deletion) deletion (two cases) spermatogram data show azoospermia phenotype. In both persons with full deletions of all three regions cytogenetic analysis revealed abnormality in karyotype – deletion in long arm of Y chromosome – respectively, 46,X,del(Y)(q). In one person with deletion of all three AZF regions karyotype analysis showed small long arm of Y chromosome with suspicion to short-arm isochromosome, which was not confirmed by methods applied in this study (analysis of Y chromosome microdeletions and Y chromosome microsatellites). In the course of clinical examination of this individual, central type obesity and short neck were found. Duration of infertility was three years (Table 2).

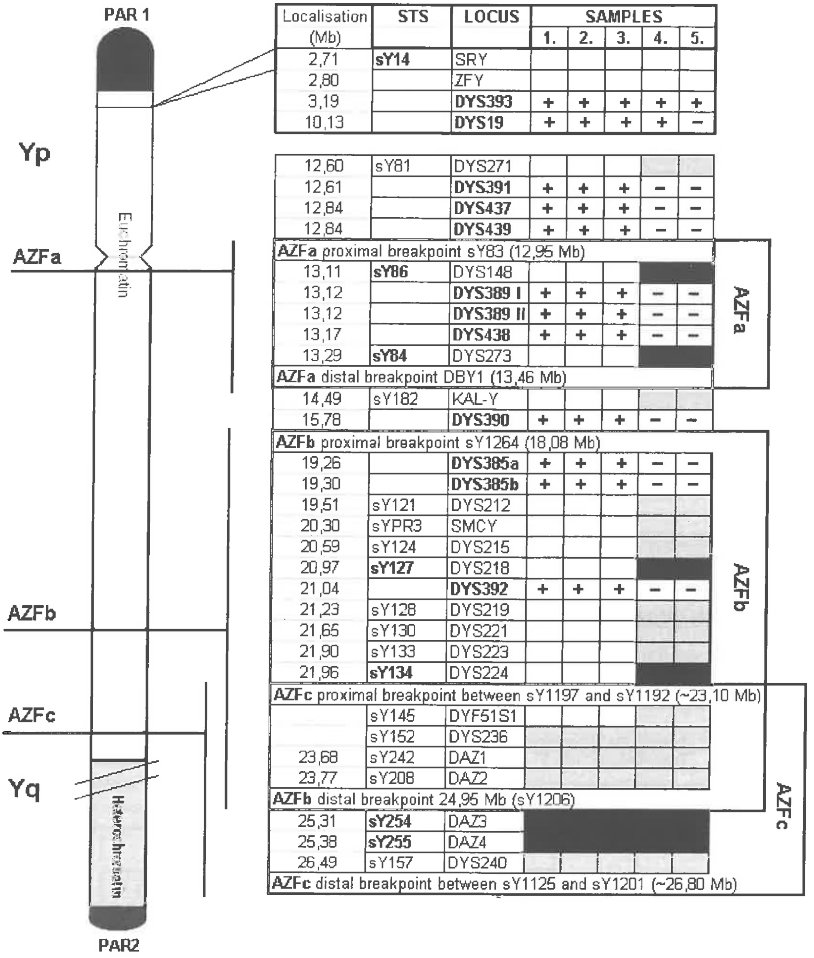
Analysing DNA samples of 15 cystic fibrosis patients, presence of Y chromosome microdeletions was not confirmed.

To compare existing Y-chromosome microdeletion analysis methods DNA samples from patients having microdeletions were analysed repeatedly with the commercial kit (Promega 2.0). The acquired results are summarised in Figure 1. (grey

squares). This analysis confirm deletions in STS regions, analysed with multiplex A and B PCR method (Figure 1. STS marked in bold type), and showed deletions in other STS, spread over all three AZF regions. Data of the commercial kit revealed the same as multiplex PCR reactions – in three of five samples with microdeletions on Y chromosome deletions were localised in AZFc region, in the rest two samples all three AZF regions were deleted, respectively, AZFa+b+c deletion

Table 2. **Genotype – phenotype associations in patients with Y chromosomal microdeletions**

Nr.	Deleted region	Spermatozoa in spermogramm	Clinical diagnosis	Karyotype	Phenotypic data
1.	AZFc	0	azoospermia	46,XY	Android adiposity, gynecomastia, brachydactyly
2.	AZFc	5 motionless spermatozoa in spermogramm	severe oligozoospermia	46,XY	Epidemic parotitis in childhood, in testis biopsy only spermatids found
3.	AZFc	1 – 2 immobile spermatozoa in spermogramm	severe oligozoospermia	46,XY	Epidemic parotitis in childhood
4.	AZFa, AZFb, AZFc	0	azoospermia	46,X, del (Y)(q)	Android adiposity, gynecomastia, small testis in right side
5.	AZFa, AZFb, AZFc	0	azoospermia	46,X, del (Y)(q)	No data



Localisation (Mb)	STS	LOCUS	SAMPLES 1.	2.	3.	4.	5.
2,71	**sY14**	SRY					
2,80		ZFY					
3,19		**DYS393**	+	+	+	+	+
10,13		**DYS19**	+	+	+	+	–
12,60	sY81	DYS271					
12,61		**DYS391**	+	+	+	–	–
12,84		**DYS437**	+	+	+	–	–
12,84		**DYS439**	+	+	+	–	–
AZFa proximal breakpoint sY83 (12,95 Mb)							
13,11	**sY86**	DYS148					
13,12		**DYS389 I**	+	+	+	–	–
13,12		**DYS389 II**	+	+	+	–	–
13,17		**DYS438**	+	+	+	–	–
13,29	**sY84**	DYS273					
AZFa distal breakpoint DBY1 (13,46 Mb)							
14,49	sY182	KAL-Y					
15,78		**DYS390**	+	+	+	–	–
AZFb proximal breakpoint sY1264 (18,08 Mb)							
19,26		**DYS385a**	+	+	+	–	–
19,30		**DYS385b**	+	+	+	–	–
19,51	sY121	DYS212					
20,30	sYPR3	SMCY					
20,59	sY124	DYS215					
20,97	**sY127**	DYS218					
21,04		**DYS392**	+	+	+	–	–
21,23	sY128	DYS219					
21,65	sY130	DYS221					
21,90	sY133	DYS223					
21,96	**sY134**	DYS224					
AZFc proximal breakpoint between sY1197 and sY1192 (~23,10 Mb)							
	sY145	DYF51S1					
	sY152	DYS236					
23,68	sY242	DAZ1					
23,77	sY208	DAZ2					
AZFb distal breakpoint 24,95 Mb (sY1206)							
25,31	**sY254**	DAZ3					
25,38	**sY255**	DAZ4					
26,49	sY157	DYS240					
AZFc distal breakpoint between sY1125 and sY1201 (~26,80 Mb)							

Figure 1. **Data of Y chromosome microdeletion analysis in five patients with AZF region microdeletions**

AZF regions and Y-chromosomal markers (sequence target sites – STS; Y chromosome microsatelites – Y-STRs or DYS) are listed above. Black boxes, STS marker absent – microdeletion of corresponding AZF region/locus (Multiplex A and B PCR results). Bold – STS used in Multiplex A and B PCR. Grey boxes, STS marker absent – microdeletion of corresponding AZF region/locus (Promega 2.0 kit results). Pluss (+) Y-STR marker present, minuss (-) Y-STR marker absent. PAR1 and PAR2 - pseudoautosomal regions.

2. Y chromosome haplogroups and male infertility

To determine which Y chromosome haplogroups (Hg) may have predisposition to the male infertility, DNA samples from 79 infertile males were analysed. Of 79 analysed DNA samples 56 contained Y chromosome macrohaplogroup M9 G allele variant, and using the corresponding biallelic markers, this Y chromosome lineage was divided into smaller groups in K cluster (K*), which divided into N3a1 haplogroup (Hg N3a1; markers Tat and P21) and R1a1 haplogroup (Hg R1a1; markers SRY1532 and M17). Y macrohaplogroup M9 C allele variant was determined in 23 (Hg I and other M9 C) cases of 79 analysed patients' samples. The samples corresponded to F cluster (F*). In this group of samples, haplogroup was determined in two samples only, which corresponded to I haplogroup (Hg I; marker M170). Unfortunately, 21 sample did not fall into any of the analysed haplogroups, thus these samples represent other haplogroups (M9-C; DE* and F*: G, J) (Table 3). Therefore further study would be necessary, however, it will not hinder understanding of the main tendencies, observed in our "infertility haplogroups" studies.

In order to understand relation of Y Hg with male infertility, Y chromosome haplogroup data, obtained from Y Hg analysis in ethnic Latvians (males, Latvians in three generations or control group) were used.

Table 3. **Incidence of Y chromosome haplogroups among infertile males and control group**

Haplogroups (Hg)	**Incidence of haplogroups (%)**		*p*
	Infertile males n = 79	**Control group** n = 153	
N3a1	24 (30.4%)	65 (42.5%)	0.223
R1a1	12 (15.2%)	60 (39.2%)	0.005
I	2 (2.5%)	13 (8.5%)	0.097
K* (without N3 and R1a)	20 (25.3%)	10 (6.5%)	< 0.001
Other (M9 C; DE* un F*: G, J)	21 (26.6%)	5 (3.3%)	< 0.001

Four main haplogroups: N3a1, R1a1, K* (without N3 and R1a) and I in both infertile males and control group comprise ~ 90% of all Latvian Y chromosome genofund, and are the dominating haplogroups in north-east part of Europe. This

division of haplogroups in different European populations as well as in both analysed groups suggests their common pre-history and origin.

