REAL-TIME PCR: NEW CAPABILITIES OF THE TECHNOLOGY IN SOLVING REPRODUCTIVE PROBLEMS

In recent years, the capabilities of real-time PCR – one of the most precise and advanced methods – have widened significantly: apart from identification of microorganisms, the technology can now be used to perform quantitative analysis of micro biome composition, to calculate viral load and for genotyping. Innovative approaches in the study of hereditary predisposition to various diseases and predictive diagnosis of cancer pathology, which became available for practical health care, allows shifting the focus gradually from diagnosis and treatment to very early diagnosis and prevention of diseases. Study data obtained in addressing reproductive problems are particularly topical: such data would be useful in prevention of pregnancy complications, genetic screening of pregnant women, identification of causes of male and female infertility and cancer prediction.

This manual is prepared by a team of authors from DNA-Technology, LLC and designed for obstetricians/ gynecologists, dermatologists, clinical laboratory diagnosticians and other related specialists.

INNOVATIVE TECHNOLOGY FOR DIAGNOSIS*
INFECTIOUS-INFLAMMATORY DISEASES
OF FEMALE REPRODUCTIVE SYSTEM

FEMOFLOR®

Identifying disorders dysbiotic and STIs – risk factors premature birth, intrauterine infections, complications followed childbirth – on pregravid phase and during pregnancy.

Femoflor® — winner of the National Medical Award in 2014

IMMUNOQUANTEX®

NEW

Evaluation of local inflammation of the reproductive tract of women: differential diagnosis of vaginitis and vaginosis.

ImmunoQuantex® — winner of the The International Award «Prix Galien Russia 2016» in nomination «Best Russian Product»

* Development and clinical testing of the technology was conducted by «DNA Technology» and FSBI «Research Center for Obstetrics, Gynecology and Perinatology» Ministry of Healthcare of the Russian Federation.

CONTENTS

INTRODUCTION

The main trends in modern medicine include a personalization approach, a shift towards early diagnosis and prevention of diseases. The breakthrough in laboratory technologies, which was witnessed in the last decade, has made innovation techniques that help to perform quantitative analysis of micro biome composition, assess risks and establish the role of hereditary factors in the development of diseases, thereby significantly increasing the information content of an inspection, available for practical health care.

Reproductive health care for the Russian population was declared by the government as the most important state objective and priority component of the National project «Health». Identification of polymorphisms associated with thrombophilia (one of the main factors of reproductive losses) during preconception preparation, genetic screening of pregnant women and identification of causes of male and female infertility have proven clinical value and can be widely applied by obstetricians/gynecologists, andrologists and other related health professions in their practice.

Cancer prediction is a new and perspective direction that includes prevention of cervical cancer and preventive diagnosis of hereditary forms of breast and ovarian cancers. Inclusion of such studies in medical examination can promote the health and quality of life, while reducing health care costs.

DIAGNOSIS OF INFECTIOUS AND INFLAMMATORY DISEASES OF FEMALE REPRODUCTIVE SYSTEM «FEMOFLOR®»

The spread of socially significant infections, including sexually transmitted infections (STIs), plays a key role in reduction of the reproductive potential of the population.Therefore, introducing a highly sensitive and highly specific diagnosis based on molecular genetic investigation approaches is a strategically important decision.

The main priority in outpatient care is to improve diagnosis and treatment of genital infections, which negatively affects women's reproductive health, life quality and increases the risk of obstetric pathology.Erased clinical picture, the lack of differential pathognomonic symptoms, asymptomatic diseases make it impossible to formulation etiological diagnosis based on clinical and anamnestic data and require that applications of laboratory methods.

Routine tests and criteria that are widely used in the diagnosis vulvo-vaginitis and bacterial vaginosis (BV), – smear microscopy, the Amsel criteria, tests for the detection of certain microorganisms, microbiological culturing – is not always possible to verify the originator, and therefore put an etiological diagnosis and reasonable treatment. The empirical therapy results in widespread recurrence of disease and chronicity of inflammation.

Classical methods of pathogen detection, which allow to identify the fact of exciter (culture method, qualitative determination of micro organisms by DNA or serodiagnosis by antigens and antibodies), are low-informative in the diagnosis of diseases caused by opportunistic microorganisms, due to the lack of quantitative capabilities accounting and evaluation criteria of pathological conditions. It is worth noting that conditionally pathogenic microflora, which is the most frequent cause of urogenital disorders in women, is represented mainly by anaerobic microorganisms. The vast majority of medical institutions of health care currently do not have the conditions for culturing such microorganisms. Another disadvantages of the culture method is long-term cultivation of microorganisms (average 5 days) and the necessity to preserve their viability until the receipt of biomaterial into the lab.

The use of PCR technology is urgent and valid also due to the fact that urinary tract infection is currently characterized by absence of specific pathognomonic symptoms, blurred clinical manifestations and multicentricity of the infection, thus presenting considerable difficulties for routine clinical diagnostics and, therefore, timely adequate therapy. Long oligosymptomatic diseases greatly increase the risk of complications with reproductive dysfunction.

Along with STIs, the structure of urogenital diseases has a stable trend on increase in the proportion of infections caused by opportunistic pathogens (OPs). Of particular interest are the processes associated with mycoplasma and ureaplasma, because, currently, their role in development of genital tract pathology has not been fully identified.

There is fundamental difference between diagnosis of infections caused by opportunistic pathogens and diagnosis of infections caused by obligate pathogens.

Extraction of opportunistic pathogens from pathological urogenital secretions of men and women is not evidence of their role in urogenital tract disease, since these microorganisms can also colonize the urogenital system under normal conditions. *M. hominis* and *U. urealyticum* are found in the urethra, vagina and rectum in 20-75 % of healthy people. The problem here is that it still remains unclear which factors of the microbial environment and organism of the host are crucial for achievement of the pathogenic potential of opportunistic mycoplasmas.

The study of complicated microbial complex of the urogenital tract has established that emergence of a pathological process requires association of several OPs (anaerobic and/or aerobic and/or Candida fungi), while attempts to identify and assign leading etiological role to certain microorganisms without assessing the microbial landscape of the urogenital tract as a whole, and also without quantitative analysis of the state of normal flora (Lactobacillus bacteria) in women can often lead to prescription of insufficient/excess drug therapy.

The issue of expediency of quantifying *Ureaplasma spp.* and *M. hominis* for clinical interpretation of results requires special attention. Clinical guidelines for management of patients with sexually transmitted infections and reproductive system infections state that **no treatment is carried out** if more than 10^4 CFU/sample (or GE/g) of *M. hominis* and/or *Ureaplasma spp.* are detected and that there are no clinical and/or laboratory signs of urogenital inflammation (local inflammation is assessed by leukocyte reaction).

Thus, detection of *U. urealyticum* and *M. hominis* in excess of the threshold value (10^4 GE/sample), in the absence of clinical symptoms of urogenital inflammation, and complaints from the patient are currently not an argument for prescription of drug therapy. If there is clinical symptoms, before prescribing drug therapy, one needs to take into account not only the quantitative analysis of *U. urealyticum* and *M. hominis*, but also the content of other OPs and lactobacilli, which will enable to fully describe the state of microbiocenosis in the patient, prescribe etiologically directed treatment and avoid polypharmacy.

Development of Femoflor® based on real-time PCR became a real breakthrough in the study of the state of micro biome composition of the urogenital tract and differential diagnosis of dysbiotic disorders.

Currently, several options of the Femoflor® test, taking into account the possibility of addressing a wide range of tasks (Table. 1), have been developed and being applied in practice.

Table 1. Composition of microorganisms in Femoflor® tests

Group	Specific components	Femoflor®-8	Femoflor®-16	Femoflor® Screen
Control	Positive control	*	*	*
	Sample intake control	*	*	*
TBM	Total bacterial mass	*	*	*
	Normal flora – Lactobacillus spp	*	*	*

Group	Specific components	Femoflor®-8	Femoflor®-16	Femoflor® Screen
Aerobic microorganism (facultative anaerobes)	Enterobacterium spp.*	*	*	
	Streptococcus spp	*	*	
	Staphylococcus spp.		*	
	Streptococcus agalactia		*	
Anaerobic microorganism (obligate anaerobes)	Prevotellabivia/ Porphyromonas spp.	*	*	*
	Gardnerellavaginalis	*	*	*
	Enterococcus spp.	*	*	
	Euterococcus spp.		*	
	Sneathia spp./ Leptotrihia spp./ Fusobacterium spp.		*	
	Megasphaera spp./ Veillonella spp./ Dialister spp.		*	
	Lachnobacterium spp./ Clostridium spp.		*	
	Mobiluncus spp./ Corynebacterium spp.		*	
	Peptostreptococcus spp.		*	
	Atopobiumvaginae		*	
Mycoplasma group	Mycoplasma hominis	*	*	*
	Mycoplasma genitalium	*	*	*
	Ureaplasmaurealyticum		*	*
	Ureaplasmaparvum		*	*
Fungi	Candida spp.	*	*	*
Pathogens	Trichomonasvaginalis			*
	Neiserriagonorrhoeae			*
	Chlamydia trachomatis			*
	Herpes simplex virus 1			*
	Herpes simplex virus 2			*
	Cytomegalovirus			*

Based on the outcome of over 3,000 studies, an algorithm used to assess the state of vaginal microbiome composition in women of reproductive age was developed. At the heart of this algorithm is differentiation of dysbiotic disorders and identification of the etiology of the process (Fig. 1). This can be used by obstetricians/gynecologists for diagnosis and reasoned selection of further tactics for patient management.

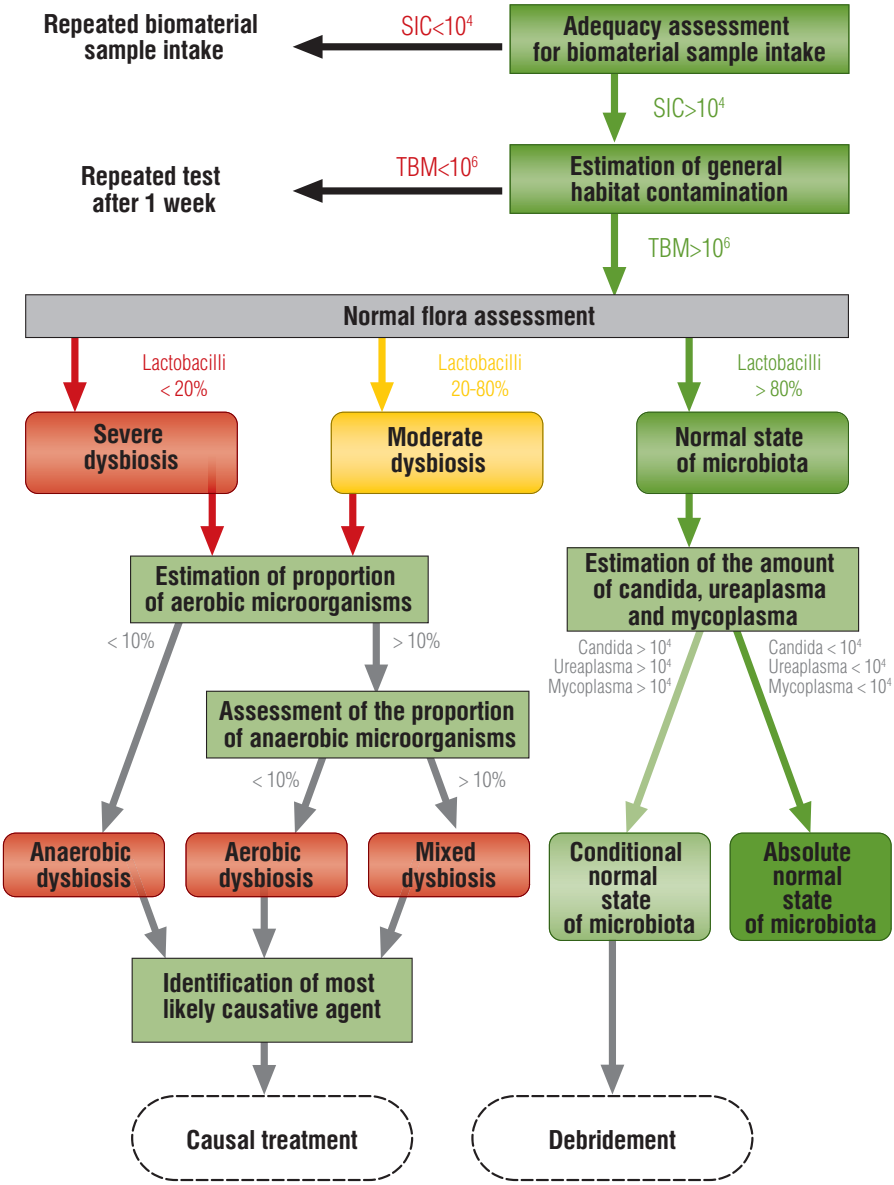


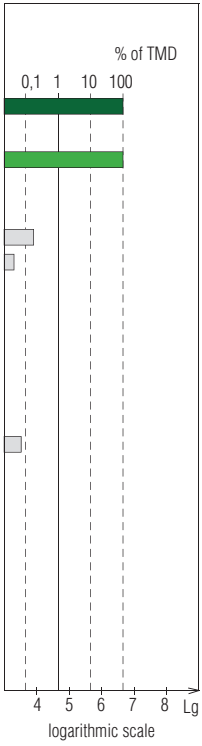
Fig. 1. Algorithm for urogenital microbiocenosis analysis in women of reproductive age

Femoflor® study enables to:

- Assess the reliability of analysis result based on a benchmark – SIC (biomaterial adequacy)
- Compare the amount of lactobacilli with the total amount of bacteria (total contamination) to assess the state of microbiocenosis and severity of its disorder: *absolute normal state of microbiota, conditional normal state of microbiota, moderate dysbiosis and severe dysbiosis* (Fig. 2);
- Compare the number of representatives of the microorganism groups with the total number of bacteria to determine their etiologic significance in the development of dysbiosis: *aerobic, anaerobic and mixed dysbiosis*.

