Background of the project

Premature ovarian failure (POF) defined as a secondary hypergonadotropic (FSH > 40IU/I) amenorrhoea occurring before the age of 40, affects approximately 1 % of females and its aetiology is still unknown is most cases. In particular, an association between POF and abnormalities of the X chromosome has been reported for several times and the balanced X-autosome translocations have been reported to be associated with POF despite their generally neutral clinical effect. In fact, the breakpoints of these aberrations are distributed over the whole X chromosome but in many cases they cluster in a critical region between Xq13 and Xq26. Deletion of the X chromosome reduce both fertility and reproductive lifespan and the basis of studies two loci for Xq-linked POF have been postulated: deletion in POF patients have been localised to chromosome Xq21.3-Xq27 (POF1), while balanced X/autosomal translocations have been localised to Xq13.3 – q21.1 (POF2). However, it is interesting that there are several genes have consistently found to be involved in POF on the X chromosome and autosome but the POF1 region deletions are by far more common as being associated with POF phenotype. Other abundant reason of the POF is the single gene association which is also can involve the POF phenotype. Out of the single genes group the FMR1 gene is almost most significant cause of POF. The association between FMR1 premutation and POF has been previously investigated at a molecular level by analysing FMR1- related factors such as the repeat tract size. Expansion of (CGG) triplet repeats in the FMR1 is associated with several disorders, including fragile X syndrome (FRAXA), fragile X-associated tremor/ataxia syndrome (FXTAS), and fragile X-associated primary ovarian insufficiency (POF). POF (age at cessation of menses <40 years) occurs in approximately 20 % of females who have an FMR1 premutation gene (with the premutation alleles, which may have 55 to 200 CGG). The known association between POF and premutation alleles in the FMR1 gene prompted us to initiate fragile X testing. The risk for premature ovarian failure would be assiciated with CGG range and it suggesting that high normal (under 40/45) and intermediate-gray zone (40/45-55) range repeats can also been associated with the risk for milder forms of premature ovarian failure. The FMR1 gene encodes the protein FMRP, which is an RNA binding protein and plays a significant role in the transport processes of RNA, stabilization of RNA molecules, and mRNA translation. The FMRP protein is expressed in all tissues, but it was detected especially in the brain, ovary and testis tissues in significant amounts. Studies proved in fruit flies and mice, that due to the lack of the FMRP protein some translational disturbances occur, which affect the early neuronal development, neurotransmission processes and synaptic connections. The premutation and full mutation cases are not only different in respect of the phenotype, but also the expression of the FMRP protein. The hyper-expression of the FMRP protein was confirmed in cases of premutation allele carriers, and the presence of the increased, more than normal level CGG repeat transcripts have a significant role in the development of the

phenotype. In cases of the full mutation carrier FRAXA patients, the high CGG repeat numbers are in association with the heterochromatin condition, which results in the reduction/absence of the FMRP protein transcription. Experiments have shown that in the case of the premutation allele, the transcription is characterised by active histone modification, while the full mutation allele is characterised by the hypermethylated condition, which is associated with hypoacetylated histone proteins. The premutation involvement of the FMR1 gene was described in 3-15% of the premature ovarian failure (POF), more recently known as primary ovarian insufficiency (POI) cases.

The risk of POF increases if the CGG repeat number of the FMR1 gene exceeds 40, or if it falls in the so-called grey zone (41-54) .The involvement of the defects of certain genes in the POF disease shows wide variation, the involvement of the FMR1 gene was described most frequently.

The possible links between the premutation status of the POF/POI and the FMR1 gene were explained with two theories.

1. One theory is that the protein FMRP, which is an RNA binding protein, encoded by the FMR1 gene, could be expressed in larger quantities in the premutation carriers, and could have a suppressor effect on the translation of mRNAs encoded by a variety of other genes. Based on these, it is also believed, that the reduced transcription of the genes involved in the oocyte maturation might result in a reduced follicle stock [8, 9].

2. According to the other theory, the accumulating mutant FMR1 mRNA could have a long lasting toxic effect on the ovary and this results in the premature destruction of the follicles. The background of the pathogenicity of the other phenotype associated with the premutation status, the tremor/ataxia syndrome (FXTAS), is still not fully understood.