The prevalent haplogroup in the population of infertile men is N3a1, which represents 30.4% of all analysed samples (24 samples of 79). Whereas, frequency of it in control group is 42.5%. This haplogroup is represented in both analysed groups, and making statistical analysis (χ^2 analysis), relation of Hg N3a1 to male infertility was not statistically significant (p=0.223).

Other most common Y haplogroup in both control and infertile male group is Hg R1a1. Comparing frequency of Hg R1a1 in both groups it was found that Hg R1a1 is less frequent in DNA samples of infertile males (15.2%, or 12 samples of 79), but in control group its frequency is 39.2%. Making χ^2 analysis, p value was determined, which was statistically significant (p=0.005), and it shows that Hg R1a1 may be more characteristic for males without disturbances of spermatogenesis than for infertile males.

Y chromosome Hg I occurred in 2.5% (2 samples of 79) of infertile males, but in control group – 8.5% (13 cases of 153). Both these groups were compared, making χ^2 analysis, and it was found that p=0.097. Thus, Hg I does not reveal connection with male infertility.

The study showed that cluster K* occurs in 25.3% Y-chromosomes of infertile males (20 samples of 79). However, in control group frequency of this K* is much less, 6.5% only. Making χ^2 analysis confidence p value was determined, which was <0.001, and it shows that K* cluster may be more characteristic to Y chromosome lineage of infertile males.

2.1. Analysis of Y chromosome haplotypes in infertile males with Y chromosome microdeletions

Y-chromosome microsatelite (Y-STR or DYS) genotyping method was used in this study to understand Y chromosome haplotypes (allelic/gene or polymorphism group, which inherits completely related; haplotypes form haplogroups) in patients with observed Y microdeletions. In addition, this method repeatedly confirmed Y chromosome microdeletions, since in patients with these microdeletions in the corresponding Y chromosome regions Y chromosome microsatellites were not detected.

Y-STR genotyping results are revealed in Figure 1 and Table 4. Samples of DNA with AZFc microdeletions (no. 1–3) reveal different Y-STR pattern and differences in number of repeats of corresponding microsatellite.

Table 4. **Data on microsatellites in patients with Y chromosome microdeletions**

No.	Type of microdeletion	Y chromosome haplotype (Y-STR haplotype)												Y Hg
		DYS 393	DYS 19	DYS 391	DYS 437	DYS 439	DYS 389I	DYS 389II	DYS 438	DYS 390	DYS 385a	DYS 385b	DYS 392	
1.	AZFc	14	15	11	13	9	13	29	10	23	11	14	13	N3a1
2.	AZFc	14	15	11	13	10	13	29	10	23	11	14	13	N3a1
3.	AZFc	14	15	10	13	10	14	30	10	23	11	14	13	K* (N)
4.	AZFa,b,c	14	15	-	-	-	-	-	-	-	-	-	-	M9 C (F*)
5.	AZFa,b,c	13	-	-	-	-	-	-	-	-	-	-	-	K* (R1b)

AZFc, AZFa,b,c – microdeletions in AZF regions
DYS – microsatellites (Y-STR)
Numbers – number of repeats of corresponding microsatellite
Minus (-) – microsatellite not found

Since all Y-STR, available in the commercial kit, are localised all over Y chromosome except AZFc region, the acquired results do not give additional information on individuals in whose chromosomes this type of microdeletions was found. Whereas, in both samples of infertile males (samples 4 and 5) with all three AZF region deletions Y-STR genotyping data reveal presence of only two microsatellites DYS393 and DYS19. Both microsatellites are located in the short arm of chromosome (Figure 1). However, sample no.5 differs from sample no.4 as it lacks DYS19 microsatellite. The acquired data reveal that determining lack of microsatellites in the long arm of Y chromosome, it is confirmed that in the analysed DNA samples deletions have occurred in the corresponding AZF regions and outside them.

In addition, data on Y-STR analysis add information, and in some cases make it more precise, on belonging of DNA samples from infertile men to certain Y chromosome haplogroup. For example, using biallelic markers it was determined that sample nr.3 belongs to M9 G macrohaplogroup cluster K*, but with help of Y-STR data and prorgamm (http://www.hprg.com/hapest5/) it was detected, that this sample belongs to haplogroup N.

3. Molecular study of *CFTR* gene

3.1. Analysis of *CFTR* gene mutations delF508 and R117H

In our study CFTR gene mutations delF508 and R117H were not found in analysed 82 infertile male DNA samples.

3.2. Poly-T and poly-TG polymorphisms in 8. intron of *CFTR* gene

Acquired results are revealed in tables 5 – 10.

***Poly-T polymorphism* in CFTR gene**

Comparing frequency of poly-T tract alleles and genotypes in the analysed samples, we found which allele and genotype variants show statistically significant differences in the groups of the analysed samples (Tables 5 and 6).

Table 5. **Polymorphism of poly-T tract alleles in analysed samples**

Poly-T tract **allele**	**Infertile males** n=164 (%)	**Control group** n=120 (%)	**CF patients** n=30 (%)
9T	19 (12%) [a]	18 (15%) [a]	21 (70%) [a b]
7T	142 (87%) [c]	92 (77%) [d]	8 (27%) [c d]
5T	3 (2%)	10 (8%) [e]	1 (3%) [e]

[a] statistically significant differences, comparing frequency of 9T allele in CF patient and infertile male samples (95% confidence interval CI=0.396-0.723; χ^2= 24.559, p<0.001)
[b] statistically significant differences, comparing frequency of 9T allele in CF patient and control group samples (95% CI=0.356-0.693; χ^2= 16.320, p<0.001)
[c] statistically significant differences, comparing frequency of 7T allele in infertile males and CF patients samples (95% CI=0.411-0.732; χ^2= 7.834, p=0.005)
[d] statistically significant differences, comparing frequency of 7T allele in control group and CF patient samples (95% CI=0.304-0.642; χ^2= 5.804, p=0.016)
[e] statistically significant differences, comparing frequency of 5T allele in control group and infertile males samples (95% CI=0.014-0.129; χ^2= 4.744, p=0.029)

Poly-T tract 9 T allele most commonly occurs in CF patients, comparing with samples from infertile males (p<0.001) and control group (p<0.001). In its turn, 7T allele, which is the most common poly-T variant in DNA samples of infertile males, does not show statistically significant differences (p=0.558) comparing frequency of this allele with control group, however, 7T allele is less frequent in CF patients group comparing with samples of DNA from infertile (p=0.005) and control group (p=0.016). Poly-T tract 5T allele, according to data from literature (Chillon et al., 1995), is prevalent in

cases of CBAVD (*congenital bilateral absence of the vas deferens*). In this study 5T allele was more represented in samples of control group, comparing with frequency of this allele in infertile males (p=0.029). Comparing frequency of 5T allele in samples of control group and CF patients, statistically significant differences were not found (p=0.624).

Table 6. **Polymorphism of poly-T tract genotypes in analysed samples**

Poly-T tract **genotype**	**Infertile males** n=82 (%)	**Control group** n=60 (%)	**CF patients** n=15 (%)
9T/9T	2 (2%) [a]	2 (3%) [b]	9 (60%) [a b]
7T/7T	62 (76%) [c]	37 (62%) [d]	2 (13%) [c d]
5T/5T	no	3 (5%)	no
9T/7T	15 (18%)	14 (23%)	3 (20%)
5T/7T	3 (4%)	4 (7%)	1 (7%)

[a] statistically significant differences, comparing 9T/9T genotype frequency in CF patients and infertile male samples (95% CI=0.326-0.778; χ^2= 21.475, p<0.001)
[b] statistically significant differences, comparing 9T/9T genotype frequency in CF patients and control group samples (95% CI=0.311-0.770; χ^2= 15.278, p<0.001)
[c] statistically significant differences, comparing 7T/7T genotype frequency in infertile and CF patients samples (95% CI=0.357-0.748; χ^2= 4.978, p=0.026)
[d] statistically significant differences, comparing 7T/7T genotype frequency in control group and CF patients samples (95% CI=0.052-0.462; χ^2= 4.472, p=0.034)

As it was mentioned above, 9T allele most commonly occurs in CF patients, consequently 9T/9T genotype is prevalent in samples from CF patients, comparing with those from infertile males (p<0.001) and control group (p<0.001). Similar data have been obtained also in case of 7T/7T genotype, as, due to high frequency of 7T allele in chromosomes of infertile males and control group. 7T/7T genotype least commonly occurs in CF patients' group, comparing with samples from infertile males (p=0.026) and control group (p=0.034). The rest of genotypes (5T/5T, 9T/7T, 5T/7T), which were observed in the analysed samples, did not show statistically significant differences among sample groups.

***Poly-TG polymorphism* in *CFTR* gene**

Comparing frequency of poly-TG tract alleles and genotypes in the analysed samples, we found which variants of alleles and genotypes show statistically significant differences in the analysed sample groups (Tables 7 and 8).