A Sample ID: Sample_1

№	Name of research	Results	
		Quantitative	Relative Lg (0/TMD)
	Control of sample intake	$10^{4.6}$ ■	
1	Total Bacteral Mass	$10^{6.6}$ □	
	Normal microflora		
2	Lactobacillus	$10^{6.6}$	0.0 (85-100 %) ■
	Facultative anaerobic microorganisms		
3	Enterobacteriaceae	not detected	□
4	Streptococcus spp.	$10^{3.8}$	-2.8 (0.1-0.2 %) □
5	Staphylococcus spp.	$10^{3.2}$	-3.4 (<0.1 %) □
	Obligate anaerobic microorganisms		
6	Gardnerella vaginalis + Prevotella bivia + Porphyromonas spp.	not detected	□
7	Eubacterium spp.	not detected	□
8	Sneathia spp. + Leptotrichia spp. + Fusobacterium spp.	not detected	□
9	Megasphaera spp. + Veillonella spp. + Dialister spp.	not detected	□
10	Lachnobacterium spp. + Clostridium spp.	not detected	□
11	Mobiluncus spp. + Corynebacterium spp.	$10^{3.5}$	-3.1 (<0.1 %) □
12	Peptostreptococcus spp.	not detected	□
13	Atopobium vaginae	not detected	□
	Yeast-like fungi		
14	Candida spp.*	not detected □	
	Mycoplasmas		
15	Mycoplasma hominis*	not detected □	
16	Ureaplasma (urealyticum + parvum)*	not detected □	
	Pathogenic microorganisms		
17	Mycoplasma genitalium**	not detected □	



* Quantitative Analysis Lg(X)
** Qualitative Analysis

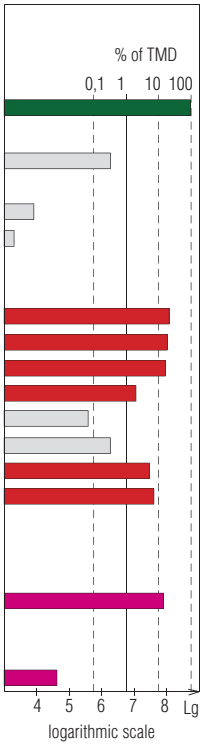
Conclusion:

Absolute normocenosis

Normal state of micro biome composition of healthy woman of reproductive age.

B Sample ID: Sample_2

№	Name of research	Results	
		Quantitative	Relative Lg (0/TMD)
	Control of sample intake	$10^{5.0}$	
1	Total Bacteral Mass	$10^{8.8}$	
	Normal microflora		
2	Lactobacillus	$10^{6.3}$	-2.4 (0.3-0.4 %)
	Facultative anaerobic microorganisms		
3	Enterobacteriaceae	$10^{3.6}$	-5.1 (<0.1 %)
4	Streptococcus spp.	$10^{3.2}$	-5.6 (<0.1 %)
5	Staphylococcus spp.	not detected	
	Obligate anaerobic microorganisms		
6	Gardnerella vaginalis + Prevotella bivia + Porphyromonas spp.	$10^{8.1}$	-0.6 (21-28 %)
7	Eubacterium spp.	$10^{8.1}$	-0.6 (20-27 %)
8	Sneathia spp. + Leptotrichia spp. + Fusobacterium spp.	$10^{8.0}$	-0.7 (18-24 %)
9	Megasphaera spp. + Veillonella spp. + Dialister spp.	$10^{7.1}$	-1.6 (2.2-2.9 %)
10	Lachnobacterium spp. + Clostridium spp.	$10^{5.7}$	-3.0 (<0.1 %)
11	Mobiluncus spp. + Corynebacterium spp.	$10^{6.3}$	-2.4 (0.3-0.5 %)
12	Peptostreptococcus spp.	$10^{7.5}$	-1.2 (5-7 %)
13	Atopobium vaginae	$10^{7.6}$	-1.1 (7-9 %)
	Yeast-like fungl		
14	Candida spp.*	not detected	
	Mycoplasmas		
15	Mycoplasma hominis*	$10^{7.9}$	
16	Ureaplasma (urealyticum + parvum)*	not detected	
	Pathogenic microorganisms		
17	Mycoplasma genitalium**	detected	



* Quantitative Analysis Lg(X)
** Qualitative Analysis

Conclusion:

Apparent Anaerobic dysbiosis
Detected DNA of: Mycoplasma_genitalium

Dysbiosis, caused by anaerobic microorganisms.

Fig. 2. Examples of how the Femoflor®-16 test results for women of reproductive age are presented

The technology is recommended for use in the following situations:

- Clinical and/or laboratory signs of urogenital inflammation;
- Disbiotic disorders amid various influences, including:
  - Treatment by antibiotics (both local and general), hormones and cytostatics,
  - Use of contraceptives, including intrauterine devices (IUDs),
  - Douching,
  - Change of sexual partner
  - Hypothermia;
- Impending surgical manipulation of the pelvic organs with a high risk of infectious complications;

- Presence of complicated obstetric or gynecological history (recurrent miscarriage, perinatal loss, infertility);
- Preconception preparation;
- Pregnancy (all the trimesters);
- Discrepancy between clinical and laboratory test results;
- Atrophic vaginitis (senile), examination of postmenopausal women;
- Analysis of the state of microbiocenosis in healthy women (it is optimal to take with gynecological smear).

Assigningthe study and obtaining Femoflor®-16 test result is extremely important for reducing the risk of adverse consequences of disorders of the vaginal microbiocenosis, leading to pregnancy pathologies and postpartum purulent-septic complications in new mothers and newborns.

In the case, when initially the patient has complaints (itching, burning genitals, abnormal vaginal discharge), it is recommended to carry out the Femoflor®Screen test (Fig. 3), which brings together testing for main pathogens – agents of STIs and quantitative study of the most important indicators of microbiocenosis state:

Identification

- Simplest (*Trichomonasvaginalis*)
- Bacteria (*Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium*)
- Viruses (*Herpes simplex virus 1andHerpes simplex virus 2, Cytomegalovirus*)

Quantitative test

- Total bacterial mass
- *Lactobacillus* microorganisms
- Opportunistic pathogens:
  - *Gardnerellavaginalis/ Prevotellabivia/ Porphyromonas spp.*
  - *Ureaplasma spp.*
  - *Mycoplasma hominis*
  - *Candida fungi*

In terms of clinical relevance of this test, the following capabilities are achieved at initial admission:

- Identification of the etiological causes of infection;
- Diagnostics of dysbiotic disorders and their degree of severity;
- Ability to determine the amount of necessary therapy;
- Ability to perform dynamic observation;
- Monitoring treatment effectiveness;
- Monitoring restoration of normal vaginal flora;
- Ability to prescribeor adjust therapy depending on the test result (analysis takes 1-2 days).

Study of urogenital tract biocenosis
Femoflor® Screen

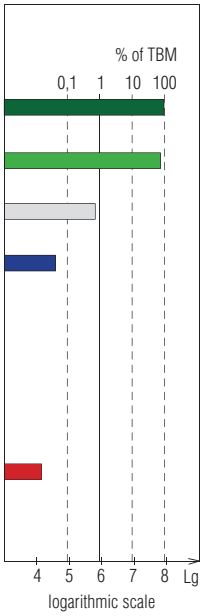
Date 4 June 2016, 17:09:24
Number of tube
Patient name
Sex
Age
Organization
Clinicain name
Comments

Logotype

Information about laboratory

Sample ID: 1

№	Name of research	Results	
		Quantitative	Relative Lg (O/TBM)
	Control of sample intake	$10^{5.5}$	
1	Total Bacteral Mass	$10^{7.9}$	
	Normal microflora		
2	Lactobacillus	$10^{7.9}$	-0.1 (70-94 %)
	Obligate anaerobic microorganisms		
3	Gardnerella vaginalis + Prevotella bivia + Porphyromonas spp.	$10^{5.8}$	-2.2 (0.6-0.8 %)
	Yeast-like fungi		
4	Candida spp.*	$10^{4.4}$	
	Mycoplasmas		
5	Ureaplasma spp.*	not detected	
6	Mycoplasma hominis*	not detected	
	Pathogenic microorganisms		
7	Mycoplasma genitalium**	not detected	
8	Trichomonas vaginalis**	not detected	
9	Neisseria gonorrhoeae**	not detected	
10	Chlamydia trachomatis**	detected	
11	HSV-2**	not detected	
12	CMV**	not detected	
13	HSV-1**	not detected	



* Quantitative Analysis Lg(X)
** Qualitative Analysis

Conclusion:

Fig. 3. Examples of how the Femoflor® Screen test results for women of reproductive age are presented

To obtain correct results of the study the situations, limiting the ability to use the "Femoflor®" technology must be taken into account as well as the basic requirements for taking biomaterial.

Situation	When taking of biomaterial is possible
Colposcopy	Not earlier than 24-48 hours after colposcopy
Ultrasound study using vaginal sensor	Not earlier than 24-48 hours after ultrasound studies using a vaginal probe
The use of antibacterial drugs	Technically, the study can be carried out on the during antibacterial drugs application, however, due to instability of microbiocenosis during this period results will provide little information. It is recommended to take the biomaterial during the next cycle, or not earlier than 2 weeks after the end of treatment.
The use of probiotics, eubiotics	Not earlier than 2 weeks after the end of application of preparations containing micro-organisms
The use of drugs – PCR inhibitors (Ultrasound gel, heparin, chlorhexidine and other chlorinated preparations)	Not earlier than 24 hours after application of these drugs
The patient after the protected sexual contact	Not earlier than 24 hours after the secure sexual contact
Patient after unprotected sexual contact	Not earlier than 48-72 hours after unprotected sexual contact
Menstruation	After bleeding ends

Compliance with these paragraphs allows avoiding typical errors of the pre-analytical stage.

Mistake	How is correctly
Taking for the research the vaginal secretions, mucus plug.	If necessary, remove excess mucus and take a scraping from posterolateral vaginal vault using a special probe.
On the day of research a patient conducts full of toilet of genitals, vaginal douching.	Do not conduct a toilet genital and vaginal douching on the day of research.
Taking biomaterial from various locations (V, C, U) to one tube with transport medium.	If necessary, obtain clinical material from several biotopes by taking biomaterial by a new probe to a new tube each time.
Violation of procedure of taking biological material - the probe breaks off and left in a test tube with transport medium, or squeezing the material in the tube from the probe is done by gloves.	The probe with taken clinical material must be placed in a tube containing transport medium, thoroughly rinsed, easily squeeze out of the top of the tube's wall, removed and discarded. The tube should be closed tightly and labeled.