At 40-45% of male premutation carrier and 8-16% of female carriers showed tremor/ataxia syndrome, wherein the first symptoms were described at over 50 years of age.In male carriers of the premutation allele the development of the tremor/ataxia syndrome (FXTAS) was described with an incidence rate of 1/800, while the rate was 1/250 in females. The classic phenotype of the FXTAS is the kinetic tremor and cerebellar ataxia, and other symptoms include cognitive impairment, psychiatric disorders, peripheral neuropathy and autonomic nervous system problems. The symptoms start about 50 years of age, and its progression can show a great variety, the life expectancy can be 5 to 25 years following the appearance of the symptoms. The additional neurological examinations of the relatives carrying the premutation status can give an opportunity to detect any possible tremor/ataxia syndrome involvement. In addition, the neurological examinations of the premutation carrier female relatives could give an opportunity for the

introduction of new test methods by mapping the differences with a potential diagnostic significance,

According to the second theory, the expression of the toxic RNA can be associated with various clinical diseases, which is caused by both the proteins bound to the RNA, and also the influencing the alternative splicing mechanism of other genes. The increased FMRP protein levels result in neuronal toxicity, which can be detected in neurons and astrocytes as eosinophilic and ubiquitin positive nuclear inclusions in the cerebrum, thalamus, basal ganglia, cranial nerves and spinal cord.

Our aim was to analyse the relationship between the paternally inherited premutation (PIP) and the maternally inherited premutation (MIP) by the examination of the family members of women with POF, carrying the premutation allele, which was confirmed by molecular genetic testing.

Project objective:

Our study aim was to analyse the relationship between trinucleotide repeat length and reproductive outcome in large cohort of POF patients. Premature ovarian failure (POF), also known as primary ovarian insufficiency (POI) can significantly influence the reproductive chances in case of women of fertile age. The diseases associated with the mutations of the FMR1 (Fragile X Mental Retardation 1) gene belong to the group of the so-called trinucleotide expansion diseases.

Our aim were the following :

i.) to design and produce reliable diagnostic panel to give risk of POF disorder at the early adult women in order to provide a good facility to protect the ability of the fertilisation

ii.) to validate the new POF and fertility problem disease diagnostic panel in characterised and uncharacterised patients

iii.) to develop new powerful patients database with clinical and molecular genetics results

iv.) to identify new correlation between the CGG repeat number of the FMR1 gene and the risk of POF

v.) to define the exact frequency of the X chromosome abnormalities (deletion and translocation) in hungarian population.

Methods (the reliable diagnostic panel)

Patients of the 1st Department of Obstetrics and Gynaecology, Semmelweis University with suspected premature ovarian failure (POF) were included in the study, after prior information and consent. The enrolment criteria were the following: secondary amenorrhoea, the ovaries stopped functioning before 40 years of age, FSH≥40 IU/l in two different measurements and low estrogen levels. Exclusion criteria were if a patient had a surgery before, significantly affecting the follicle

stock in both ovaries, or medication was used, damaging the ovarian function (photostatic treatment).

Molecular genetic and cytogenetic (G-banding cytogenetic analyses) examinations were performed in cases of women with suspected premature ovarian failure. Southern blot analysis was performed during the molecular genetic analysis and the exact CGG repeat number was determined by the Repeat Primed polymerase chain reaction (RP-PCR) method.

Southern blot analysis: DNA was isolated from blood samples of the patients, and the genomic DNA was digested with restriction endonucleases EcoRI and EagI at 37° C all night long. Southern blot analysis was performed on the samples following the digestion, the samples were run on 0.5% agarose gel for 18 hours at 40 V voltage. As a result, the DNA samples digested by restriction endonucleases, were separated by size. After the separation the sample DNA was blotted from the agarose gel to the nitrocellulose membrane by the capillary principle. The membrane was probed with the radiolabeled Stb12.3 DNA probe (FMR1 gene specific), using hybridisation at 65 ° C all night long. The principle of the method is random priming technique. The unbound radioactive probe was removed by incremental solutions (at 65°C). Using a Packard Instant Imager device we performed the rapid measurement of the radioactive signals of the fragments, their preliminary assessment; then we put a Kodak XOMAT X-ray film on the membrane, and exposed at -100°C for 4-5 days. At this rating the normal fragment size in the healthy (normal) control group can be measured at 2.8 kb, and about at 5.2 kb in accordance with the inactive X. In the premutation cases the fragment size was measured between 2.9-3.3 and 5.3-5.7 kb.

RP-PCR: The exact CGG repeat number was determined by the Repeat Primed (RP)-PCR technique. The genomic DNA sample was diluted to 20 ng/μl concentration, then we used 2 μl for the LCD reaction. The PCR reaction was performed with three primers, the FMR1 gene specific primers (forward and reverse, FAM-labeled) and a so-called CGG repeat primer together. The resulting PCR products were separated by capillary electrophoresis according to size, and the amount of the product can be determined based on the fluorescent signal intensity.