Table 7. **Polymorphism of poly-TG tract alleles in the analysed samples**

Poly-TG tract **allele**	**Infertile males** n=164 (%)	**Control group** n=120 (%)	**CF patients** n=30 (%)
12TG	15 (9%)	18 (15%)	1 (3%)
11TG	86 (52%) [a]	52 (43%) [b]	4 (13%) [a b]
10TG	59(36%)	48 (40%)	15 (50%)
9TG	4 (2%) [c]	2 (2%) [d]	8 (27%) [c d]
8TG	no	no	1 (3%)
7TG	no	no	1 (3%)

[a] statistically significant differences, comparing 11TG allele frequency in infertile males and CF patient samples (CI=0.211-0.501; χ^2=6.077, p=0.014)
[b] statistically significant differences, comparing 11TG allele frequency in control group and CF patients samples (95% CI=0.116-0.420; χ^2=4.003, p=0.045)
[c] statistically significant differences, comparing 9TG allele frequency in CF patient and infertile male samples (95% CI=0.112-0.421; χ^2=16.438, p<0.001)
[d] statistically significant differences, comparing 9TG allele frequency in CF patient and control group samples (95% CI=0.118-0.428; χ^2=15.471, p<0.001)

Table 8. **Polymorphism of Poly-TG tract genotypes in the analysed samples**

Poly-**TG** tract **genotype**	**Infertile males** n=82 (%)	**Ethnic males** n=60 (%)	**CF males** n=15 (%)
12TG/12TG	2 (2%)	no	no
11TG/11TG	18 (22%)	12 (20%)	0
10TG/10TG	8 (10%) [a]	7 (12%) [b]	6 (40%) [a b]
9TG/9TG	no	no	3 (20%)
12TG/11TG	8 (10%)	6 (10%)	1 (7%)
12TG/10TG	3 (4%) [c]	12 (20%) [c]	0
11TG/10TG	39 (48%)	21 (35%)	2 (13%)
11TG/9TG	3 (4%)	1 (2%)	1 (7%)
10TG/9TG	1 (1%)	1 (2%)	1 (7%)
8TG/7TG	no	no	1 (7%)

[a] statistically significant differences, comparing 10TG/10TG genotype frequency in CF males and infertile males samples (95% CI=0.084-0.550; χ^2= 4.332, p=0.037)
[b] statistically significant differences, comparing 10TG/10TG genotype frequency in CF males and ethnic males samples (95% CI=0.056-0.533; χ^2= 4.171, p=0.041)
[c] statistically significant differences, comparing 12TG/10TG genotype frequency in ethnic males and infertile males samples (95% CI=0.059-0.284; χ^2= 6.339, p=0.012)

Poly-TG tract allele 11 TG prevails in samples from infertile males (p=0.014) and in control group (p=0.045), comparing with CF patients sample group. In addition, comparing infertile and control group *CFTR* gene poly-TG polymorphism 11 TG variant, statistically significant differences were not observed (p=0.510). In its turn, 9TG allele, which corresponds to one of the shortest TG dinucleotide repeats, is more represented in samples from CF patients, comparing with samples from infertile males

($p<0.001$) and control group ($p<0.001$). In the rest of TG alleles (12TG, 10TG), which were observed in all analysed sample groups, statistically significant differences were not observed.

Comparing poly-TG tract TG genotypes in the analysed sample groups, statistically significant differences were observed in 10TG/10TG and 12TG/10TG genotype variants. Genotype 10TG/10TG prevails in CF patients, comparing with samples from infertile males ($p=0.037$) and control group ($p=0.041$). In addition, 12TG/10TG genotype is the dominating variant in chromosomes from control group, comparing with infertile males' group ($p=0.012$). Whereas, presence of 12TG/10TG genotype in CF patients' samples has not been observed. Analysing frequency of 11TG/10TG genotype in all three sample groups with statistical analysis computer programme CIA (Confidence Interval Analysis; proportions and their differences 95% confidence interval for the difference; Newcombe method) it was confirmed that 11TG/10TG genotype is more common in infertile males, comparing to control group (95% CI=0.075-0.489), however, making χ^2 analysis, statistically significant data were not obtained comparing incidence of this genotype variant in both above mentioned sample groups ($\chi^2= 0.647$, $p=0.421$). Acquired results suggest that 11TG/10TG genotype is more common in infertile males than in control group, though it cannot be claimed that these data are undoubtedly statistically confident, since one method reveals statistically significant differences, but other does not. The rest of TG genotypes, which were represented in all sample groups, occur equally frequently, or are unique for one of the groups (Tables 7 and 8), and statistically significant differences did not show.

***Poly-T and poly-TG polymorphisms* in CFTR gene (T/TG haplotypes)**

Comparing frequency of poly-T and poly-TG tract alleles and genotype in the analysed samples, we clarified which variants of alleles and genotypes show statistically significant differences in the analyzed sample groups (Tables 9 and 10).

The prevalent haplotype among infertile males' *CFTR* alleles is 7T/11TG. This haplotype most rarely occurs in samples from CF patients, comparing to samples from

infertile males (p=0.009) and control group (p=0.045). Whereas, comparing 7T/11TG haplotype in samples from infertile males and control group, statistically significant differences were observed making analysis with statistical analysis computer programme CIA (95% CI=0.002-0.230) only. However χ^2 analysis did not show statistically significant differences (χ^2= 1.292, p=0.256), comparing incidence of this haplotype variant in both sample groups. Acquired results suggest that 7T/11TG haplotype is more common in infertile males than in the control group, however, it cannot be claimed that these data are definitely statistically confident, as one method shows statistically significant differences, bet the other one does not.

Table 9. **Polymorphisms of poly-T and poly-TG tract alleles (T/TG haplotypes) in the analysed samples**

Poly-T and poly-TG tract alleles	Infertile males n=164 (%)	Control group n=120 (%)	CF patients n=30 (%)
9T/11TG	3 (2%)	1 (1%)	1 (3%)
9T/10TG	12 (7%) [a]	15 (13%) [b]	12 (40%) [a b]
9T/9TG	4 (2%) [c]	2 (2%) [d]	8 (27%) [c d]
7T/12TG	14 (9%)	14 (12%)	1 (3%)
7T/11TG	81 (49%) [e]	45 (38%) [f]	3 (10%) [e f]
7T/10TG	47 (29%)	33 (28%)	3 (10%)
7T/7TG	no	no	1 (3%)
5T/12TG	1 (1%) [g]	6 (5%) [g]	0
5T/11TG	2 (1%)	4 (3%)	0
5T/8TG	no	no	1 (3%)

[a] statistically significant differences, comparing frequency of 9T/10TG allele in CF patients and infertile males samples (CI=0.396-0.723; χ^2=14.233, p<0.001)
[b] statistically significant differences, comparing frequency of 9T/10TG allele in CF patients and control group samples (95% CI=0.356-0.693; χ^2=6.264, p=0.012)
[c] statistically significant differences, comparing frequency of 9T/9TG allele in CF patients and infertile males samples (95% CI=0.112-0.421; χ^2=16.438, p<0.001)
[d] statistically significant differences, comparing frequency of 9T/9TG allele in CF patients and control group samples (95% CI=0.118-0.428; χ^2=15.471, p<0.001)
[e] statistically significant differences, comparing frequency of 7T/11TG allele in infertile males and CF patients samples (95% CI=0.220-0.494; χ^2=6.829, p=0.009)
[f] statistically significant differences, comparing frequency of 7T/11TG allele in control group and CF patients samples (95% CI=0.099-0.386; χ^2=4.01, p=0.045)
[g] statistically significant differences, comparing frequency of 5T/12TG allele in control group and infertile males samples (95% CI=0.005-0.099; χ^2=3.635, p=0.022)

Similar situation is observed also in case of 7T/10TG haplotype, when infertile males' samples are compared to CF patient group (according to CIA statistically confident differences, 95% CI=0.018-0.285; according to χ^2 analysis data do not show

statistically confident differences χ^2=2.289, P=0.130), as well as comparing control group (95% CI=0.003-0.283; χ^2=1.967 P=0.161) with CF male 7T/10TG haplotype results. But 9T/10TG (40%) and 9T/9TG (27%) are the prevalent haplotypes among alleles of cystic fibrosis patients. 9T/10TG haplotype is more common in CF patients comparing to samples from infertile males ($p<0.001$) and control group ($p<0.001$). Similarly, 9T/9TG haplotype is more common in CF patients, comparing to samples from infertile males ($p<0.001$) and control group ($p<0.001$). Whereas, variants of *CFTR* gene haplotypes in infertile males do not differ much from *CFTR* haplotypes in control group, statistically significant differences in both groups were not observed, except 5T/12TG haplotype, which more occurs in control group ($p=0.022$).

Table 10. **Polymorphism of poly-T and poly-TG tract genotypes (T/TG haplotypes) in the analysed samples**

Poly-T and poly-TG tract genotypes*	Infertile males n=82 (%)	Control group n=60 (%)	CF patients n=15 (%)
9T/11TG; 9T/11TG	1 (1%)	no	no
9T/10TG; 9T/10TG	no	no	5 (33%)
9T/9TG; 9T/9TG	no	no	3 (20%)
7T/12TG; 7T/12TG	2 (2%)	no	no
7T/11TG; 7T/11TG	17 (21%)	10 (17%)	0
7T/10TG; 7T/10TG	8 (10%)	7 (12%)	1 (7%)
5T/11TG; 5T/11TG	no	2 (3%)	no
9T/11TG; 9T/10TG	1 (1%)	1 (2%)	0
9T/10TG; 9T/9TG	no	1 (2%)	1 (7%)
9T/11TG; 7T/12TG	no	no	1 (7%)
9T/10TG; 7T/12TG	2 (2%)	6 (10%)	0
9T/10TG; 7T/11TG	9 (11%)	7 (12%)	1 (7%)
9T/9TG; 7T/11TG	3 (4%)	1 (2%)	1 (7%)
9T/9TG; 7T/10TG	1 (1%)	no	no
7T/12TG; 7T/11TG	8 (10%)	5 (8%)	0
7T/12TG; 7T/10TG	no	3 (5%)	no
7T/11TG; 7T/10TG	27 (33%)	12 (20%)	1 (7%)
5T/12TG; 7T/10TG	1 (1%)	3 (5%)	0
5T/11TG; 7T/10TG	2 (2%)	1 (2%)	0
5T/8TG; 7T/7TG	no	no	1 (7%)
5T/12TG; 5T/11TG	no	1 (2%)	no

* statistically significant differences, comparing poly-T and poly-TG genotype variants in the analysed sample groups were not observed

The rest of T/TG haplotypes, which are represented in all sample groups, occur equally frequently, or are unique for one of the groups (described above, or see Table

9 and 10), and statistically significant differences did not show. Equally, analysing poly-T and poly-TG tract genotypes, statistically significant differences among sample groups used in the study were not observed (Table 10).