This technology can be effective in preparing patients for gynecological surgery to prevent purulent-septic complications caused by a wide range of obligate anaerobic opportunistic pathogens.

In 2014, an author's team of doctors and scientists, led by Dmitriy Trofimov, CEO, DNA-Technology LLC, won the National Medical Award "PRIZVANIE" among the best doctors of Russia in the category "For development of a new diagnosis method" for creating Femoflor®.

The development of the concept of study of complex multi microbial communities ("Femoflor®") was the work on research of the local inflammation, since the intensity of the inflammatory process at the same causative agent can vary widely among different patients.

To a large extent the development of the infection depends on the state of mucosal (local) immunity and the immunological reactivity depends of the microorganism (Serov V.N., 2006). In this regard, the study mucosal immunity is extremely interesting, in particular for purposes of differential diagnosis of nonspecific vaginitis, and BV and also of vulvovaginal candidiasis and Candida carriage. Until recently the only way to routine study of local immunity remained a microscopic evaluation of leukocyte reaction.

A world first for this purpose DNA-Technology Company in collaboration with FSBI "Scientific Center for Obstetrics, Gynecology and Perinatology" n.a. Academician Kulakov V.I. – have designed **"ImmunoQuantex®"**, allowing on the basis of integrated assessment of mRNA gene expression of innate immunity (IL1B, IL10, IL18, TNFa,TLR4, GATA3, CD68 , B2M) to carry out an objective diagnosis of the inflammatory process.

Both tests (**"Femoflor®"** and **"ImmunoQuantex®"**) can be conducted from one sample (scrapping of epithelial cells from vagina and cervical channel) by means of Real-time PCR, which allows to shorten the time of diagnosis up to 4 hours and reduce load on laboratory. The use of molecular genetic methods for operation with compliance with complex conditions for cultivation of microorganisms is not required, greatly simplifies the implementation and scaling of techniques in health care facilities, and reduces the probability of human factors errors.

The use of sensitive molecular genetic markers can detect development of the disease at an early stage, allowing carrying out early and very early diagnosis of reproductive diseases, to shift the focus from treatment to prevention of disease, and minimize the risk of recurrence.

Result of research by Real-time PCR method.

№	Name of research	Result	Unit	Interpretation	Interval
Evaluation of vaginallocal in flammation by method of reverse transcription followed by real-time PCR					
1	IL1B	5.6	Lg		
2	IL10	1.5	Lg		
3	IL18	5.4	Lg		
4	TNFA	3.5	Lg		
5	TLR4	2.8	Lg		
6	GATA3	4.4	Lg		
7	CD68	4.5	Lg		
8	B2M	4.8	Lg	valid	>>4
9	TLR4/GATA3	0.021	relative units	<--x]	<<0.070]
10	TNFA/IL18	0.010	relative units	<-x-]	<<0.050]
11	IL10/IL18	0.11	relative units	<-x-]	<<4.0]
12	IL1B/CD68	13.9	relative units	<--]x-	<<12.7]
13	Inflammation index	3.5	%	no inflammation	<50

Fig. 4. An example or the answer form "ImmunoQuantex".

Obstetric and gynecological problems cannot be solved from isolated point of view of the analysis of microorganisms inhabiting the lower reproductive tract, or evaluation of local inflammation. This approach to the interpretation of the individual components of a complex system can lead to diagnostic errors. The performed research have proved the importance and clinical need for a new concept – a comprehensive assessing the state of the vaginal flora, and local inflammation of female reproductive tract ("Femoflor®" + "ImmunoQuantex") by molecular-genetic diagnostic method. Further development of this innovation can lead to changes in the traditional view of diagnostics and, as a result, the clinical paradigm and attitudes towards treatment and prevention of reproductively significant diseases, lead to update of national and international clinical guidelines for treatment.

«Prix Galien Russia 2016» – DNA-Technology is award winner in the category "The best Russian product".

DIAGNOSIS OF MALE UROGENITAL SYSTEM DISEASES «ANDROFLOR®»

Currently, topical direction in the study of microbome composition is to compare the microbial composition of the urogenital tract of sexual partners for the purpose to develop an effective algorithm of laboratory examinations couples and selection of therapy in case disease of reproductive system of infectious etiology or violations productive function of one / both partners.

For example, when examining sexual partners of patients with bacterial vaginosis morphotypes of associated with bacterial vaginosis microorganisms were detected in 25 % of men, and clinical manifestations (balanoposthitis) were seen in 3 % of patients. In addition, conditional pathogens - potential pathogens of inflammatory diseases of urogenital tract can enter the urethra during anal and oral-genital contacts, creating a risk of infection for both sexual partners.

Diseases of the male genital tract are the leading cause of male reproductive function impairment. This defines their social and economic significance, especially taking to account current decrease of birthrate.

The combined infection and inflammation process, which longevity and intensity defines the extent of fertility impairment, is considered the main reason of male genital tract diseases onset. Chronic inflammation provides prolonged toxic action on germinal epithelium, disrupts blood-testis barrier, affects rheological properties and chemical composition of the seminal fluid and can provoke development of autoimmune reactions like formation of antisperm antibodies.

The progression of inflammation provokes growth of activated immune cells level, which is accompanied with excessive production of oxygen free radicals and increased secretion of lymphokines and monkines promoting secondary inflammation in reproductive tissues.

According to Tenth Revision of International Classification of Diseases (ICD-10) the list of male genital tract diseases which can be caused by combined infection and inflammation include following diseases:

Obligate pathogens and viruses are considered the main etiologic factors of combined infection and inflammation process. Most frequently recognized pathogens are *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, Mycoplasma genitalium, Herpes Simplex Virus* type 1 and 2, but in the last few years there have been many reported the increased disease-causing role of opportunistic microorganisms: *Ureaplasma urealyticum, Ureaplasma parvum, Haemophilus, Candida* etc.

Considering the clinical and social significance of the male genital tract diseases, their slight symptoms or asymptomatic clinical course as well as importance of equivalent medical research of both partners having reproductive system impairment, DNA-Technology Company developed and implemented the unique technology Androflor® that allows diagnosing combined infection and inflammatory disease of male genital tract.

Androflor® is:

- Detection of infectious agents causing sexually transmitted diseases (STD's)
- Detection of opportunistic microorganisms inhabiting male genital system and having a potency to cause combined infectious-inflammation diseases
- Identification of infectious process's etiology
- Prognosis of therapy intensity
- Dynamic monitoring
- Sample intake control (human genomic DNA quantitative evaluation)

The result interpretation flowchart is shown on Figure 5.

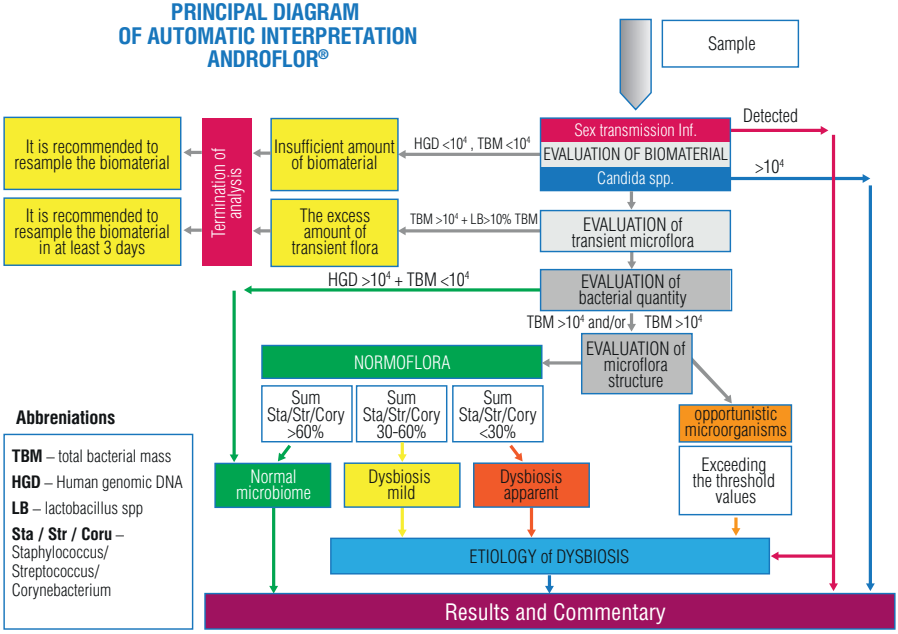


Fig. 5. Principal diagram of automatic interpretation of results.

The variants of Androflor® kit are indented for different clinical purposes and include different number of microorganisms detected (Fig. 6).

Table 2. Analytes detected by Androflor® REAL-TIME PCR Detection Kit and Androflor® Screen REAL-TIME PCR Detection Kit

Analyte	Androflor®	Androflor® Screen
Human DNA	+	+
Total bacterial mass (TBM)	+	+
Lactobacillus spp.	+	+
Staphylococcus spp.	+	+
Streptococcus spp.	+	+
Corynebacterium spp.	+	+
Gardnerella vaginalis	+	+
Atopobium cluster	+	–
Megasphaera spp./ Veilonella spp./ Dialister spp.	+	–
Sneathia spp./ Leptotrihia spp. / Fusobacterium spp.	+	–
Ureaplasma urealyticum	+	+
Ureaplasma parvum	+	+
Mycoplasma hominis	+	+
Bacteroides spp./ Porphyromonas spp./ Prevotella spp.	+	–
Anaerococcus spp.	+	–
Peptostreptococcus spp./ Parvimonas spp./ Eubacterium spp.	+	–
Pseudomonas aeruginosa/ Ralstonia spp./ Burkholderia spp.	+	–
Heamophilus spp.	+	–
Enterobacteriaceae/ Enterococcus spp.	+	+
Candida spp.	+	+
Mycoplasma genitalium	+	+
Trichomonas vaginalis	+	+
Neisseria gonorrhoeae	+	+
Chlamydia trachomatis	+	+

- Diagnostics and treatment efficiency monitoring of any combined infection and inflammation diseases of the male genital tract (Androflor® REAL-TIME PCR Detection Kit)
- Diagnostics and treatment monitoring of acute infection and inflammation diseases of the male genital tract (Androflor® Screen REAL-TIME PCR Detection Kit)

Material for the study:

scrapings from the balanus, urethral swabs, urine, prostatic fluid, ejaculate, biopsies of prostate tissue.