Cytogenetics analyses: Chromosome analysis was performed on stimulated peripheral blood cultures on metaphase cells with trypsin and Wright Giemsa stain. In some cases Fluorescence in Situ Hybridization (FISH) analysis were carried out on methanol/acetic acid-fixed suspensions. Slides preparation for FISH was made according to standard techniques. X, Y centromere specific probes as well as X Painting probe (Cytocell, United Kingdom) were used for evaluation of sex chromosomes and their possible hidden structural abnormalities. Spectrum Green CEP X and Spectrum Red SRY gene specific probe (Abbott, Germany) was used to detect chromosome X copy

number and to control the presence of SRY gene on chromosome Y respectively. The samples and probes were denatured and hybridized using the ThermoBrite Denaturation/Hybridisation System by programming 10 minutes of denaturation at 82°C, followed by overnight hybridization at 37°C. Post hybridization wash was performed in 0.4X SSC/0.1% NP-40 (72°C, 2 minutes) followed by a wash in 2X SSC/0.1% NP-40 (room temperature, 2 minutes). The slides were air dried in the dark, then counterstained with 10 micro liters of DAPI (4,6-diamidino-2-phenylindole). The fluorescence microscope (Axioskop 2 Mot Plus, Carl Zeiss MicroImaging GmbH Heidelberg, Germany) equipped with a Hamamatsu C 4800 CCD camera and PSI Cytovision 3.6 (Scientific Systems, UK) computer analysis systems were used for the evaluation of the results). Karyotypes and FISH results were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2009).

Array CGH analysis: Array CGH analysis was performed according to the manufacturer's protocol on genomic DNA ISCA plus design array of Nimblegen Roche containing 1.4 M probes per sub array. This microarray provides a mean average resolution of approximately 15-20 Kb on chromosomes to detect chromosomal imbalances throughout the whole genome. The CGH protocol involves independently labelling of the patient (test DNA) and the reference genomic DNA (Human Genomic DNA, Promega Madison, WI U.S.A.) with Cy3 and Cy5 dyes using a NimbleGen Dual-Color DNA Labelling Kit (Roche NimbleGen Inc.). Cohybridization of these DNAs to a NimbleGen CGH array were performed for 72 h at 42 C°. Following hybridisation the array are washed and dried at room temperature using the wash buffer kit (Roche NimbleGen Inc.). Array CGH were scanned on NimbleGen MS 200 microarray scanner and data are extracted and analysed using NimbleScan software and SignalMap and Deva 1.1 software (Roche NimbleGen Inc.). DNA CNVs were mentioned as gain or loss as a linear ration, and the length of the variation was given in megabase (Mb).

Project results

Due to premature ovarian failure, a total of 210 patients underwent genetic testing. We examined the FMR1 gene trinucleotide repeat number in DNA samples of the patients, and in 28cases (13%) we detected deviations (CGG repeat number corresponding to premutation or grey zone). In 7 cases out of the 28 cases the CGG repeat number fell within the range of the so-called grey zone (41-54 CGG repeat). In 4 cases out of 15 cases we found differences in both alleles, one was a premutation allele (55-200 CGG repeat), and the other allele showed a repeat number belonging to the grey zone. Out of 210 cases 9 patients had detectable cytogenetic abnormalities (7.2%).

At the confirmed premutation and grey zone cases we completed the determination of CGG repeat number of the FMR1 gene of the family members, and studied the maternal-paternal inheritance process. Out of 28 cases, only maternal inheritance (MIP) was detected in only 2 cases, in one case the premutation allele (91 CGG repeat number), while in the other case an allele belonging to the grey zone (41 CGG repeat number) were inherited from their mothers. During the inheritance there was no expansion (CGG repeat number increase) observed.

In 5 cases, the premutation allele was inherited from the father, and the repeat number ranged from 55 to 133. Expansion of the repeat number could be observed in 4 of these cases during the inheritance process (75→79, 88→90, 70→74, 87→89 CGG repeat number expansion). Among the tested male relatives, in 3 cases we confirmed the neurodegenerative differences in the presence of premutation or grey zone alleles.

In 5 cases out of 10 cases (i.e. 50% of cases) the patient inherited a paternal allele with a repeat number within the grey zone. The repeat number varied between 41 and 46. Interestingly, in one case, the normal paternal allele showed mosaic expansion (40 repeat number), the patient had repeat numbers 22/42/52 in a mosaic form, that means that most likely 40→42 and 40→52 repeat number expansion occurred during the inheritance of the paternal allele.