Similar methodological approach and interpretation of analysed data was showed in Italian (Tamburino et al., 2008) and German (Gallati et al., 2009) research. German research showed that alleles 5T/12TG and 5T/13TG are associated with disturbances of spermatogenesis in case of nonobstructive azoospermia. In this research haplotypes 5T/12TG and 5T/13TG were found only in DNA samples of infertile males, however our data and Italian research (Tamburino et al., 2008) could not confirm this association.

3.3. Poly-T and poly-TG polymorphism analysis of *CFRT* gene in males with severe form of infertility

Due to the acquired results on correlated similarity of poly-T and poly-TG tract allele and genotype variants in infertile male and control groups (described in chapter 3.2.), it was decided to make a separate analysis in order to find out if there is any relation in *CFTR* gene polymorphisms and certain form of male infertility. To perform this task two sample groups were chosen – samples from azoospermia (12 of 100 infertile males' samples) and oligozoospermia (26 of 100 infertile males' samples) patients. Comparing frequency of polymorph alleles T and TG of *CFTR* gene in both groups it would be possible to assess if in samples from Latvian infertile males exists any relation between *CFTR* gene polymorphism and male infertility. Unfortunately, comparison poly-T and poly-TG polymorphism in analysed sample groups and statistical analysis of data (χ^2 analysis; *Yates'* correlation), did not show statistically significant differences in frequency of poly-T and poly TG variants in case of azoospermia un oligozoospermia.

Discussion

1. Significance of introduction of Y chromosome microdeletions diagnostics method in Latvia

Microdeletions of the Y chromosome are the second most frequent genetic cause of spermatogenetic failure in infertile men after the Klinefelter syndrome (Simoni et al., 2004). Determination of Y chromosome microdeletions is a routine diagnostic method in a number of world and European molecular genetics laboratories. In addition, European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) support the improvement of the quality of the diagnostic assays by publication of the laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions and by offering external quality assessment trials. Since up to now Y chromosome microdeletion analysis in Latvia has not been offered as a method of diagnosis. Therefore one of the main tasks of this study was to approbate and introduce this precise and sensitive method of analysis in Latvia.

Why is it necessary to introduce Y chromosome microdeletion detection method in Latvia? Firstly, Y chromosome microdeletions cannot be determined basing on cytogenetic (chromosome staining and microscopic analysis) methods, clinical anamnesis or sperm analysis. Therefore molecular diagnostic methods, such as polymerase chain reaction (PCR), are needed The other aspect which determines necessity to introduce this method in Latvia is to show in European context that in Latvia as well it is possible to make analysis of Y chromosome microdeletions precisely and qualitatively. Accuracy of diagnosis of Y chromosome microdeletions was checked in 2009 (in the framework of A. Puzuka's promotion work), participating in external laboratory quality control testing, organized by EMQN. In the Laboratory of Molecular Genetics of Riga Stradins University in anonymous samples (sent by EMQN), precisely were determined genotypes – presence or absence of microdeletions in concrete AZF regions, as well as a qualitative patient report form was developed, which precisely revealed recommendations in case of microdeletions. According to assessment, made by EMQN specialists, accuracy of diagnosis and interpretation of Y chromosome microdeletions made in RSU Laboratory of

Molecular Genetics was evaluated with 2 points (maximum number of points for precise determination of genotype) and 1.67 points for interpretation.

What is the significance of determination of Y chromosome microdeletions? Since up to now Y microdeletions in Latvia have not been determined, and introduction of this method enabled us to detect frequency of Y chromosome microdeletions in males with idiopathic infertility, that corresponds to 5%. In addition, the approbated method can be offered as a routine screening method in case of idiopathic male infertility. Thus, for part of patients with Y chromosome microdeletions, detection of microdeletions could establish diagnosis of infertility; evaluate possibility of in vitro fertilisation and assessed potential risks, as well as facilitate genetic counselling of a couple.

2. The suggested algorithm of determination of Y chromosome microdeletions

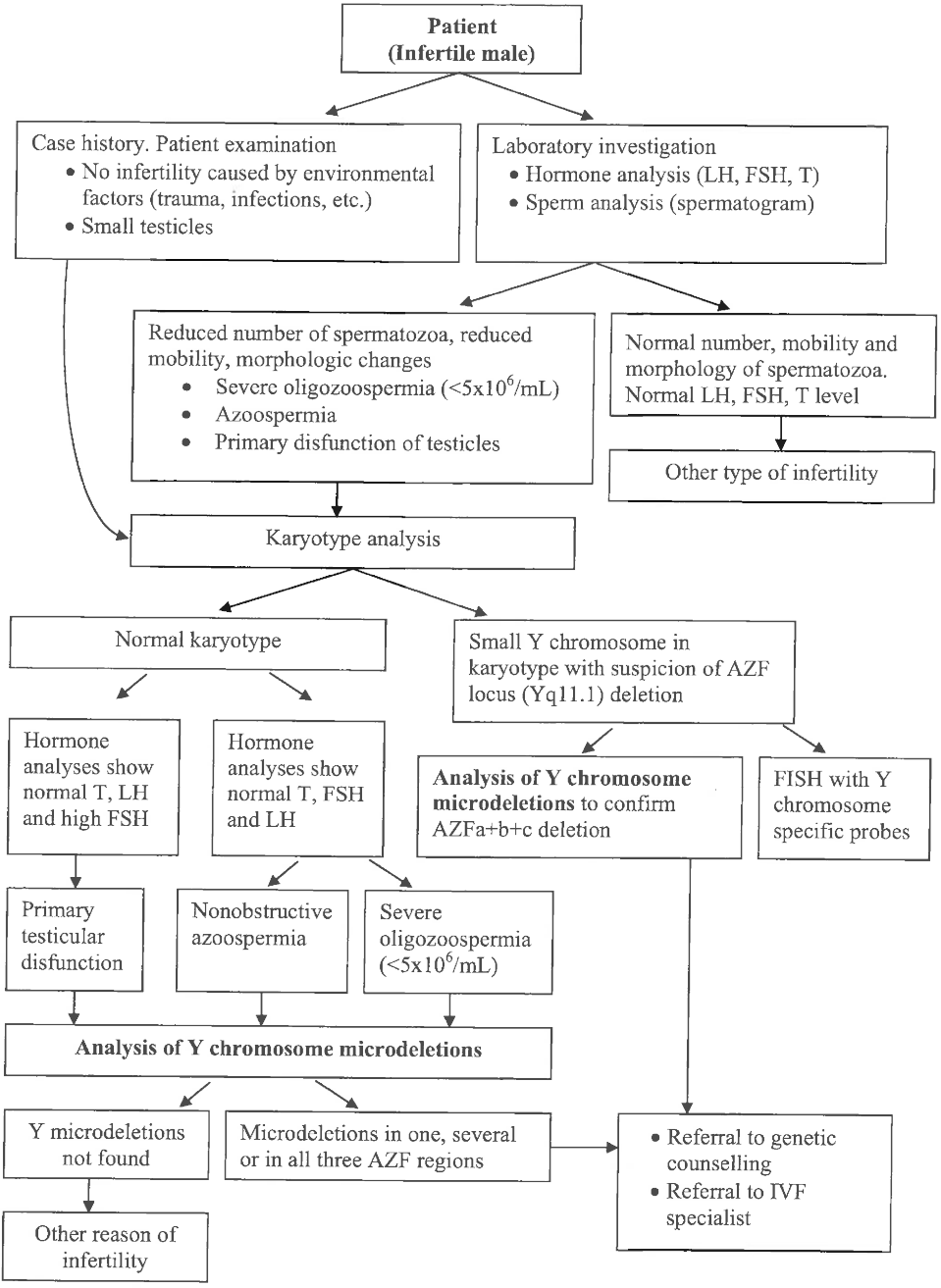
In which cases analysis of Y chromosome microdeletions should be made? According to other authors' data and results obtained in the study, analysis of Y chromosome deletions should be made for infertile males having reduced number of spermatozoa ($<5x10^6$/mL) in case of severe oligozoospermia or nonobstructive azoospermia (absence of spermatozoa in ejaculate), as well as in case of infertility that is not caused by environmental factors, hormonal disturbances or cytogenetic pathology (see algorithm).

There are several reasons to consider testing for Y chromosome microdeletions:

1) To aid in the diagnosis and determining the aetiology of the disorder;
2) To guide decision-making about the advisability of testicular sperm harvest;
3) For genetic counselling.