A Urogenital microbiome composition test Androflor®

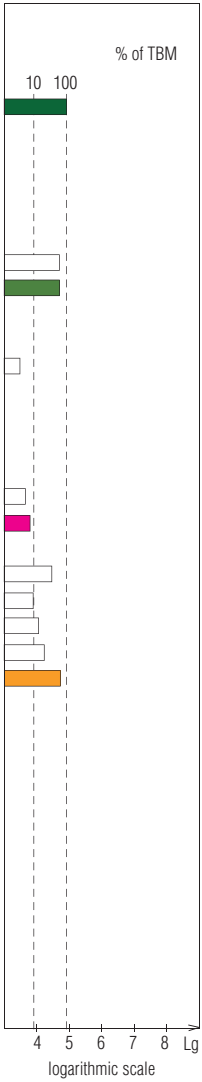
Date 21 January 2016, 12:18:03
Number of tube
Patient name
Sex
Age
Organization
Clinicain name
Comments

Logotype

Information about laboratory

Sample ID: Sample_1

№	Name of research	Results	
		Quantitative	Relative Lg (O/TBM)
	Human DNA	$10^{5.0}$	
1	Total Bacteral Mass	$10^{4.9}$	
	Lactobacillus spp.		
2	Lactobacillus	not detected	
	Normal microflora		
3	Staphylococcus spp.	not detected	
4	Streptococcus spp.	$10^{3.0}$	-1.9 (1.2-1.6 %)
5	Corynebacterium spp.	$10^{4.6}$	-0.3 (46-62 %)
6	Commensals, sum	$10^{4.6}$	-0.3 (47-64 %)
	BV-associated opportunistic microorganisms		
7	Gardnerella vaginalis	not detected	
8	Megasphaera spp./ Veillonella spp./ Dialister spp.	$10^{3.4}$	
9	Sneathia spp./ Leptotrichia spp./ Fusobacterium spp.	not detected	
10	Ureaplasma urealyticum*	not detected	
11	Ureaplasma parvum*	not detected	
12	Mycoplasma hominis*	not detected	
13	Atopobium cluster	$10^{3.4}$	-1.3 (4-5 %)
14	BV-associated opportunistic microorganisms, sum	$10^{3.4}$	-1.1 (7-9 %)
	Opportunistic anaerobes		
15	Bacteroides spp./ Porphyromonas spp./ Prevotella spp.	$10^{4.3}$	-0.6 (21-28 %)
16	Anaerococcus spp.	$10^{3.9}$	-0.1 (9-12 %)
17	Peptostreptococcus spp./ Parvimonas spp.	$10^{4.1}$	-0.8 (14-19 %)
18	Eubacterium spp.	$10^{4.2}$	-0.7 (17-23 %)
19	Opportunistic anaerobes, sum	$10^{4.7}$	-0.1 (61-82 %)
	Opportunistic microorganisms Haemophilus spp.		
20	Haemophilus spp.	not detected	
	Opportunistic microorganisms Pseudomonas aeruginosa/ Ralstonia spp./ Burkholderia spp.		
21	Pseudomonas aeruginosa/ Ralstonia spp./ Burkholderia spp.	not detected	
	Opportunistic microorganisms Enterobacteriaceae spp./ Enterococcus spp.		
22	Enterobacteriaceae spp./ Enterococcus spp.	not detected	
	Yeast-like fungl		
23	Candida spp.*	not detected	
	Sexually transmitted infections		
24	Mycoplasma genitalium**	not detected	
25	Trichomonas vaginalis**	not detected	
26	Neisseria gonorrhoeae**	not detected	
27	Chlamydia trachomatis**	not detected	



* Quantitative Analysis Lg(X)
** Qualitative Analysis

Conclusion:

Dysbiosis minor with opportunistic anaerobes predominance

B **Urogenital microbiome composition test Androflor® Screen**

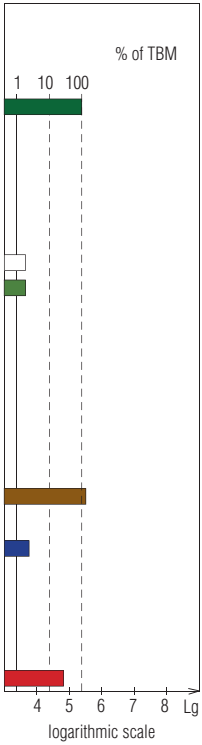
Date 21 September 2016, 13:34:20
Number of tube
Patient name
Sex
Age
Organization
Clinicain name
Comments

Logotype

Information about laboratory

Sample ID: –

№	Name of research	Results	
		Quantitative	Relative Lg (O/TBM)
	Human DNA	$10^{4.5}$	
1	Total Bacteral Mass	$10^{5.4}$	
	Lactobacillus spp.		
2	Lactobacillus	not detected	
	Normal microflora		
3	Staphylococcus spp.	not detected	
4	Streptococcus spp.	not detected	
5	Corynebacterium spp.	$10^{3.7}$	-1.6 (1.9-2.6 %)
6	Commensals, sum	$10^{3.7}$	-1.6 (1.9-2.6 %)
	BV-associated opportunistic microorganisms		
7	Gardnerella vaginalis	not detected	
8	Ureaplasma urealyticum*	not detected	
9	Ureaplasma parvum*	not detected	
10	Mycoplasma hominis*	not detected	
11	BV-associated opportunistic microorganisms, sum	not detected	
	Opportunistic microorganisms Enterobacteriaceae spp./ Enterococcus spp.		
12	Enterobacteriaceae spp./ Enterococcus spp.	$10^{5.5}$	0.1 (85-100 %)
	Yeast-like fungl		
13	Candida spp.*	$10^{3.6}$	
	Sexually transmitted infections		
14	Mycoplasma genitalium**	not detected	
15	Trichomonas vaginalis**	detected	
16	Neisseria gonorrhoeae**	not detected	
17	Chlamydia trachomatis**	detected	



* Quantitative Analysis Lg(X)
** Qualitative Analysis

Conclusion:

Dysbiosis apparent with Enterobacteriaceae spp./ Enterococcus spp. DETECTED: Trichomonas vaginalis, Chlamydia trachomatis.

Fig. 6. Examples of how the Androflor® (A) and Androflor® Screen (B) test results for are presented.

One test of male urogenital tract microbiome composition by Real-time PCR «Androflor®» allows you to fully replace the complex of methods offered in the aid of medical standards, which are approved by Russian Ministry of Health in 2012, and to expand the abilities of diagnosis due to the additional diagnosis of obligate anaerobes.

MOLECULAR GENETIC TESTING

HLA class II typing

According to WHO, infertility affects 10-12 % of married couples in the world. At the same time, about 5 % of infertility is caused by anatomical, genetic, endocrinological or immunological factors. The frequency of infertile marriages in Russia exceeds 15 %, which, according to WHO, is in a critical level. The country has registered cases of over 5 million infertile married couples. Moreover, 1.63 million are in need of assisted reproductive technologies.

An important aspect of establishing the causes of infertility among married couples is to identify the immune component, first of all, the effect of HLA genes.

HLA (**H**uman **L**eukocyte **A**ntigen) is the major histocompatibility complex (MHC) in humans – immune response genes.

MHC class I molecules (A, B, C) are found on the surfaces of all cell types except erythrocytes and trophoblast cells.

MHC class II molecules (DP, DM, DQA, DQB, DQ, DR) are found on the surface of antigen-presenting cells (dendritic cells, macrophages, B-lymphocytes).

MHC class III molecules encode the components of the complement system and proteins present in the blood.

HLA-typing is widely used in the following areas of medicine: identification of tissue compatibility of the donor and recipient in organ and tissue transplantation, differential diagnosis and prognosis of autoimmune diseases, diagnosis of the causes of reproductive disorders of unknown cause.

The difference between a man and woman (a couple) in terms of HLA gene variants is one of the important conditions for a successful advent and carrying of pregnancy. The similarity between a couple in HLA gene variants increases the likelihood of emergence of an embryo with a double set of identical gene variants, i.e. HLA homozygote, which is an adverse factor that may lead to reproductive losses. Therefore, HLA-typing of a couple is used for diagnosis of the causes of reproductive failures to establish similarities in the couple's HLA gene variants.

Besides, it was established that out of 60 % of known causes of spontaneous abortion, autoimmune factors account for 20 %. Very low fertility was detected in patients with systemic lupus erythematosus, rheumatoid arthritis, diffuse toxic goiter, type 1 diabetes mellitus (T1DM) and endometriosis.

For diagnostic purposes, the most commonly used is HLA typing of the DRB1 gene at low resolution or at the level of 13 allele groups. In some cases, it is also necessary to type HLA-DQA1 and DQB1 genes. In most cases, the required level of typing is the allele group: 8 groups for the DQA1 gene and 12 groups for the DQB1 gene (Table 3).

Table 3. HLA-DRB1 variants and HLA DRB1-DQA1-DQB1 haplotypes

DRB1*01	DQA1*0101–*0104–*0105–*0107	DQB1*0501
DRB1*15 (02)	DQA1*0102-0103/*0103	DQB1*0602-0602/*0601
DRB1*16 (02)	DQA1*0102	DQB1*0502/*0504/*0505
DRB1*03	DQA1*05	DQB1*02
DRB1*04	DQA1*03/*04	DQB1*02/*03/*04
DRB1*11 (05)	DQA1*0501	DQB1*0301
DRB1*12 (05)	DQA1*0501/0601	DQB1*0301
DRB1*13 (06)	DQA1*0102-*0103/*0501	DQB1*0602-0620/*0301
DRB1*14 (06)	DQA1*0101/*0501	DQB1*0503/*0301
DRB1*07	DQA1*0201	DQB1*0201/*0303
DRB1*08	DQA1*04/*06	DQB1*03/*04
DRB1*09	DQA1*03	DQB1*0303
DRB1*10	DQA1*0101	DQB1*0501

Involvement of HLADR genes in the reproductive failure structure is associated with chronic miscarriage in early pregnancy (DRB1*01, DRB1*03, DRB1*04 and DRB1*10) and idiopathic premature ovarian failure (DRB1*03, DRB1*04).

Identification of the role of HLA in the reproductive failure structure of a married couple is one of the important steps in overcoming this problem.

Diagnosis of male infertility: AZF deletion, CFTR gene mutations

Today, it is known that about half of all cases of infertility in a couple is caused by the male factor – isolated or in combination with the female factor. Infections in the etiology of male infertility are sidelined. The leading are tissue proliferative responses, autoimmune component, neuroendocrine responses and genetic disorders.

Genetic disordersare present in 30-50 % of cases where oligozoospermia, azoospermia and other severe disorders are detected via semen analysis (baseline study in a barren marriage).

According to Letter No. 2510/3797-03-32 from the Russian Ministry of Health, dated April 11, 2003, genetic diagnosis is recommended to identify the genetic factors of male infertility.

Y chromosome deletion in the AZF (azoospermia factor) region is the most common genetic factor of male infertility (Fig. 7).

In men with azoospermia or oligozoospermia, microdeletions can be present in the three loci of the Y chromosome – **AZFa**, **AZFb** and **AZFc**. These deletions are extremely rare for normospermia and for sperm concentration > 5 million/ml.

Y chromosome *microdeletions* are not determined via cytogenetic analysis and this makes their molecular genetic search reasonable. The European Academy of Andrology (EAA) recommends testing all men with azoospermia and severe oligozoospermia (< 5 million sperm per milliliter of semen) for the presence of AZF deletion.

AZFa contains three genes named USP9Y, DBY and UTY, whose deletions lead to azoospermia with the Sertoli cell-only syndrome type 1 (absence of germ cells in the seminiferous tubules), which is typical for complete AZFa deletion.

Procedures for surgically isolating spermatozoa are not effective in men carrying these deletions.

It should be noted that the sub-region does not contain repetitive sequences and its deletions occur with a low frequency (about 5 % of Y chromosome microdeletions). STS markers sY84, sY86 and sY615 are sufficient to identify AZFa deletions. The use of at least two markers – sY84 and sY86 – is diagnostically significant.

AZFb contains sequences that are represented both as a single copy and in the form of high-repeating direct and inverted palindromic sequences. AZFb deletions occur in about 16 % of all the Y chromosome microdeletions. Complete AZFb deletion, which leads to a delay in maturation of sperm during spermatogenesis, is of major clinical significance. In this case, TESA forecast is poor.

The multi-copy RBMY gene, whose deletions are detected in men with azoospermia or severe oligozoospermia, is mapped in this subregion. STS markers in the subregion are sY127 and sY134. In line with the guidelines by the European Academy of Andrology

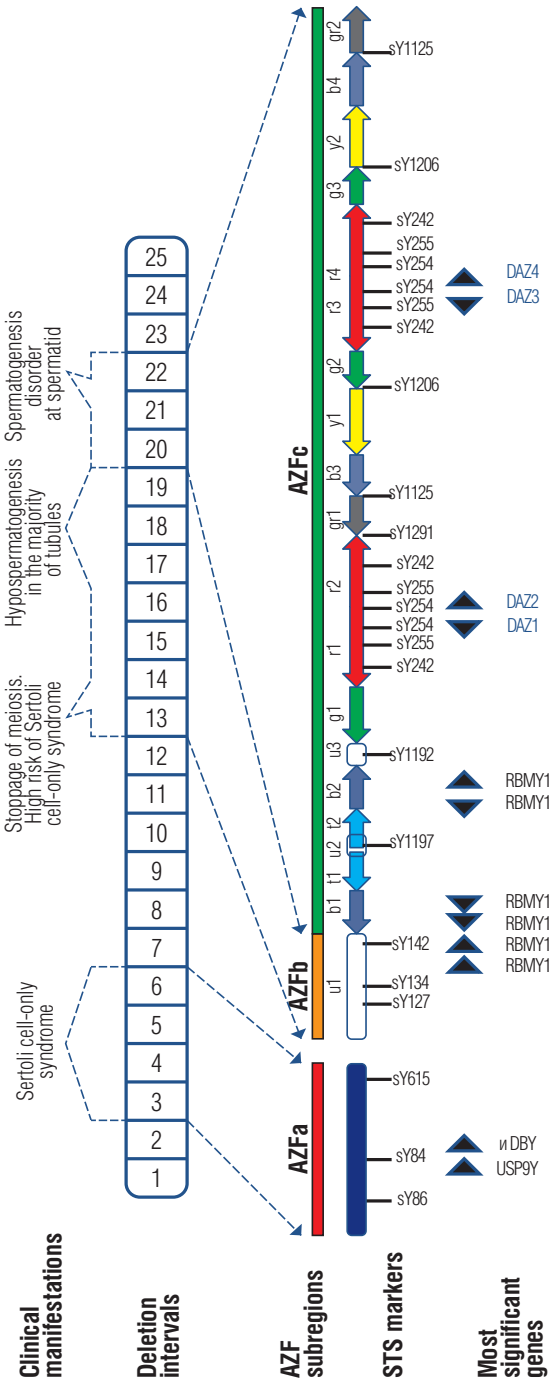


Fig. 7. Schematic representation of AZF

(EAA) and the European Molecular Genetics Quality Network (EMQN), loss of these markers goes with severe disorders of spermatogenesis with high risk of the Sertoli cell-only syndrome type I.