In 3 cases out of 15 cases maternal and paternal inheritance was simultaneously observed (PIP and MIP). In each case, the inherited paternal allele was a premutation allele, while the patient inherited the maternal allele with repeat numbers within the grey zone.

Interestingly, in one case we detected a mosaic form in the father, with the 52/64/76 CGG repeat numbers. The patient inherited the paternal allele with the 76 CGG repeat number, while the inherited maternal allele was the 52 CGG repeat number allele.

In 3 cases the expansion rate of the CGG repeat number observed in the family exceeded 200, so full mutation occurred. In one case, the patient's son, in another case his brother and in one case other family members were diseased.

We investigated the presence of AGG interruptions playing a role in the repeat number expansion. The number of AGG interruptions was 3 in almost 50% of the cases, only in one of these cases occurred CGG repeat number expansion. If the number of AGG interruption was 0-2 (total of 8 cases), there were 4 cases of CGG repeat number expansion of the examined patients, and if the patients and their family members were taken into consideration together, the overall incidence of CGG repeat expansion was 7.

At one patient we detected one FMR1 allele of X chromosome which was the 2.8 Kb unmethyleted and the 5.2 Kb methylated allele was failed. The following role was to make a molecular cytogenetic assay which was a specific cytogenetics array CGH analysis. At this case we identified a large deletion on the X chromosome (measure: 67.355 Mb) and exact breakpoints location ChrX:87842016-155255380 (ChrX q21.31-q28) based on the Human genome GRCh37/hg19 assembly. In order to verifying the array CGH results we perfomed a specific FISH analysis. The whole painting chromosome X FISH probe did not disclose X chromosome balanced translocation and identified a normal and smaller X chromosome with this justified the array CGH results.

We have made the RP-PCR analysis at 40 control female samples also in order to define the normal CGG trinucleotide repeat range at the Hungarian population.

<u>Towards goals</u>

Project objective

The present challenge lies in increasing detection rate as well as abbreviating the time-to-diagnosis for patients and families via characterising the exact CGG repeat number the FMR1 gene and X chromosome abnormalities (deletion and translocation) and reducing analysis costs by using platforms with new molecular genetics technique Reapet Primed-PCR, FISH. These two goals will allow us to characterize the genotype also in those cases which carrying 42-55 CGG repeat of the FMR1 gene (grey zone) and obvious premutation status and in those cytogenetic aberration whose pathophysiology is multigenic.
One of the main objectives of the project result is to greatly reduce the time of the detecting POF/POI disease and verifying whether those genetic mutations or chromosomal aberrations impact the patient conditions.

Scientific objective:

The primary aim of our research group was the clinical and molecular genetic analysis of women involved in the POF/ POI disease, and the determination of the exact CGG repeat numbers with the new RP-PCR method. In the confirmed premutation cases we mapped the maternal and paternal inheritance process by the examination of the relatives.

Based on our results, in case of paternal inheritance the FMR1 gene premutation CGG repeat number in inherited to the next generation with about the same number of repeats. In our studies we did not detect any CGG repeats rising to full mutation status in the offspring generation in paternal inheritance cases. In the examined samples we detected premutation status in both the male and the female offspring.

In case of maternal inheritance, the full mutation status is much more likely to occur in the offspring, depending on the premutation CGG number; and the number of the so-called AGG interruptions. The higher the premutation repeat number of repeats and the lack of AGG interruptions, the more likely is the full mutation status occurring in the next generation. The difference between the paternal and maternal inheritance is due to the difference between the male and female gamete maturation.

The determination of the number of AGG interruptions within the CGG repeat has a key role. If the number of AGG interruptions is 1 or 0, we have to consider the increase of the CGG repeat number occurring in the offspring, which definitely increases the risk of the FRAXA premutation or full mutation in the case of a mother carrying a grey zone (41-54 CGG repeat number). So we can say

that during family planning the clinical geneticist should always consider the exact CGG repeat number, and also the number of AGG interruptions to give reliable results for a personalized risk assessment. Using the RP-PCR technique, these interruptions can be detected and quantified, for which we could predict the risk of CGG expansion possible in further generations. Therefore, in the premutation cases with higher CGG repeat numbers, the prenatal CGG repeat number diagnosis is definitely justified in order to determine the possible symptomatic fragile X syndrome.

Based on our experience, among the molecular genetic test methods the Southern blot analysis is suitable to detect the full mutation cases, higher premutation status and to isolate the methylated and unmethylated X chromosome in the female specimens. The RP-PCR method can be used to define the smaller premutations and the exact CGG number. Due to the quantitative nature of the RP-PCR, it is possible to detect the mosaicism as well.