Abbreviations used in algorithm:
LH – luteinising hormone;
FSH – follicle - stimulating hormone;
T – testosteron;
Azoospermia – no spermatozoids in ejaculate
Oligozoospermia – reduced number of spermatozoids in ejaculate
FISH – fluorescent in situ hybridisation
IVF – in vitro fertilisation

Algorithm for detection of Y chromosome microdeletions



2.1. Analysis of Y chromosome microdeletions to aid in the diagnosis and determining the aetiology of the disorder

Assessing data from literature (Krausz & McElreavey, 1999; Simoni et al., 2004; Bhasin, 2007) and results obtained in this study on presence of microdeletions and number of spermatozoa, one can conclude that microdeletions in Latvian infertile men population occur in cases when spermatogram data suggest severe infertility, i.e., spermatozoa are not present in ejaculate, or microscopically only few spermatozoids can be observed. Therefore, we would suggest to make microdeletion analysis similarly to that suggested by Simoni et al (2004), however, not to miss any of microdeletions and consequently make wrong diagnosis, the criteria could be analysis of all corresponding infertility cases if number of spermatozoids is $<5x10^6$/mL. Microdeletion analysis in males with less severe infertility forms should not be recommended, as frequency of Y chromosome microdeletions in this group is very low. To clarify whether microdeletions have any more correlation apart from above mentioned reduction in the number of spermatozoa, different investigations have been conducted and possible relation to other disturbances of the reproductive system, for example, hormonal changes (Oates et al., 2002), anatomical defects (Reyes-Vallejo et al., 2006) searched for. In theses studies positive correlation was not found. It indicates that in males with identified Y chromosome microdeletions, clinically they manifest as infertility only. Thus, it confirms again that in males with known reason of infertility, for example, cryptorchidism or orchitis caused by epidemic parotitis, analysis of Y chromosome deletions is not necessary. The same refers to patients with obstructive azoospermia, since in these cases; other reasons should be looked for, such as anatomical defects or other genetically determined reasons, for example, mutations in *CFTR* gene.

2.2. Analysis of Y hromosome microdeletions in azoospermia patients before testicle biopsy

In case of idiopathic azoospermia (nonobstructive) molecular analysis of Y chromosome microdeletions can be used as an alternative – patient-friendly method to find out presence or absence of mature spermatozoa in testicles before performance of testis biopsy. The molecular analysis for complete AZF microdeletions in the Y chromosome of idiopathic azoospermic men is an attractive prognostic tool for the

finding of mature spermatozoa in the patient's testis tissue without the need to extract an RNA sample from his testicular tissue biopsy and expression analysis of a post-meiotic marker gene. Existence of this expression suggests presence of mature spermatozoa in testicles. If an infertile male chooses procedure of intracytoplasmic sperm injection (ICSI), testicle biopsy or testicular sperm extraction (TESE) is needed. Firstly, testicle biopsy is inconvenient procedure for the patient, secondly, it may be useless, as mature spermatozoa will not be found by express analysis, and, finally, the patient will have to undergo testicle biopsy repeatedly to obtain mature spermatozoa for further in vitro fertilisation manipulations. Thus, applying Y chromosome microdeletions analysis and finding out AZF deletion variant before testicle biopsy, the patient can avoid unnecessary/useless procedure as well as expensive and time consuming expression analyses. Testicle biopsy to extract spermatozoa and perform ISCI is not advised for patient group with AZFa and AZFb microdeletions, since usually these procedures are unsuccessful and do not achieve the desired result (Vogt, 2005). Microdeletions, which affect more than one AZF region (AZFa+c un AZFa+b+c), are also related with situation when testicle biopsy does not allow obtaining spermatozoa (Brandel et al., 1998). However, if a partial AZFb, AZFc or complete AZFc deletion is diagnosed in the patient's Y-DNA, testicular mature spermatozoa are often found (~50% of cases) at least in single testis tubules because residual local complete spermatogenesis is typical for these patient groups, eventually also resulting in the presence of some mature spermatozoa in the patient's ejaculate (i.e. cryptozoospermia; sperm concentration <1million/ml) (Vogt, 2005).

2.3. Analysis of Y chromosome microdeletions and genetic counselling of a couple before in vitro fertilisation

Analysis of Y chromosome microdeletions in infertile males who conform to above described criteria is significant not only for making diagnosis, but also for genetic counselling before *in vitro* fertilisation procedure. Y chromosome changes, detected in patient's Y chromosome (most commonly microdeletions in AFZc region) will be 100% transmitted to the male offspring if assisted reproduction is performed. It is known that male descendants, having fathers with Y chromosome AZFc microdeletion, will have the same Y chromosome genotype (Silber et al., 1998; Mau-

Kai et al., 2008; Poongathai et al., 2009). However there are reports on cases when deletions of AZFc region, were becoming larger in male descendants Y-chromosomes comparing to the size of fathers AZF microdeletion. Explanation of that is, that AZF regions are affected by *de novo* mutations, based on homologue re-combination (Kuroda-Kawaguchi et al., 2001; Chan et al., 2002). To summarize, during the genetic counselling a couple should be informed that using spermatozoa from infertile male (father), positive by Y chromosome microdeletions, using ICSI procedure all male descendants will inherit this genetic defect and also will be infertile. However, positive is the fact that Y chromosome microdeletions are not related with other health problems. All female offspring will be healthy and fertile as will not inherit Y chromosome from the father.

3. Comparison of Y chromosome microdeletion detection methods

According to definition microdeletions are deletions whose size is less than light microscope resolution (Shaffer, 1997). Analysing human karyotype using cytogenetic analysis it is possible to resolve 400 – 500 regions on metaphase chromosomes. At this level deletions of 5 – 10 Mb size can be found. Deletions less than 5 – 10Mb can be detected applying molecular methods – PCR (polymerase chain reaction), FISH (fluorescent *in situ* hybridisation), PRINS (Primed *IN Situ* labeling) and others.

Several PCR based molecular methods exist, which help to determine Y chromosome microdeletions. These methods are based on the same principle, and use multiplex PCR format. Goole of the method is to determine presence or absence of DNA sequence (markers, STS - sequence target sites), localised on Y chromosome AZF regions. The methods differ in ability to apply the corresponding technique simply, fast and without errors (Table 11). In our study two methods were used for comparison: commercially available kit for determination of Y chromosome microdeletions (Promega 2.0) and, recommended by EMQN (the European Molecular Genetics Quality Network), multiplex PCR for analysis of Y chromosome microdeletions (Simoni et al., 2004). The commercial kit includes 21 STS markers of which two STS are for reaction control but the rest 19 are located in certain AZF regions. EMQN recommendations for microdeletion determination suggest to apply eight markers, two of which are used for control of the method, bet the rest six are

located in AZF regions (two in each region). The commercial set may seem to be better, as several markers are used and therefore analysis might be more specific, nevertheless it is not true, since the authors of EMQN recommendations have managed to prove that the six markers chosen for the analysis are unique for AZF regions, and with them complete deletions of AZFa, AZFb and AZFc regions can be determined unmistakably. The most significant point in comparison of these two methods is to prove which of the methods is more suitable for fast and qualitative detection of Y chromosome microdeletions in laboratory (Table 11).

Table 11. **Comparison of commercially available and EMQN recommended methods for determination of Y chromosome microdeletions**

Parameters of comparison	**Commercially available method of determination of microdeletions**	**EMQN recommended method of determination of microdeletions**
Performance	Range of complicated procedures for preparation and analysis of a sample.	Easy and simple scheme of preparation and analysis of a sample.
Time	Time consuming.	Fast.
Number of multiplex PCR	Each sample should be analysed with five multiplexes; risk of mixing reagents and samples is high.	Each sample should be analysed with two multiplexes only; risk of mixing reagents and samples is little.
STS markers	19 STS are used; great number of markers does not give better results, as a lot of markers are not informative.	Six STS are used, all markers are informative.
Marker of internal control	*SMCX/SMCY*, which is not proper for internal PCR control.	*ZFX/ZFY*, which is more suitable for internal PCR control.
Interpretation of results	Complicated interpretation of gel picture of the analysed samples.	Easily understandable interpretation of gel picture of the samples.
Costs	Expensive (~88 EUR a sample, not including charge for making of analysis).	~ 10 x cheaper (~6 EUR a sample, not including charge for making of analysis).

Other inaccuracy of commercial Promega technique is that analysis includes the so-called fourth AZF region – AZFd region. This region was firstly described in 1999 and it is located between AZFb and AZFc regions. It was thought that deletions of AZFd region are asociated with severe teratozoospermia (Kent-First et al., 1999). However, clarifying sequence of AZF region (Skaletsky et al., 2003), presently accepted division of AZF regions is AZFa, AZFb and AZFc. Furthermore, AZFb and AZFc regions overlap, and there is no need to separate this overlapped region as the fourth region, since there does not exist a clinically different (for example, teratozoospermia) patient group (Simoni et al., 2004). Thus the commercial kit still

analyzes genetically unaccepted AZF region and uses markers, which are localised in this region, bet are not informative for infertile males (Kuruda-Kawaguschi et al., 2001; Skaletsky et al., 2003). On the contrary, EMQN recommends for a cheaper, more convenient, faster, adjusted to structural specifics of Y chromosome AZF regions and patient's clinical characteristics standardised method of Y chromosome microdeletion analysis.

There exist also other Y hromosome microdeletion determination methods, such as molecular cytogenetics method PRINS (Primed *IN Situ* labelling) (Kadandele et al., 2002), DNA chip technology (Lee et al., 2004), or genotyping (Yeom et al., 2008). These protocols are interesting, mainly based on EMQN scheme, but are time consuming, complicated to perform or require the latest technology – acquisition of expensive analyzers for analysis in routine diagnosis.