AZFc deletions are the most common (over 60 % of Y chromosome microdeletions). One of the key genes in this sub-region is the DAZ gene. The **b2/b4** deletion leads to loss of all its copies. Markers sY254, sY255, sY1291, sY1206, sY1197 and sY1125 are used to identify it.

The histologic picture of the testicle in b2/b4 deletion may be different as spermatogenesis is blocked much less frequently than in AZFa and AZFb deletions. The Sertoli cell-only syndrome type II (absence of germ cells in the seminiferous tubules)is rarely detected. Therefore, spermatozoa can be detected both in the testicle and in the semen.

One more Y deletion type – **gr/gr deletion** (sY1291 deletion) – is described in the AZFc locus. In this case, half of the AZFc subregion drops out, which changes the number of copies of genes located in this region. The risk of oligozoospermia is seven times higher in carriers of gr/gr deletions and such men may develop testicular germ cell tumors.

Microdeletions of several subregions of the Y chromosome are found in 15 % of cases and they almost always lead to azoospermia and Sertoli cell-only syndrome. Moreover, disorder in spermatogenesis in the case of distal deletion of AZFb and AZFc may be less severe.

ATTENTION!

Among the identified markers, there are some groups associated by rigid coupling:

- sY84, sY86,
- sY127, sY134,
- sY254and sY255.

In identifying the genetic causes of infertility, particularly incomplete AZF deletions, infertility can be overcomed by intracytoplasmic sperm injection (ICSI). It should be noted that in the case of conception, Y chromosome deletion will surely be passed to all the sons of the man, and the size of their microdeletions may be more extensive up to complete deletion. In this regard, it is necessary to observe the boys born via ICSI to assess their fertile status.

If AZF microdeletions are detected in the father through **assisted reproductive technology**, preimplantation genetic diagnosis and transfer of female embryo are recommended.

Indications for genetic analysis:

- Examination of infertile couple in the complex of diagnostic methods;
- Selection of adequate infertility overcoming methods;
- Assessment of the probability of sperm release during TESE, MESA, TESA or RESA;
- Assessment of the risk of fertility disorders in sons.

DNA-Technology, LLC developed a kit of reagents for identification of AZF deletions associated with male infertility. The analytical panel included 13 nonpolymorphic markers that allow identifying deletions in all AZF loci (Fig. 8).

№	Name of Marker	Loci	Result
1	sY86	AZFa	Norm
2	sY84	AZFa	Norm
3	sY615	AZFa	Norm
4	sY127	AZFb	Norm
5	sY134	AZFb	Norm
6	sY142	AZFb	Norm
7	sY1197	AZFc	Norm
8	sY254	AZFc	Norm
9	sY255	AZFc	Norm
10	sY1291	AZFc	Deletion
11	sY1125	AZFc	Norm
12	sY1206	AZFc	Norm
13	sY242	AZFc	Norm

Fig. 8. Form for presenting the "Identification of AZF deletions" study results

Nevertheless, AZF deletion is not the only cause of genetically caused male infertility. An important component is the **CFTR** (Cystic Fibrosis Transmembraneconductance Regulator) gene mutations associated with development of **mucoviscidosis** (cystic fibrosis) – a hereditary disease (OMIM: 219700), involving a disorder of epithelial ion transport.

Depending on the degree of damage to the protein, CFTR gene mutations are divided into classes (Table 4). Class I–III mutations are categorized under phenotypically severe mutations. Cases of cystic fibrosis caused by these mutations are more often severe in nature and are characterized by early onset of serious complications and exocrine pancreatic insufficiency. Identification of such mutations could be the basis for correction of patients' management tactics.

Table 4. Classes of CFTR gene mutations and their phenotypes
(Zielenski&Tsui, 1995,Greenetal, 2010)

CLASS	DEGREE OF PROTEIN DAMAGE	PHENOTYPES
Class I	**Protein synthesis with altered primary structure** Gene mutations lead to critical reduction in number of chloride channels on the cell surface or to total absence. This may be due to defective mRNAs (unstable form), disorder in mRNA splicing process and/or disorder in the synthesis of amino acid sequence of the protein. This leads to formation of an unstable protein structure, which is further degraded in the cytoplasm, or to a truncated CFTR protein that does not reach the cell membrane. The latter is associated with premature termination of translation of stop codons	Mostly severe
Class II	**Defective protein maturation (formation of its secondary and tertiary structures). The protein does not reach the cell membrane and is destroyed in the cytoplasm** CFTR mutations lead to formation of small defective channels or to complete absence on the cell surface due to defective protein processing and transportation	Mostly severe
Class III	**Defective chloride channel response to cAMP stimulation** Normal amount of non-functional protein (almost not detected in the Russian population) is formed in the cell membrane	Mostly severe
Class IV	**Chlorine channel narrowing** CFTR mutations cause formation of a protein with a normal response to cAMPstimulation, but low amplitude of the ion current and shorter residence time of the channel in open state	Varies / mild
Class V	**Reduced amount of functionally active protein, defective protein transportation to the cell membrane** CFTR mutations lead to a reduction in the amount of mRNA and disruption in the translation process. As a consequence, insufficient amount of chloride channels is formed for maintenance of ion homeostasis	Mostly mild

Men with mucoviscidosis (cystic fibrosis) almost always have azoospermia and infertility due to bilateral aplasia of vas deferens (OMIM: 277180 – congenital bilateral absence of vas deferens, CBAVD).

In some cases, men are diagnosed with “genital” form of cystic fibrosis that leads to infertility with almost total absence or existence of minimal clinical manifestations of cystic fibrosis (CF).

It is believed that up to 70 % of men, who are suffering from infertility and have azoospermia (absence of sperm in the semen) or severe oligozoospermia, are carriers of CFTR mutations. Moreover, the mutations are often detected only in one allele.

Clinically absence of vas deferens is often not detected. Therefore, all patients with azoospermia, especially those whose sperm count is less than 1.5 million and pH<7 are recommended to be screened for CFTR mutation carrier in order to avoid CBAVD.

For genetic causes of infertility, treating patients with conservative methods (hormone therapy) was ineffective, but application of IVF and ICSI allowed these patients to have their own children. Moreover, given the high population frequency of carriage of CFTR mutations, the risk of having a child with CF in such a situation is significantly high. In this regard, genetic diagnosis of CFTRmutation carrier in women is necessary. If a mutation is detected in a woman in a couple, it is necessary to schedule preimplantation or prenatal genetic diagnosis, as there is a high probability of having a sick child (25 %) (Table 5). Genetic diagnosis may also be recommended for the patient's close relatives.

A conference of the National Institute (USA) in 1997 recommended that all pregnant couples and those planning pregnancy, even those without a family history of CF, be offered screening for CFTR gene mutations.

In the US in 2005, the FDA recommended molecular diagnostic tests for diagnosis of cystic fibrosis. According to Order No.185 of the Russian Ministry of Health, dated March 22, 2006 "On mass screening of newborns for hereditary diseases", cystic fibrosis diagnosis was included in neonatal screening. According to Order No.1605n dated December 28, 2012 "On approval of the standard for specialized medical care in cystic fibrosis (mucoviscidosis)", regulations on identification of genes associated with mucoviscidosis were introduced in the list of laboratory studies.

Indications for genetic analysis:

- In a complex of diagnostic methods for establishing or verification of CF diagnosis;
- For pre-implantation and prenatal diagnosis of CF in known mutations in a proband and the proband's parents;
- Given the high frequency of CFTR mutation carriage, it is recommended to identify carriage in relatives of patients (degree I and II) and their husbands/wiveswhen planning for pregnancy;
- As part of a package of measures during planned pregnancy, especially in consanguineous marriages;
- In a complex of diagnostic methods to establish the causes of infertility in a man (especially at bilateral or unilateral aplasia of the vas deferens and/or obstructive azoospermia);
- When deciding on the use of assisted reproductive technologies to fight infertility.

Table 5. CFTR gene mutations associated with cystic fibrosis

GENE	GENE FUNCTION	MUTATION	GENOTYPE	CLINICAL MANIFESTATIONS
CFTR – cystic fibrosis transmembrane conductance regulatorr	Encodes a protein functioning as a cAMP-dependent chloride channel	F508del	**NN**	Without features
			Nm	Degree of protein damage corresponds to class II
			mm	
		dele2,3 (21kb)	**NN**	Without features
			Nm	Degree of protein damage corresponds to class I
			mm	
		2143delT	**NN**	Without features
			Nm	Degree of protein damage corresponds to class I
			mm	
		W1282X	**NN**	Without features
			Nm	Degree of protein damage corresponds to class I
			mm	
		N1303K	**NN**	Without features
			Nm	Degree of protein damage corresponds to class II
			mm	
		3849+10kbC>T	**NN**	Without features
			Nm	Degree of protein damage corresponds to class V
			mm	
		2184insA	**NN**	Without features
			Nm	Degree of protein damage corresponds to class I
			mm	
		G542X	**NN**	Without features
			Nm	Degree of protein damage corresponds to class I
			mm	

*** Note:** N – normal, m – mutation, NN – homozygote (normal), mm – homozygote (mutation), Nm – heterozygote (mutation)

The set of data that can be obtained through genetic testing for the presence of AZF deletions and CFTR gene mutations in a man allows to take a reasoned decision on patient management tactics and to choose the optimal ART scheme (Table 6).

Table 6. Algorithm of genetic testing when abnormalities of the spermogram are detected

Genetic testing	Results of spermiological analysis		
	Azoospermia	Severe oligozoospermia (sperm count < $10*10^6$)	Moderate oligozoospermia (sperm count < $10-20*10^6$)
Cytogenetic analysis	During diagnosis. At the beginning of the VRT program	During diagnosis. At the beginning of the VRT program	Infertility for 1 year. At the beginning of the VRT program
Search for AZFmicrodeletions	During diagnostics (**no obstruction**). At the beginning of the VRT program	During diagnosis. At the beginning of the VRT program	
Molecular genetic analysis of CFTR	**Obstructive azoospermia.** At the beginning of the VRT program	During diagnosis. At the beginning of the VRT program	

Diagnosis of female infertility

Infertility in marriage is an important factor that reduces the reproductive potential of the population. It is observed in 4-5 million women. In Russia, there are 6.5 million registered women suffering from infertility. Moreover, prevention of diseases, including diseases that lead to infertility, is clinically more effective and economically more feasible than treatment.

Besides, there is a steady increase in the frequency of pregnancy complications (80 %) and delivery complications (70 %); the proportion of normal birth does not exceed an average of 33 % (2006).

The problem of miscarriage is one of the urgent problems of obstetrics. At present, almost 20 % of pregnancies are terminated before a viable fetus is born. Not every pregnancy can be saved as sporadic early losses are a manifestation of natural selection, due to the abnormal karyotypes of 60 % of abortuses.

Nevertheless, up to 40 % of early miscarriages and up to 80 % of late miscarriages occur with embryo/fetus with normal karyotype and can be quite preventable. 35 % of all pregnancies proceed with threat of miscarriage at all stages of pregnancy – the most frequent obstetrical pathology.

Identifying the causes of recurrent pregnancy loss requires high-tech examination methods: genetic, immunological, thrombophilic, endocrine, microbiological, virological and functional methods.

Contribution of inherited (genetically determined) thrombophilia in the structure of reproductive failures and gynecological complications deserves special attention.

IDENTIFICATION OF GENE POLYMORPHISMS ASSOCIATED WITH THROMBOPHILIA

Thrombophilia (from two Greek words: thrombus, meaning "clot," and philo, meaning "lover of") is the state of the blood system, which manifests itself in hemostasis disorder and has a propensity to develop recurrent vascular thrombosis (mostly venous) of different localization and often occurring in connection with pregnancy, after surgery, injury or physical overstrain. 30-50% of the disease is caused by genetic disorders in the blood cells, as well as defects in the blood coagulation system.

Structurally, the following are singled out in the hemostasis system:

- plasma (clotting factors and fibrin formation – **F2**, **F5**, **F7**, **F13**, **FGB** and **PAI-1** genes);
- platelet (platelet adhesion to the vascular wall, vasoconstriction, platelet aggregation, thrombus formation – **ITGA2** and **ITGB3** genes).

Functionally, the following systems are singled out: *coagulation*, *anticoagulation* and *fibrinolysis*.

Certain polymorphisms make different contributions to development of pathological conditions. The most important are F2 and F5 gene mutations. Defect of coagulation factor V (Leiden mutation) is transmitted over autosomal dominant inheritance. As a result, factor V mutation becomes resistant to the effects of activated protein C, which leads to uncontrolled thrombosis in the venous and arterial beds, microcirculation zones, and including in the placenta vessels. The defect of coagulation factor II is also transmitted through autosomal dominant inheritance and causes high risk of thrombosis. The influence of other genetic factors is less pronounced. However, a combination of several polymorphisms significantly increases the risk of developing thrombophilic states.

It should be noted that in women, the genetically determined tendency to develop thrombophilia is often clinically manifested during pregnancy in the form of thrombosis and obstetrical complications due to the features of the hemostasis system during physiologically proceeding pregnancy.During pregnancy, the tendency to develop blood stasis, combined with hyper coagulation, contributes to development of thrombosis and thromboembolism; in this case, venous thrombosis dominate (80 %). Genetic factors increase the risk of thromboembolism in pregnant women by many times. Therefore, genetic diagnosis of thrombophilia for the prevention of obstetric complications should be performed before pregnancy.

However it should be remembered that genetic predisposition to thrombophilia is not a diagnosis, but a tendency to develop thrombosis and other cardiovascular diseases in certain situations. In order to understand whether genetic predisposition is occurring, the state of patients should be monitored via routine laboratory tests (Table 7).

Indications for genetic analysis:

a. Repeated thrombosis.
d. Thrombosis of unclear etiology after 50 years.
b. Thrombosis at any age if there is a family history.
d. Thrombosis of unusual localization (portal, mesenteric and cerebral vein thrombosis)
a. High-risk situations:
- Massive surgical intervention;
- Pregnancy and postpartum period;
- Use of hormonal contraception or hormone replacement therapy;
- Prolonged immobilization.

a. Belonging to the risk group
b. Family-run nature of the disease
c. Early start
d. Atypical severe course
e. Tolerance to therapy

Apart from the factors considered, prescription of oral contraceptives and menopausal hormone therapy (MHT) is an extremely important issue. It is shown that hormonal contraception and MHT themselves slightly increase the risk of thrombosis. However, the danger increases sharply with carriage of a certain genotype.

According to the Medical Eligibility Criteria for Contraceptive Use released in 2012 and the fourth edition of the Medical Eligibility Criteria for Contraceptive Use developed by WHO in 2009, to prevent thrombosis and thromboembolic complications in those taking oral contraceptives, it is recommended to identify thrombogenic mutations (F2 – prothrombin mutation, and F5 – Leiden factor).

According to the Eligibility Criteria, the use of drugs containing estrogen in women with Leiden thrombogenic mutation is contraindicated (fourth category of the Eligibility Criteria). In prescribing a contraception based on progestin or copper-bearing IUDs, or hormone-bearing IUDs, women with thrombogenic mutations (F2 and F5) fall into the second category of the Eligibility Criteria (drug intake under medical supervision).

Genetic analysis reveals gene polymorphisms of factors and components of the hemostasis system, which lead to their (factors and components)abnormal synthesis or disruption of functional activity. This allows to assess the risks of cardiovascular disease and obstetrical complications, thromboembolism, venous and arterial thrombosis.

Screening of the genetic features of thrombophilia helps to early identify patients at risk and make appropriate adjustments in the management tactics.

Table 7. Polymorphisms of genes associated with risk of thrombophilic states, their possible manifestations and laboratory control

Gene	Polymorphism	Protein product	Prevalence in the population	Laboratory control	Manifestation and risk, or*
Plasma hemostasis unit. Coagulation system					
FGB	**455 G>A**	Fibrinogen, beta subunit	A allele: 20-30 %, AA genotype: 5-10 %	• Identification of fibrinogen • Thrombin time (shortening) • Integrated assessment of hemostasis – thromboelastogram (hypercoagulable changes in formation of the platelet-fibrin clot structure)	• Tendency to hyperfibrinogenemia • Myocardial infarction • Lacunar infarcts of the cerebral vessels OR>2.6
F2	**20210 G>A**	Prothrombin	A allele: 1–4 %	• PR • INR • Prothrombin time • Thromboelastogram	• Increase in plasma prothrombin levels • Venous thrombosis OR=2.5–3.8 • Early myocardial infarction OR5>[10] Myocardial infarction in smokers OR>40 • Ischemic stroke OR=1.4. For children OR>4 • Pelvic organ prolapse **During pregnancy:** • Venous thrombosis in the III trimester and 3 months after birth OR=30 • Loss of fetus in the first trimester OR=4.6 • Fetal hypotrophy • Recurrent miscarriage

Gene	Polymorphism	Protein product	Prevalence in the population	Laboratory control	Manifestation and risk, or*
F5	**16916 G>A** (Arg506Gln, Leiden mutation)	Proaccelerin	A allele: 2–6 %	• Protein C • Protein S • Calculation ofantithrombin III levels • Thromboelastogram • APTT (activated partial thromboplastin time)	• Venous thrombosis of the lower extremities, OR=3.8-9.45 • Risk of thromboembolism, OR>18 for AA homozygote • Repeated episodes of venous thromboembolism OR=4 • Risk of cerebral vascular thrombosis and ischemic stroke, angiopathy • Myocardial infarction in patients without acute coronary stenosis OR=2to8 • Venous thrombosis during use of COC, OR=10-15 (for heterozygotes); OR>30 for AA homozygotes **During pregnancy:** • Recurrent miscarriage • Fetal death in the later stages: in the second trimester OR=6, in the III trimester OR=9 • Reduced risk of fetal death throughout the first trimester OR=0.16 • Success in IVF embryo transfer OR=2
F7	**0976 G>A** (Arg353Gln)	Factor VII	A allele: 14–16 %	• Thromboelastogram • Calculation of clotting factor VII levels • Activity of factor VII (for treatment with indirect anticoagulants) • APTT	• Reduced risk of myocardial infarction, even at noticeable coronary atherosclerosis OR=0.47 • Risk of bleeding during anticoagulant therapy • Severe hemophilia

Gene	Polymorphism	Protein product	Prevalence in the population	Laboratory control	Manifestation and risk, or*
F13A1	**103(163) G>T** (Val34Leu)	Plasma transglutaminase, fibrin-stabilizing factor	T allele: 40 % (TT homozygotes: 2–3 %)	• Thromboelastogram • Calculation of clotting factor XIII levels • Activity of factor XIII • APTT	• Reduced risk of venous thrombosis OR=0.7-0.8 • Reduced risk of myocardial infarction and stroke, in the case of high fibrinogen levels • Increased risk of delayed bleeding • Subarachnoid hemorrhage **During pregnancy:** • Recurrent miscarriage
Plasma hemostasis unit. Coagulation system					
SERPINE1 (PAI-1)	**675 5G>4G**	Plasminogen activator inhibitor-1	4G allele: 50–60 % (4G/4G homozygotes: 58 %)	• Thromboelastogram with test-tube activation of fibrinolysis • Activity of plasminogen • Protein S level • Thromboelastogram with test-tube activation of fibrinolysis	• Reduced blood fibrinolytic activity • Thrombosis OR=1.7 • Myocardial infarction (with ITGB3:1565C - variant) OR=6.4 for men; OR=4.5 for women • Cardiovascular disease OR=1.5 for 4G/4G homozygotes • Venous thrombosis in protein S deficiency for 4G/4G **During pregnancy:** • Recurrent miscarriagefor 4G/4G • Preeclampsia OR=1.62 for 4G/4G; OR=1.49 for 5G/4G • Reduced likelihood of IVF embryo implantation for 4G/4G homozygotes

Gene	Polymorphism	Protein product	Prevalence in the population	Laboratory control	Manifestation and risk, or*
Platelet unit of hemostasis					
ITGA2 (VLA-2 receptor, GpIa)	**807 C>T** (F224F)	Platelet glycoprotein Ia	T allele: 40 %	• Calculation of platelet count • Platelet aggregation test	• Cardiovascular disease OR=3 • Aspirin resistance OR=3.76 **During pregnancy:** • Risk of fetal loss in the early stages for TT homozygotes
ITGB3 (GpIIIa, GP3A)	**1565 T>C** (L33P, PIA1/PIA2)	Platelet glycoprotein IIIa, platelet fibrinogen receptor	C allele: 15-18 %	• Calculation of platelet count • Platelet aggregation test	• Myocardial infarction OR=2.8-6; in PAI-1; 4G - variant; OR=6.4 for men; OR=4.5 for women • Resistance to aspirin therapy **During pregnancy:** • Early fetal loss OR=2.7-4.4 • Recurrent miscarriage

IDENTIFICATION OF GENE DEFECTS IN FOLATE CYCLE ENZYMES

Genetic defects in folate cycle enzymes – **MTHFR**, **MTR** and **MTRR** – serves as a thrombogenic risk aggravating factor, as well as an independent factor in fetal abnormalities and reproductive failures.

Folate cycle defects lead to accumulation of homocysteine in the cells and increase in the overall level of homocysteine in the plasma. Homocysteine has a strong toxic, atherogenic and thrombophilic effect. Polymorphisms in folate cycle genes are associated with the following phenomena (Table 8):

- Pregnancy complications (placental insufficiency, premature detachment of normally situated placenta, late preeclampsia);
- Fetal development defects (defective closure of the neural tube, anencephaly, deformation of the facial skeleton);
- Intrauterine fetal death, recurrent miscarriage;
- Homocystinuria (recurrent thrombosis and thromboembolism, ectopialentis, early osteoporosis and multiple bone deformities);
- Cardiovascular diseases (ischemic heart disease, myocardial infarction, atherosclerosis, atherothrombosis);
- Carcinogenesis (colorectal adenoma, breast cancer and ovarian cancer);
- Increased side effects of chemotherapy.

Table 8. Genetic polymorphisms associated with folate cycle defects

Gene	Polymorphism	Risk allele, prevalence in the population, %	Possible polymorphism manifestations
MTHFR (methylenetetrahydrofolatereductase)	677C>T (Ala222Val)	TT – 7–1 3 % CT – 35–40 %	• Additional thrombophilia risk factor • Late preeclampsia, other pregnancy complications • Early miscarriage risk • Congenital disorder • Cancer risk • TT homozygotes: recurrent miscarriage
	1298 A>C (E429A)	CC – 10–11 % AC – 40 %	• Increased need for folate • Congenital disorder • When receiving folic acid antagonists in the I trimester, increased risk of congenital disorder especially neural tube defects or cardiovascular defects • Cancer risk

Gene	Polymor-phism	Risk allele, prevalence in the population, %	Possible polymorphism manifestations
MTR (methionine synthase)	2756 A>G (D919G)		• CC homozygotes: recurrent miscarriage • Hyperhomocysteinemia • Risk of cardiovascular diseases • Obstetric forms of pathology • Placental insufficiency related to vitamin B12 deficiency • Defective closure of the medullary canal, Down syndrome
MTRR (methionine synthase reductase)	66 A>G (I22M)		• Increased risk of breast cancer in carriers of BRCA gene mutations • Hyperhomocysteinemia • Risk of cardiovascular diseases • Neural tube defects in the fetus • Association with low B12 plasma levels

The following are dietary factors (folate deficiency in a diet) of folate deficiency states and hyperhomocysteinemia:

- Prolonged use of methotrexate, anticonvulsants and other drugs (folic acid antagonists, aspirin, Biseptol), oral contraceptives and estrogens;
- Diseases of the stomach and intestines with B12 vitamin malabsorption;
- Malignant tumors of the pancreas and intestines;
- Long-term chronic infections;
- Renal diseases.