To summarize the work done in this study, as well as comparison of both above described methods, one can conclude that in molecular genetics laboratory for routine diagnosis of Y chromosome microdeletions more suitable method, which reveals precise results, is the analysis suggested by EMQN. Thus, in Latvia, a simple PCR protocol for determination of Y chromosome microdeletions has been approbated and introduced.

3.1.Detection of complete AZFa+b+c deletions – cytogenetic methods versus molecular PCR based methods

Sometimes it is important to define the size of deletion/microdeletion in order to find changes in genetic material on Y chromosome more accurately. In this study, in two of five patients (Figure 1, samples 4 and 5), had deletions of all the three AZF regions (AZFa+b+c deletions). Cytogenetic analyses showed small Y chromosome, which could be explained by almost all long-arm deletion of Y chromosome or it might be isochromosome of short arm of Y chromosome. Both aberration variants of Y chromosome frequently occur in azoospermia patients, as they usually develop after a break in q11 region of Y chromosome long arm, thus losing all for spermatogenesis responsible genes localised in AZF regions (Vogt et al., 2005).

To specify boundaries of Y chromosome microdeletions, EMQN recommendations suggest touse additional STS, to find out breakage points of deletion. In this study, an alternative approach was applied - AZFa+b+c deletion

samples were analysed with Y chromosome microsatellite analysis kit, which is used for determination of Y chromosome haplotype. Microsatellites applied in this method are informative; their presence may suggest existence of certain Y chromosome fragments in samples of AZFa+b+c deletions. Unfortunately, analysis of these microsatellites did not give the expected results – presence of microsatellites localised in the long arm, and with this we did not manage to prove that Y chromosome in these patients is not isochromosome. In this case also neither multiplex PCR nor Y microsatellite analysis is able to detect this genetic change. In this case the first choice is molecular cytogenetic methods (FISH, fluorescent *in situ* hybridization), where with help of specific fluorescent marks (probes) it is possible to determine whether Y chromosome in these infertile males has two short arms (isochromosome).

Regardless the fact that multiplex PCR and Y chromosome microsatellite analyses in case of AZFa+b+c deletions (Figure 1, samples 4 and 5) in our study did not exclude possibility of isochromosome, the acquired data enable us to make conclusions on AZFa+b+c deletions. Definitely, in both AZFa+b+c deletions patients' Y-chromosomes have short arm, which is suggested by presence of STS corresponding to *SRY* and *ZFY* genes (results of multiplex PCR), as well as presence of microsatellite DYS393 and, in sample 4, also microsatellite DYS19. However, both methods have a disadvantage – it is not possible to know number of short arms in corresponding Y chromosome precisely. Though, one could use the fact that in both samples microsatellite DYS393, and in one sample microsatellite DYS19 occur in their classic form: DYS393 microsatellite recurs 14 times (tetranucleotide $[AGAT]_{14}$) and DYS19 microsatellite recurs 15 times (tetranucleotide $[TAGA]_{15}$). If number of recurrence of these microsatellites were doubled, we could definitely claim that analysed Y-chromosomes had two short arms (isochromosomes of short arm). Whereas, in AZFa+b+c deletion samples, we did not found on Y chromosome long arms any of the markers (all of the methods showed occurrence of deletions). Markers that could be able to prove presence of long arm of Y chromosome have to be localised on Y chromosome long arm closely to centromere. Those markers could be DYS391 microsatellite (Y microsatellite analysis kit) as well as DYS271 microsatellite (Promega 2.0 microdeletions determination kit). In our study, both microsatellites were not observed on Y-chromosomes of both patients (no. 5 and no. 4); however, presence of these microsatellites in other cases could prove presence of Y chromosome long arm. Thus, we can put forward one more hypothesis: since

microsatellites DYS391 or DYS271 are located on long arm of Y chromosome closely to centromere, they can possibly indicate presence of Y chromosome long arm in cases where deletion of Y chromosome long arm would have occurred. So, to clarify changes of genetic material of Y chromosome in both patients, one of molecular cytology analyses should be made. FISH analysis with specific *SRY* fluorescent probes could solve the situation, however, the patients did not agree to it. However, FISH would only prove the type of Y chromosome aberration; it would not be therapeutically significant, as all the three AZF region deletions were already determined with multiplex PCR method, thus finding out cause of azoospermia.

4. Y chromosome haplogroups and their relation with male infertility

The worldwide distribution of Y lineages is thought to be a consequence of random evolutionary forces, such as genetic drift, population expansion, and migrations. The influence of natural selection in these processes is unknown, but usually regarded to be of little significance. However, one cannot dismiss natural selection so easily, because the Y chromosome carries important genes involved in spermatogenesis, which can be targets for adaptive processes (Carvalho et al., 2003). If any of the genes is functionally significant, this gene is expected to be evolutionary conservative and occur in individuals of related species. Thus, frequency of reproductively successful genes or genetic variations should increase in the course of evolution of human population. Taking into consideration this fact, opposite process should affect frequency of genes or genetic variants that decrease fertility – it should decrease among the population. Above mentioned hypothesis directly refers to Y chromosome. Thus, unfavourable mutation (e.g., decreasing fertility) may be preserved on Y chromosome (haplogroup), favourable by other factors (e.g., suitable for certain environmental conditions), and its frequency may even grow or remain constant in the population. On the contrary, favourable mutation in unfavourable Y chromosome variant may be lost (negative selection). Frequency of useful Y chromosome variants would grow if positive selection of these variants occurred. The most extreme variant would be, for example, the variant which results in complete loss of spermatogenesis, and which will not be inherited by next generation. Its frequency among population will be very low – it will occur in genotypes of infertile persons only (Tyler-Smith, 2008). Several studies have indicated to relation of Y

chromosome variant or haplogroups with reduced number of spermatozoa. (Krausz et al., 2001a; Arredi et al., 2007; Yang et al., 2008). These studies demonstrate selective processes, in which Y chromosome genes are involved.

Latvian genofund of Y chromosome is comprised by four main Y chromosome haplogroups – N3a1 (K* cluster N sub-cluster N3 haplogroups sub-haplogroup), R1a1 (K* cluster R sub-cluster R1 haplogroups sub-haplogroup), K* (K* cluster without Hg N3a un Hg R1a) and I (F* cluster IJ sub-cluster haplogroup). They are represented in both infertile males and control (Latvians, three generations) groups. They are also the dominating haplogroups in northeast Europe, which, in its turn, indicates to common origin and pre-history of above mentioned haplogroups.

The prevailing haplogroup in Latvia in both infertile males (30,4%) and control (42,5%) population is N3a1 haplogroup, which does not show statistically significant differences (p=0,223) in both analysed groups, therefore Hg N3a1 does not have relation with male infertility. It could suggest that Hg N3a1 is reproductively successful, and cause of male infertility is other than genes localised in this Y chromosome variant. However, studying Y chromosome variant characteristic to Hg N3 (haplogroup to which sub-haplogroup N3a1 belongs) more carefully, it has been found that this haplogroup involves all Y-chromosomes with deletion of two *DAZ* genes – *DAZ3* and *DAZ4*, (Fernandes et al., 2004; Machev et al., 2004, Yang et al., 2008). It is explained by partial deletions of AZFc region, which may be inherited and not always manifest as infertility. Complete AZFc deletion removes nine genes including all members of the three families *BPY2*, *DAZ* and *CDY1*, almost always resulting in spermatogenic failure. In contrast, the partial AZFc deletion removes five genes leaving one *BPY2*, two *DAZ* and one *CDY1* (Fernandes et al., 2004; Repping et al., 2004; Fig. 2), but is found in haplogroup N3 men with normal spermatogenesis who make up half the population of northern Europe (Zerjal et al., 1997, 2001). In this relatively simple genetic background, a survey of haplogroup N men with spermatogenic failure might reveal point mutations in the remaining single-copy genes (Tyler-Smith, 2008).

Nevertheless, a well-known fact is that Y chromosome microdeletions have a tendency to arise in the way of homologue recombination, thus, harmless partially deleted variants of AZFc Y chromosome (*DAZ3/DAZ4* deleted N3 haplogroup variants) may be changed. De novo deletions in partially deleted AZFc region could lead to loss of more genes and complete deletion of AZFc region may appear. Thus,

such partially deleted AZFc variants are inherited in future generations in changed form and result in infertility in male descendants. With this, part from *DAZ3/DAZ4* deleted N3 haplogroup variants could be lost due to gene drift. As it is known, *DAZ3/DAZ4* deletion is characteristic to N3 haplogroup, and knowing Hg N3 incidence in the world, it is possible to clarify incidence of this variant as well. As mentioned before, N3 haplogroup is widespread in northern Europe and Asia, where it comprises 12% of all Y haplogroups. In addition, in some populations it is one of the most common haplogroups, e.g., in Finnish population its frequency is 52%, Yakut 86% (Zerjal et al., 1997), and Latvian 42.5%. The possible common ancestor of N3 haplogroup existed 8800 – 3200 years ago (Hammer un Zegura, 2002). Therefore N3 haplogroup is an ancient and successful Y hromosome lineage, and *DAZ3/DAZ4* deletions do not affect harmfully fertility of the carriers of this Y chromosome variant. In addition, there is an opinion that structural changes of Y chromosome, characteristic to N3 haplogroup variants, as our study reveals (Hg N3a1), are able to prevent infertility, therefore this haplogroup is reproductively successful. It is also suggested by other studies which have observed the so called compensating mechanism of partial deletions of AZFc region, i.e., after deletion of one gene in certain gene family duplication of other gene of this gene family could occurred (Repping et al., 2003; Lin et al., 2007; Giachini et al., 2008; Navarro-Costa et al., 2010).