Indications for genetic analysis:

- Scheduled preparation for pregnancy;
- Elevated homocysteine blood levels (hyperhomocysteinemia);
- Miscarriage, fetal death in the II and III trimesters of pregnancy;
- Birth of a fetus with isolated defects of the neural tube, heart or urogenital tract;
- Antiphospholipid syndrome;
- Prescription of oral contraceptives and hormone replacement therapy;
- Family history of cancer;
- Chemotherapy;
- Coronary artery disease, hypertension, atherosclerosis and atherothrombosis.

Practical guidelines for genetic polymorphisms associated with folate cycle defects:

- Folate-rich diet. High levels of folic acid (vitamin B9) stabilize the changed enzyme or promotes activation of alternative remethylation paths. Folic acid-containing products: dark green leafy vegetables (spinach, lettuce, asparagus), beets, carrots, Brussels sprouts, broccoli, tomato juice, yeast, liver, egg yolk, cheese, melon, apricots, pumpkin, avocados, beans, whole wheat and dark rye flour.
- If polymorphisms associated with folate cycle defects are detected in a child's genetic passport, a lifetime ban on vegetarian diet and support for weakened vitamin exchange are of great importance.
- Avoid coffee abuse (consuming more than 5 cups per day).
- Be wary of drugs that influence folate metabolism. Folic acid levels in the serum are lowered by a number of drugs: aspirin, Biseptol, anticonvulsants, estrogens, contraceptives, etc.
- When using COCs, prophylactic use of folic acid, vitamins B6 and B12 is recommended.
- Use of 400 micrograms of folic acid daily within 3 months before pregnancy and the first 3 months of pregnancy. Patients carrying the 677T allele are recommended to consume 4 mg/day of folic acid during the preconception period and throughout the pregnancy. During use of folic acid, relative vitamin B12 deficiency can occur. Therefore, prescription of folic acid should be combined with vitamins B12 and B6.
- It is important to draw the attention of the doctor to the fact that for a patient with unsuspected hyperhomocysteinemia, standard therapy at the hospital aimed at addressing preeclampsia manifestations may not be only ineffective but even worsen the patient's condition. This applies to drugs such as methionine and aminophylline very often used to treat preeclampsia. Methionine and eufillin significantly increase the homocysteine levels in the blood, which may include or complement the cascade of pathological reactions that lead to development of generalized microangiopathy and thrombophilic states.
- In the first trimester of pregnancy, it is not recommended to take dihydrofolatereductase inhibitors that block conversion of folic acid to its active form (for example, trimethoprim and sulfasalazine) and other folic acid antagonists (for example, carbamazepine, phenytoin, valproic acid and cholestyramine).

Additional tests:

- Homocysteine test;
- Folic acid test;
- Vitamin B12 and methylmalonic acid tests;
- Protein C activity test.

An integrated approach to identification of genetic polymorphisms associated with thrombogenic risks and folate cycle defects is justified also from the point of view that the genetic features of parents are inherited and affect the development of the child. Currently, Russian and foreign literature state that genetic disorders in the fetus and/or conditions at birth and postnatal period cause a high risk of neonatal stroke.

Observation of children born to mothers with Leiden mutation found that the risk of neonatal stroke is higher by 8.5 times than the population risk, and 2.1 times higher than when the mother has prothrombin gene mutation. In 68 % of cases (when testing 60 mother-child pairs), presence of C677T polymorphism in the MTHFR gene in a homo- or heterozygous state in the fetus, in combination with hyperhomocysteinemia in the mother, neonatal stroke was observed (Simchen M. J., 2009).

The cumulative effect of genetic polymorphisms of the hemostasis system and folate cycle on increased risk of developing thrombophilic states is reflected in current regulatory documents and standards of health care delivery.

DIAGNOSIS OF REPRODUCTIVE SYSTEM CANCERS

BREAST AND OVARIAN CANCER

The average morbidity rate of malignant tumors among the female population in Russia is 309.8 per 100,000 women. First on the list are breast tumors (19.4 %), followed by endometrial cancer (6.7 %), cervical cancer (5.2 %) and ovarian cancer (5.1 %).

In Russia, strategies for identification of malignancies are absolutely inadequate to modern capabilities of medicine.This shows the need for special screening programs.The mortality rate of patients with malignant diseases of the breast (died within the first year after diagnosis) in Russia is 11.9 %. The figure reaches 20.3 % for cervical cancer.

In Russia, medical care for women to detect breast diseases is provided by obstetricians/gynecologists, who have passed through thematic improvement on breast pathology (mammology). Women with identified cystic and nodular changes in mammary glands are sent to an oncology clinic to verify the diagnosis. After exclusion of malignant tumors, women with benign breast diseases are placed under medical observation by an obstetrician/gynecologist.

Thus, screening programs aimed at preventing and detecting breast disease in the early stages, with possibility of highly specific differential diagnosis of pathological states, is a priority in obstetrics and gynecology.

A significant number of cancer cases are hereditary and linked to carriage of mutations in certain genes received from one parent. Carriage of oncogenic mutations is widespread and recorded in 1-2 % of people in all populations.

Inherited mutations are characterized by their frequency of occurrence in the general population, as well as by their degree of penetrance (Table 9). Penetrance reflects the probability that the carrier of that genetic marker will develop the disease, cancer in this case. The higher the penetrance, the higher the probability.

- **Class I** mutations are rare in the general population, but have high penetrance. Example: BRCA1 and BRCA2 gene mutations in patients with breast cancer and/or ovarian cancer. About 50-70 % of inherited cases of such cancer are caused by mutations in one of these genes (often BRCA1).
- The **second class** of inherited oncogenic mutations has an average risk of disease. These mutations are also quite rare in the general population.
- The **third class** (low-risk mutations) is widespread in the population. The clinical significance of detection of this class of mutations is largely dependent on the presence of additional risk factors.

Table 9. Genetic predisposition to breast cancer

PENETRANCE	INCREASED RISK	GENES
High-penetrance	5–20 times	BRCA1, BRCA2
Moderate-penetrance	1.5–5 times	CHEK2, ATM, PALB2, NBN, RECQL3
Low-penetrance	Up to 1.5 times	CHEK2, ATM, PALB2, NBN, RECQL3

It is known that 5-10 % of cases of breast cancer and ovarian cancer are hereditary, and can be attributed to mutations in the **BRCA1** and **BRCA2** genes. Both genes increase the risk of breast cancer by 80-85 % in women as they approach 80 years of age.

BRCA1 and BRCA2 genes encode proteins involved in the regulation of DNA repair and thus maintain genome integrity. Families carrying BRCA1 and BRCA2 mutations show autosomal dominant inheritance of tumors.

It was shown that BRCA1 is an associated breast cancer. Unlike sporadic cancer, it has a higher degree of malignancy, a high incidence of estrogen and progesterone-negative tumors, incidence of medullary cancer expressed as lymphoid infiltration, pronounced therapeutic pathomorphism up to complete regression.

It is established that the survival rate of patients with hereditary cancer of the female reproductive system are significantly higher than in the total group of patients, regardless of the stage of treatment: 5-year survival rate of patients with hereditary breast cancer is 75% (and 43 % for all other forms of cancer).

BRCA1 and BRCA2 genes are not strictly specific for breast cancer. Pathological genotype BRCA1/2 increases the risk of developing ovarian cancer, stomach cancer, colon cancer, pancreatic cancer, bladder cancer, head and neck tumors, endometrial cancer, biliary tract cancer, as well as melanoma.

DNA-Technology LLC, together with the Blokhin Russian Cancer Research Centre, conducted joint research to determine the frequency of occurrence of BRCA1 and BRCA2 gene mutations described in the literature in an unselected sample of breast cancer patients in a Russian population on a sample of 1091 people.

The two organizations developed a set of reagents by real-time PCR for identification of polymorphisms associated with risk of breast cancer (Table 10).

Table 10. Genetic polymorphisms associated with breast cancer

Gene	Polymorphism (mutation)	Risk allele	Incidence		Risk assessment for different genotypes
BRCA1	185delAG	delAG	0.1%	Overall incidence of about 5.9 % in an unselected sample (1091 people)	Ins/Ins – population risk Ins/Del – high risk
	4153delA	delA	0.7%		Ins/Ins – population risk Ins/Del – high risk
	5382insC	insC	4.0%		Del/Del – population risk Del/Ins – high risk
	3819delGTAAA d	delGTAAA	0.2%		Ins/Ins – population risk Ins/Del – high risk
	3875delGTCT	delGTCT	0.1%		Ins/Ins – population risk Ins/Del – high risk
	300T>G (Cys61Gly)	G	0.4%		TT – population risk TG – high risk
	2080delA	delA	0.2%		Ins/Ins – population risk Ins/Del – high risk
BRCA2	6174delT	delT	0.2%		Ins/Ins – population risk Ins/Del – high risk

An important aspect of introducing screening programs on identification of carriers of BRCA1 and BRCA2 gene mutations in the activities of obstetricians/gynecologists is that breast cancer is observed in pregnant women aged 32-38 years with a frequency of 1 in 3,000 pregnancies. Moreover, early diagnosis of breast cancer in pregnant women or breastfeeding women is difficult because of physiological changes in the breast tissue (engorgement and increase in size). Therefore, breast tumors are often detected in pregnant women at a late stage and are characterized by adverse prognosis. At the same time, the incidence of ovarian tumors in pregnancy is 0.076-1.14 %. In these cases, there is a question about the appropriateness of surgery during pregnancy, which is associated with a high risk of abortion.

It is necessary to consider not only the predictive importance of test, but also the fact that carriers of BRCA mutations associated with breast and ovarian cancer have certain sensitivity to a number of specific drugs:

- Not sensitive to taxanes,
- Highly sensitive to anthracyclines and cisplatin.

Indications for molecular genetic testing:

- Oncological burdened family history (two or more cases of breast/ovarian cancer in the family of first and second degree relatives, breast cancer before the age of 50, ovarian cancer at any age, bilateral breast cancer, breast cancer in men);
- Breast cancer at a young age (before 45 years, or before 50 years where there is no opportunity to gather informative family history);
- Synchronous and metachronous bilateral breast cancer;
- Primary/multiple malignancies, including combination of breast cancer and ovarian cancer;
- Morphological features of breast cancer: three times negative (tumor ER-, PR-, HER2/neu-) and medullary breast cancer;
- Ovarian cancer, fallopian tube cancer, peritoneal metastases at any age;
- Breast cancer in men in personal and family history;
- Ethnic origin (Ashkenazi Jews).

A model for presentation of the test results is presented in the form for identified patient's genotype for the studied parameters and is accompanied by a comment (Fig. 9).

№	Name of research	Genotype	Note
1	BRCA1:185delAG	Ins/Ins	morm
2	BRCA1:4153delA	Ins/Ins	norm
3	BRCA1:5382insC	Del/Del	norm
4	BRCA1:3819delGTAAA	Ins/Del	norm
5	BRCA1:3875delGTCT	Ins/Ins	Normal
6	BRCA1:300 T>G (Cys61GLy)	T/T	Normal
7	BRCA1:2080delA	Ins/Ins	Normal
8	BRCA2:6174delT	Ins/Ins	Normal

Conclusion:

Mutations in gene (185delAG, 4153delA, 5382insC, 3819delGTAAA, 3875delGTCT, 300 T>G (Cys61Gly), 2080delA), BRCA2 (6174delIT) are not detected.