The other most common Y chromosome haplogroup in both analysed sample groups is Hg R1a1. Furthermore, Hg R1al less occurs (p=0,005) in DNA samples of infertile males (15.2%) comparing with the control group (39.2%). R1a1 haplogroup can be considered as one of the reproductively most successful lineages of Y chromosome, as it is more characteristic to fertile than infertile males (p=0.005). As results of our study suggest, similarly to N3a1 haplogroup, R1a1 haplogroup is preserved due to positive selection. Hg R and Hg N are not closely related, and their common ancestor, possibly, existed 36000 – 6000 ago (Hammer & Zegura., 2002). Different facts have been reported on R haplogroup (haplogroup to which sub-haplogroup R1a1 belongs). Scientists have showed that on Y-chromosomes, belonging to this haplogroup, deletions of genes are observed (partial AZFc region deletions), but loss of these genes does not affect fertility (Fernandes et al., 2004; Vogt, 2005). Its possible molecular explanation is the occurred inversion in pre-N Y chromosome (Vogt, 2005), which has resulted in R haplogroup, that, in its turn,

prevent this haplogroup from deletions. Since the inverted sequence of AZFb and AZFc region does not allow deletions of AZF regions to occur so this Y chromosome variant manifests more in control group comparing to the studied infertile male Y-chromosomes. In other scientists' works as well are mentioned data similar to those obtained in Latvia, i.e., that R haplogroup more commonly occurs in fertile males than in infertile males. For example, Gomes et al (2008) also claims that Y-haplogroup R was higher in fertile than in infertile men seemingly to be favorable to fertility.

Conversely, haplogroup K* (macrohaplogroups M9 G allelic variant, without Hg N3a and Hg R1a) occurs in 25.3% of infertile males Y-chromosomes, though in control group frequency of this haplogroup is much less (6.5%). Therefore, K* haplogroup could be more characteristic to Y chromosome lineages of infertile males ($p<0.001$) and K* haplogroup could be called "infertility haplogroup". Haplogrop K* was found on Y chromosomes in representatives of Danish population as well (4.6%), and its frequency, similarly to our study, was much higher among infertile Danish males (28%) compared to control group (Krausz et al., 2001a). In Europe, K* cluster haplogroups (Hg L, Hg N, Hg O, Hg P, Hg R) occur frequently. Particularly frequent they are in northern Europe, for example, in Saami population its frequency is 46%, in Finnish population 63%, in Baltic countries > 70% (Latvia 80%). High incidence of the cluster in northern and eastern Baltic regions contrasts with the rest part of Europe. In northern Europe, among the populations speaking Germanic languages, K cluster occurs more rarely: in population of Germany 6%, northern Sweden– 8%, Gothland – 8%, Denmark– 4.6%, Norway – 4%. Incidence of the cluster in Finno-Ugric, Baltic, and Germanic speaking languages suggests that the found association could be primarily geographic. According to Krausz et al (2001) high incidence of K* haplogroup among Danish infertile males (28%) can be explained in two ways. Firstly, this Y chromosome lineage may really be connected with reduced number of spermatozoa, secondly, differences in the analysed sample groups are possibly related to the local geographical specifics of incidence of K* Y chromosome lineage. Effect of geographical factors in case of this association should be considered and its exclusion proved. Literature reveals cases of erroneous interpretation of relation of Y haplogroups with male infertility, although the real cause of Y chromosome distribution in those analysed populations was geographical aspect (Kuroki et al., 1999; Previdere et al., 1999). Since in Latvian study samples of infertile males represent all population of Latvia, and samples of control group used for comparison

also represent all population of Latvia, and differences in incidence of K* haplogroup in regions of Latvia do not exist, the acquired results support association of Y haplogroup K* with male infertility. Relation of K* haplogroup with male infertility has been found by other scientists as well. For example, in study of China population, it was discovered that K* haplogroup is more common among infertile males (2.8%) in comparison to fertile males (0.78%), and making statistical comparison, significant and statistically significant differences were determined (0.028) (Lu et al., 2007). Furthermore, Danish study proved, that majority of infertile males, belonging to K* haplogroup, had reduced number of spermatozoa (azoospermia, oligozoospermia). From this one can conclude that K* lineages have low reproductive success. If *in vitro* fertilisation were not applied, such Y-chromosomes would disappear rather fast from the population due to negative selection. Krausz et al (2001) expresses hypothesis on Danish population: if Hg K frequency among fertile males is 5%, and assuming that average selective adjustment of this Y chromosome lineage is 0.5, it is possible that K* lineage would disappear from Danish population within 12 generations. Similarly could be considered about Hg K* in Latvia (6.5%), and negative selection to this haplogroup. Nevertheless, reproductive success of K* haplogroup is difficult to determine with simple calculations, as it is restricted by several factors, such as lack of migration of Hg K* Y chromosome variant from surrounding populations, and not all K* sub-haplogroups are equally sensitive to this effect. As it was mentioned above, K* includes several sub-haplogroups, of which N3 and R1 are with good reproductive success. However, as it was proved in our study, these are other K* cluster sub-haplogroups of Y chromosome which are characteristic to patients with spermatogenesis disturbances, which make some K* sub-haplogroups predisposed to the male infertility.

In addition, in our study statistically significant differences ($p<0,001$) were observed on Y chromosome variants containing M9 C allele, comparing DNA samples of control group and infertile males. Variants of M9 C allele are characteristic to two Y chromosome clusters – DE* and F* (Hg G, Hg H, Hg I, Hg J). The exception is F* clusters Hg I, that occurs in infertile patients, but does not show statistically significant differences comparing with control group. DNA sample group of M9 C allele infertile males is very heterogeneous, and additional studies are needed. Though basing on data from literature, one can conclude that DE* and F* clusters as well have certain relation with male fertility.

To assess which of DE* and F* Y chromosome haplopgroups predispose to infertility or prevent infertility, a number of studies have been conducted in populations of northern Italy and China. These observations prove that DE* clusters E haplogroup is related with infertility. Molecular analysis of structure of Hg E Y chromosome suggests that this Y chromosome lineage is more affected by b2/b4 (full AZFc region) microdeletions than other haplogroups (Arredi et al., 2007). It might be assumed that selection to haplogroup should occur, nevertheless this negative selection is not observed. E haplogroup is widespread in East- and North-Africa, Middle East and Europe (Cruciani et al., 2004). Frequency of microdeletions of Y chromosome AZFc region, causing spermatogenesis disturbances, in deleted Y chromosomes is less than 0.03%, which approximately corresponds to development frequency of *de novo* deletions, however, it is not sufficiently high for domination of selection over gene drift (Repping et al., 2003). Furthermore, assessment of selection effect is hindered by existence of different infertility variants, all of which are related with AZFc deletions (from oligozoospermia to azoospermia and in case of partial AZFc deletions even up to normospermia).

Opposite to the fact that frequency of E haplogroup is increased in case of Y chromosome microdeletions, J haplogroup is less common in north Italian infertile male group (3%, Arredi et al., 2007), whereas in control population frequency of J haplogrop is 15%. Though the mentioned difference is not statistically confident, molecular structure of Hg J variant shows that Y-chromosomes, belonging to Hg J, prevent Y microdeletions. Hg J lineages do not contain L1PA4. L1PA4 is LINE element (*long interspersed nuclear element*), inserted on Y chromosome HERV (human endogenous retroviral DNA sequences), which, in its turn, is situated in AZFa locus. As in the course of evolution Hg J Y chromosome variant arose due to L1PA4 deletion (proved by deletion of biallelic Y chromosome marker 12f2), intrachromosomal homologue re-combinations, which would result in lost AZFa region, in these lineages cannot occur (Jobling et al., 1998; Kamp et al., 2000).

In China study (Yang et al., 2008) it was found out that haplogroups C (C* cluster) and DE* are more affected by partial deletions of AZFc region, whereas O3* (haplogroup, that belongs to K* cluster NO* sub-cluster) prevents the deletions. Molecular studies of Y-chromosomes, represented by these haplogroups, suggest presence of certain partial AZFc region variants in these Y lineages. For example, Hg DE* might be more susceptible to *DAZ1/DAZ2/CDY1a* gene deletion in AZFc region,

whereas O3* haplogroup prevents of this deletion. But Hg C lineages are more affected by *DAZ1/DAZ2/CDY1b* deletions (Yang et al., 2008). Since Y haplogroup composition is the main factor dictating the population-dependent response, a thorough analysis of their distribution in our increasingly globalized society may offer an ever more gentrified outlook on the impact of partial AZFc deletions for male fertility (Novaro-Costa et al., 2010). Therefore it can be concluded that most likely certain structural changes of Y chromosome exist, which represent certain Y haplogroups and are related to male infertility.

Conclusions

1. To determine molecular genetic causes of male reproductive pathology, detection of Y chromosome microdeletions, Y chromosome haplogroup analysis and study of CFTR gene mutations and polymorphisms were performed.
2. In Latvia, frequency of Y chromosome microdeletions of AZF regions in males with idiopathic infertility is 5% and AZF microdeletions are observed in case of severe forms of infertility – azoospermia or oligozoospermia.
3. An algorithm has been developed, which can help doctors, having patients with infertility problem, select those infertility cases, when detection of Y chromosome microdeletions is purposeful.
4. Method of molecular analysis of spermatogenesis disturbances has been approbated and introduced, which will enable in Latvia to diagnose microdeletions on Y chromosome AZF regions.
5. Statistically significant differences have been found in incidence of Y chromosome haplogroups of infertile males and control group, which suggests that some Y chromosome lineages may be at increased risk of developing infertility.
6. The analysed *CFTR* gene mutation delF508, mutation R117H, as well as 8 intron poly-T and poly-TG polymorphisms do not show relation with male infertility, which indicates that these mutations and polymorphisms do not affect process of spermatogenesis directly.
7. Suggestions for genetic counselling of patients with infertility have been developed.