Fig. 9. Form for presenting the "Identification of mutations in BRCA 1, 2 genes" study results

If BRCA1 or BRCA2 gene mutations are detected in patients with breast and/or ovarian cancer, doctors are advised to:

- Consider the possibility of carrying out contralateral mastectomy and bilateral salpingo-oophorectomy;
- Consider the use of specialized drugs for targeted therapy in breast cancer and ovarian cancer (PARP inhibitors: olaparib, veliparib and others);
- Discuss the possibility of carrying out genetic testing to detect germinal mutations in BRCA1 or BRCA2 gene in adult first-degree relatives.

Absence of BRCA1 and BRCA2 mutations do not exclude the possibility of genetically caused breast cancer and other oncopathologies associated with genetic disorders. In this regard, patients that fall into the risk group are recommended to go for the second stage of genetic testing to identify moderate-penetrance predisposition genes in breast cancer.

The **CHEK2 gene** (cell-cycle checkpoint kinases 2) encodes a protein called cell cycle checkpoint regulator, which is involved in DNA repair processes and in cell division regulating processes. Mutations in the gene are related to the second class of mutations in genes that participate in DNA replication, transcription, recombination and repair; these genes encode various enzymes (e.g. polymerase, helicase, topoisomerase, etc.) and cell cycle regulating proteins. Defects in these genes lead to chromosomal instability and therefore can lead to different cancer diseases.

The clinical significance of detection of CHEK2 gene mutation is not lower than that of BRCA1 and BRCA2 genes, especially in the case of familial cancers. It is recommended to carry out test for a number of mutations in these genes as a second-line test if a negative result for carriage of BRCA1 and BRCA2 mutations is obtained. Furthermore, a number of genetic syndromes, whose main characteristic is a predisposition to chromosomal instability,is described. For some of such syndromes, association with heterozygous carriage of CHEK2 gene mutation has been established.

In the CHEK2 gene, three mutations are the most important: 1100delC, IVS2+1G>A and 470T>C (Ile157Thr).

Mutation 1100delC in the CHEK2 gene is prevalent in many countries. Mutant allele frequency is 1.1-1.4% in the European population. It was shown that 1100delC mutation is associated with breast cancer. A large-scale study by the International consortium Breast Cancer Case-Control Consortium, which included over 10,000 breast cancer patients and 9,000 healthy women from five countries, found that the hazard ratio for carriers of 1100delC mutations in the CHEK2 gene is 2.34.

Apart from a clear association with development of breast cancer, it was established that 1100delC mutation is associated with prostate cancer.

The **IVS2+1G>A mutation** in the CHEK2 gene is rarer than 1100delC and leads to formation of a non-functional protein. Breast cancer is first and foremost associated with emergence of oncological diseases of different localization. It is more common among residents of Eastern Europe and North America. In a large sample of Eastern European popula-

tion (about 2000 patients), it was established that IVS2+1G>A allele is clearly associated with development of prostate cancer.

Missense mutation 470T>C (Ile157Thr) in the CHEK2 gene is associated with Li–Fraumeni syndrome, breast and prostate cancers, cancer of the colon and rectum, both sporadic and familial. This mutation is more frequent than 1100delC and IVS2+1G>A and it is found in the population (with a 4-5 % frequency). On average, the presence of Ile157Thr mutations of the CHEK2 gene increases the risk of breast cancer to a lesser extent than other carriage of other CHEK2 mutations.

A model for presentation of the test results is presented in fig. 10.

№	Name of research	Genotype	Characteristic
1	CHEK2:1100delC	Ins/Ins	norm
2	CHEK2:IVS2+1G>A	G/G	norm
3	CHEK2:470T>C(Ile157Thr)	T/C	Pay attention

Conclusion:
470T>C(Ile157Thr) substitutionwas detected in CHEK2 gene in a heterozygous state.
CHEK2 gene mutation (1100delC, IVS2+1G>A) is not detected.
It is recommended to consult a geneticist and oncologist.

Fig. 10. Form for presenting the "Oncogenetics CHEK2" study results

Indications for genetic testing:

- Family history (breast cancer, prostate cancer, or colorectal cancer in first-degree relatives);
- One or more relatives with the same type of tumor;
- Atypical proliferative breast diseases;
- Multiple primary tumors in the same organ;
- Multiple primary tumors in different organs;
- Multiple primary tumors in paired organs;
- Multifocality within one organ;
- Tumor manifestations at an early age;
- Two or more relatives with rare forms of cancer;
- Two or more relatives with tumor related to family cancer;
- Three or more relatives in two generations with tumors of one localization;
- Negative test result for BRCA1 and BRCA2 gene mutations.

CERVICAL CANCER

An important aspect in the diagnosis of malignant tumors of the reproductive system is the timely detection of cervical cancer development factors. This disease is the second most prevalent (after breast cancer) and accounts for about 14 % of all malignancies in women.

Russia has a high level of advanced stages of cervical cancer – over 40 % of all diagnosed cases. More than 6,000 women in Russia die of cervical cancer every year. In the 16-40 age group, cervical cancer is the second leading cause of death in cancer patients after breast cancer.

Studies of Georgios Papanicolaou (Γεώργιος Παπανικολάου) showed that using cytology (Pap smear, PAP Smear Test), taken from the cervix and cervical canal can identify the stage of precancer (dysplasia) and early stages of cervical cancer. In 40-s of XX century it was the first formulated theoretical basis of cytological screening of cervical cancer.

Cervical cancer (CC) - virtually is the only oncological disease which satisfies all the conditions recommended by the WHO for screening of population.

Numerous studies have shown that the proportion of screening coverage of the female population is a priority in order to prevent CC. Unfortunately, no country cannot provide 100 % cytological screening today. According to WHO experts (Csaky-Szunyogh Of M., 2014), cervical cytology screening strategy should be based in the **most rational distribution of available resources which are always limited** to maximize the effectiveness of screening.

Today, it is established that human papillomavirus infection is the primary etiologic agent of cervical cancer. It has been shown that the DNA of human papillomavirus (HPV) is detected in over 99.5 % of all biopsies of cervical cancer patients.

There are more than 120 types of HPV, of which about 30 can infect the urogenital tract epithelia. In terms of ability to induce malignant transformation of the epithelium, HPVs are divided into two groups: **low-oncogenic risk** and **high-oncogenic risk** types.

The high-oncogenic risk group includes the following HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, 82. Here, type 16 is the most prevalent in Europe and is detected in more than 50 % of all cervical cancer cases.

The low-oncogenic risk HPV types include 6, 11, 36, 42, 43, 44, 46, 47, 50. Infection caused by these types usually occurs in the form of benign cervical lesions and warts in the throat.этого нет в русском варианте.

Thus, cervical cancer – one of the few malignancies neoplasms with established etiology of the disease. This discovery enabled a completely new approach to the problem of cervical cancer screening. The genetic material of the virus – is an excellent target for laboratory diagnostics, allowing use for joint screening by molecular genetic techniques. The main advantages of these methods: objectivity, manufacturability and affordability. Exactly the use of molecular genetic techniques allowed to provide the main condition for effective cervical cancer screening – e.g. maximum coverage population. Accordingly, for the purposes of screening-test system should identify the maximum number of the most common highly oncogenic HPV types in the population.

A large number of studies conducted in different countries have shown that molecular genetic methods have a higher clinical sensitivity, whereas cytology method is characterized by high clinical specificity for the detection of neoplastic changes (Czeizel A. E., 2009). This means that the use of molecular genetic method for screening identifies more women with symptoms of neoplastic transformation (less likely "miss" the cancer), but the number of false-positive screening results (healthy women us mistakenly included in the risk group) will be higher than when using cytology method. **Thus, screening by identifying HPV provides 60-70 % better 5-year protection against cervical cancer compared to cytological screening.**

Liquid cytology has a number of advantages compared to conventional cytology methods. Although it is an expensive technology that requires advanced techniques and highly qualified personnel, but even when using liquid-based cytology, diagnostic sensitivity of cytology does not reach that of HPV screening.

Recommended by the WHO a key issue for the choice of strategies for screening of cervical cancer is the availability of resources for the HPV test (Comprehensive cervical cancer control. A guide to essential practice. Second edition. / World Health Organization, 2014). As a priority, are considered two possible strategies:

- HPV test, then test with acetic acid.
- HPV test, colposcopy then.

Both of these strategies are consistent with the concept of a **two-stage screening**.

The experience of many years of observations of the 176 464 women aged 20-64 years in Sweden (Swedescreen), in Netherlands (POBASCAM), in England (ARTISTIC) and in Italy (NTCC) clearly showed that the most effective is **use of molecular genetic techniques as a first step of screening** (Czeizel A. E., 2009). Moreover, all the **HPV-positive patients necessarily require additional (cytology or other) study** for a choice of tactics of further observation and, if necessary, treatment. **All HPV-negative patients do not need any following observation within the next 5 years**, which allows for 80-90 % release the cytological laboratory from unnecessary work.

Particularly noted that the holding of so-called "simultaneous" screening when all patients immediately examined by molecular genetic and cytological study is inappropriate because it does not provide the best protection against cervical cancer, compared with only HPV study, but essentially overloads cytological laboratory with meaningless work.

In the absence of resources for HPV screening WHO recommends the following strategy: cytology, colposcopy then (Comprehensive cervical cancer control. A guide to essential practice. Second edition. / World Health Organization, 2014). Importantly, if the cytological screening is selected as the primary method, HPV-test is not used at all.

Detection of HPV by PCR method also allows establishing viral load.

A significant advantage of the approach proposed by DNA-Technology LLC is the ability to maintain **simultaneous implementation of qualitative and quantitative multiplex analysis** (Fig. 11).

Quantification of HPV DNA is represented by two types of analysis: **absolute** and **relative**.

Under absolute type of analysis, after amplification, the amount of copies of the DNA virus in the sample is automatically calculated. For relative type of analysis, normalization of the amount of DNA virus in the human genomic DNA is used.

№	Name of research	Results		
		Relative, (X/SIC)	Quantitative, Lg (copies/sample)	Qualitative
1	HPV 31	not detected	not detected	
2	HPV 35	not detected	not detected	
3	HPV 16	1.6	7.4	
4	HPV 52	not detected	not detected	
5	HPV 33	not detected	not detected	
6	HPV 68	–3.4	2.4	
7	HPV 45	not detected	not detected	
8	HPV 82	–1.5	4.3	
9	HPV 51	not detected	not detected	
10	HPV 6	1.2	7.0	
11	HPV 44	not detected	not detected	
12	HPV 11	not detected	not detected	
13	HPV 18	not detected	not detected	
14	HPV 39	–1.9	3.9	
15	HPV 58	not detected	not detected	
16	HPV 66	not detected	not detected	
17	HPV 26	not detected	not detected	
18	HPV 53	not detected	not detected	
19	HPV 59	not detected	not detected	
20	HPV 56	–0.6	5.2	
21	HPV 73	–2.1	3.7	
22	SIC		5.8	

Fig. 11. Form for presenting the "Quant-21" study results

CONCLUSION

The team of authors would like to thank you for your interest and hope that the innovative methods performed via real-time PCR will help obstetricians/gynecologists and doctors of related disciplines in addressing a wide range of practical tasks, helping to improve the quality of diagnosis, treatment efficacy and quality of life of patients.

A health care course on modernization, economic assessment of new medical technologies and import substitution should be an important aspect that promotes active introduction of modern examination methods. In this regard, real-time PCR techniques described in this guide are extremely of vital importance for they are entirely Russian invention, they have an optimal clinical & economic performance ratio and they attract lower costs. Replacing low-informative outdated technologies with new ones will enable to reduce patient examination time, carry out primary and secondary prevention of diseases and help address demographic problems.

Please accept our sincere respect and gratitude for a long-term and fruitful cooperation.

The DNA-Technology team.

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AUTHORS:

Baranova Elena E., Ph.D. (Medicine)
Bateneva Elena I., Ph.D. (Medicine)
Galkina Irina S., Ph.D. (Chemistry)
DonnikovAndrey E., Ph.D. (Medicine)
Zorina Victoria V., Ph.D. (Biology)
Tumbinskaya Lydia V., Ph.D. (Biology)
Shigorina Galina G.

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CONTACT OFFICE:
DNA-Technology LLC, 125 Zh, Varshavskoye Highway, bldg. 6, Moscow, Russia.
Phone / Fax: +7 (495) 640-17-71, www.dna-technology.ru, mail@dna-technology.ru

CUSTOMER SUPPORT:
8 800 200-75-15 (free call from within Russia), hotline@dna-technology.ru