Publications and reports on topic of the study

Publications:

1. L.Timsa, A. Puzuka, L. Pliss, A. Krumina et al. *Human Y chromosome and its role in human pathology and population phylogenetic studies.* – Proceedings of the Latvian Academy of Sciences, Section B, 2005, vol.59, p. 93-108.
2. A.Puzuka, A.Krumina, N.Pronina, V.Lejins, J.Erenpreiss, J.Bars, I.Grinfelde, V.Baumanis. *Analysis of Y-chromosomal microdeletions and polymorphism of CFTR gene in infertile males – a study from Latvian population.* – Proceedings of the Ukrainian Academy of Sciences; Genetics, Selection and Biotechnology, 2007, p.575-578.
3. A.Puzuka, A.Krumina N.Pronina, V.Lejins, J.Erenpreiss, J.Bars, I.Grinfelde, V.Baumanis. *Molecular analysis of male infertility in Latvia.* - RSU Collection of Scientific Papers, Research Articles in Medicine and Pharmacy, 2007, p. 90-93.
4. A.Puzuka, I.Prane, J.Erenpreiss, A.Krumina. *CFTR gene and male infertility.* - RSU Collection of Scientific Papers, Research Articles in Medicine and Pharmacy, 2008, p. 97-100.
5. A.Puzuka, N.Pronina, I.Grinfelde, J.Erenpreiss, V.Lejins, J.Bars, L.Pliss, I.Pelnena, V.Baumanis, A.Krumina. *Y chromosome – a tool in infertility studies of Latvian population. - in press* Russian Journal of Genetics, 2011, p. 1-7.

Conference theses:

1. A.Krumina, L. Pliss, A. Puzuka et al. *Latvian population and its Indo-European roots by analyses of mitochondrial DNA (mtDNA) and Y chromosme data.* – 29th FEBS journal, 2005, Abstracts, p.131.
2. L.Pliss, L.Timsa, A.Puzuka, A.Krumina, V.Baumanis, K.Tambets, R.Villems. *Phylogeography of mitochondrial and Y – chromosomal lineages and genetic structure of Latvians.* – 20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, Kyoto, Japan, 2006, Abstracts, p.198.
3. L.Pliss, A.Puzuka, S.Rozane, A.Sabule, V.Baumanis, A.Krumina. *Y-chromosomal short tandems repeats (Y-STRs) and their applications for population studies and forensic casework in Latvia.* –The 5th ISABS conference in forensic genetics and molecular antropology, Croatia 2007, Abstracts, p.127.
4. L.Pliss, A.Puzuka, L.Timsa, I.Pelnena, V.Baumanis, A.Krumina. *Genetic polymorphism of Y chromosome short tandems repeats (Y-STRs) in the Latvian population.* – European Journal of Human Genetics, Abstracts, 2007, vol.15, suppl.1, p.292.
5. A.Puzuka, A.Krūmiņa, N.Proņina, V.Lejiņš, J.Ērenpreiss, J.Bārs, I.Grīnfelde. *Analysis of Y – chromosomal microdeletions in infertile males.* – RSU Scientific conference, Riga, 2007, Abstracts, 251.lpp.
6. A.Puzuka, A.Krumina, N.Pronina, V.Lejins, J.Erenpreiss, J.Bars, I.Grinfelde, V.Baumanis. *Microdeletions in AZF region of Y chromosome in infertile males, a study from Latvian population.* - European Journal of Human Genetics, Nice, France, 2007, Abstracts p.208.-209.

7. L.Pliss, I.Pelnena, A.Puzuka, V.Baumanis, A.Krumina. *Genetic analysis of 12 Y-chromosomal STRs haplotypes in the Latvian population.* - The FEBS Journal, 2008, Abstracts, vol.275, suppl.1, p.424.
8. A.Puzuka, A.Krūmiņa, I.Prane, V.Baumanis. *Vīriešu neauglības ģenētiskie riska faktori – Y hromosomas mikrodelēcijas un mutācijas CFTR gēnā.* –RSU Zinātniskā konference, 2008, Tēzes, 239.lpp.
9. A.Puzuka, A.Krumina, I.Prane, I.Grinfelde, N.Pronina, V.Lejins, J.Erenpreiss, V.Baumanis. *Incidence of Y chromosomal microdeletions and CFTR gene mutations in infertile males in Latvia.* –The FEBS Journal, Athens, Greece, 2008, Abstracts, vol.275, suppl.1, p.100.
10. L.Pliss, I.Pelnena, A.Puzuka, A.Sabule, S.Rozane, A.Ivanovs, V.Baumanis, A.Krumina. *Y chromosomal short tandem repeats (Y-STRs) and its association with azoospermia factors' (AZF) microdeletions in infertile males.* – The American Society of Human Genetics, 58th Annual Meeting, Philadelphia, USA, 2009, Abstracts, p.166.
11. A.Puzuka, L.Pliss, N.Proņina, J.Ērenpreiss, V.Baumanis, A.Krūmiņa. *Vīriešu neauglības ģenētisko faktoru izvērtējums pēc Y hromosomas mikrodelēciju un haplogrupu datiem.* - RSU Zinātniskā konference, 2010, Tēzes, 97.lpp.
12. A.Puzuka, A.Krumina, N.Pronina, V.Lejins, J.Erenpreiss, J.Bars, I.Grinfelde, V.Baumanis. *Y-chromosome microdeletion and haplogroup patterns in Latvian population.* - 4th Utah/Florence Symposium on the Genetics of Male Infertility, Utah, USA, 2010, Abstracts, p.55.

Oral reports in conferences and meetings of Latvian Council of Science (LSC) cooperation projects No.05.0023 and No.10.0010:

1. Ziņojums "Latvijas populāciju raksturojoši Y hromosomas haplotipi" LZP sadarbības projekta Nr.05.0023 "Latvijas populācijas genofonda pētījumi un to izmantošana cilvēka patoloģijas diagnostikā un profilaksē" sēdē, 2004. gada 27. februārī.
2. Ziņojums "Populāciju ģenētika un tās praktiskā nozīme" Latvijas Universitātes 64. konferencē, 2006. gada 9. februārī.
3. Ziņojums "Vīriešu neauglības molekulāri ģenētiska izpēte" LZP sadarbības projekta Nr.05.0023 "Latvijas populācijas genofonda pētījumi un to izmantošana cilvēka patoloģijas diagnostikā un profilaksē" sēdē, 2007. gada 24. aprīlī.
4. Ziņojums "Vīriešu neauglības ģenētika" Latvijas Universitātes 66. konferencē, 2008. gada 13. februārī.
5. Ziņojums "Vīriešu neauglības ģenētisko faktoru izvērtējums pēc Y hromosomas mikrodelēciju un haplogrupu datiem" Rīgas Stradiņa universitātes zinātniskā konferencē, 2010. gada 19. martā.
6. Ziņojums - promocijas darba prezentācija "Vīriešu reproduktīvās patoloģijas ģenētiskā riska faktoru izvērtējums Latvijas populācijā" LZP sadarbības projekta Nr.10.0010 "Slimību etioloģijas, patoģenēzes un cilvēka novecošanās procesu ģenētiskā izpēte Latvijas populācijā" sēdē, 2010. gada 18. maijā.
7. Ziņojums „Evaluation of male infertility genetic aspects according Y chromosome microdeletion and haplogroup data" Ziemeļvalstu un baltijas valstu andrologu (NAFA-BAA) zinātniskā konference, Tartu, Igaunija, 2010. gada 11. septembrī.

The site and financing of research

The study has been worked out in Molecular Genetics Scientific Laboratory of Riga Stradins University and Latvian Biomedical Research and Study Centre in the framework of Latvian Council of Science (LSC) grant No. 04.1198 „ Analysis of Y chromosome applied to the detection of molecular pathologies and population characteristics", LSC cooperation project No. 05.0023 „Molecularly genetic characteristics of Latvian population" and LSC cooperation project No. 10.0010 "Genetic study of etiology of diseases, pathogenesis and human aging process in the population of Latvia". The work was carried out with support from European Social Fund (ESF) project "Support for doctoral and postdoctoral research in medical sciences and acquisition of scientific degree in Riga Stradins University" (agreement No. 2009/0147/1DP/1.1.2.1.2/09/ IPIA/VIAA/009).

Acknowledgements

I wish to thank all of those who have supported and contributed to my research work in different ways. The author wants to express special thanks to:

Professor Astrīda Krūmiņa for the supervision of research, inspiration, consulting, guidance, support and patience in the work process.

Professor Viesturs Baumanis for the supervision of research, consulting, support, constructive criticism and valuable discussions.

My colleagues at the Latvian Biomedical Research and Study Centre and Riga Stradiņš university for encouragement and support.

Doctors Juris Ērenpreiss, Voldemārs Lejiņš, Ieva Grīnfelde, Jānis Bārs, and head of Medical Genetics Clinic DNA laboratory Natālija Proņina for blood samples of patients and prosperous collaboration.

Professor Uldis Teibe for support in statistical assessment of research results.

My dear family and friends for encouragement, understanding, support and patience in the work process.