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Analysis of the genetic background of patients with Turner syndrome: karyotype-phenotype associations and pregnancy-associated complications

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Introduction

Premature ovarian insufficiency (POI) is a medical condition affecting 1-2% of women according to previous studies, but more recently international research has shown that it affects even more, 3-4% of women, and manifests itself as hypergonadotropic hypogonadism. It is characterized by early menopausal symptoms before the age of 40 and the progressive decrease followed by the cessation of ovarian function. Genetic causes have a pronounced impact on its pathophysiology: a wide range of chromosomal abnormalities and gene mutations are known to cause POI.

Identifying these genetic abnormalities is essential, because besides ovarian dysfunction, they can cause other diverse symptoms, organ abnormalities and co-morbidities, thus, detecting the exact genetic background can improve the identification of associated medical conditions and can be a key factor in providing targeted therapy for affected individuals.

Premutation of the FMR1 gene is an example of a genetic abnormality that causes early ovarian failure, which results from the expansion of the CGG trinucleotide repeat and leads to fragile X syndrome when the full mutation is present. In carriers, that is, in the presence of a lower number of the trinucleotide repeat sequence called premutation, development of POI has an almost 20% chance, therefore it is important to exclude its possibility in case of screening for primary or secondary amenorrhoea. Other genetic mutations such as BMP15, LHR, FSHR, INHA, FOXL2 gene mutations and the presence of POI have also been investigated. Turner syndrome (TS) is the most common genetic cause of premature ovarian insufficiency, affecting 1 in every 2500 births. Pseudoautosomal genes on the X chromosome are inactivated by haploinsufficiency, resulting in a severe reduction in the expression of the affected genes and loss of function of most of the proteins they produce. The syndrome itself is known for its diverse spectrum of co-morbidities, mainly in the manifestations of gynecologic, cardiologic and endocrine diseases. Several karyotypes can result in the TS phenotype: in addition to the classic 45,X karyotype, other karyotypes may be caused by mosaicism (e.g. 45,X/46,XX), structural rearrangements (e.g. Xq isochromosome, X ring chromosome), deletions (e.g. 46,Xdel(X)) or mosaic abnormalities involving the Y chromosome (e.g. 45,X/46,XY). As the majority of co-morbidities can cause long-term health problems, early diagnosis can help to ensure early intervention and improve disease outcomes and quality of life. An individualized, multidisciplinary approach to the care of patients with Turner syndrome is needed to address

the challenges arising from its complexity and diverse co-morbidity spectrum, while being able to integrate both physical and mental health concerns.

The development of Turner syndrome, as in many other recognized diseases, is characterized by multiple genetic and epigenetic variability, which is responsible for the diverse manifestations of the disease. The expression of DNA hypomethylation, post-translational modifications, or epigenetic alterations generated by microRNAs has been shown to affect cognitive function, the cardiovascular system, or metabolic pathways in the presence of TS. The most likely genetic theory for the development of the disease is the phenomenon of haploinsufficiency, which plays a role in the phenotypical features caused by chromosomal aberrations. Another theory is that epigenetic influence of imprinting may also play a role in the development of chromosomal alterations. Due to the loss of the second sex chromosome, the epigenetic regulation of gene expression is essentially modified, which manifests in DNA methylation processes and gene downregulation, affecting mostly autosomal genes.

In recent years other karyotypes have become the focus of research, as several data have supported the hypothesis that the differences between classical and non-classical karyotypes in Turner syndrome patients may be expressed in a broad spectrum of symptoms and co-morbidities. However, the highest number of cases remain to have the classical 45,X karyotype with the absence of one sex chromosome. In case of mosaicism, two or more cell lines with different chromosomal features can be found in the same individual, hence in TS cases, additional karyotype variation can arise through mosaicism, which can result in different phenotypes. This has encouraged clinicians to completely change screening and treatment strategies. The most pronounced disease features are usually seen in classical karyotypes, while mosaicism or structural abnormalities (ring chromosome, isochromosome, deletion) usually result in a milder manifestation. The presence of the Y chromosome in some karyotypes may be associated with an increased risk of malignancies such as gonadoblastoma, which may justify elective surgery for risk reduction.

Besides specific phenotypic features such as short stature, delayed puberty, epicanthus, flat face, low-set ears, gothic palate, pterygium, shield-shaped chest, widely spaced nipples, micrognathia or pigmented naevi, concomitant cardiovascular, gynecologic, endocrine, gastroenterologic, neuropsychiatric and autoimmune diseases are seen with an increased prevalence in TS patients compared to the general population, therefore, these abnormalities should be targeted, diagnosed, treated and followed up as early as possible, aiming at the best possible quality of life.

International data suggest that not only may the aforementioned phenotypic features differ, but also the presence of co-morbidities can vary depending on the genetic background, including karyotype and mosaicism variations. Cardiovascular complications are the most common malformations, the best-known being hypertension, tachycardia, aortic stenosis, mitral valve prolapse, tricuspid valve insufficiency, atrial septal defect, coarctation of the aorta, ductus arteriosus, aortic insufficiency. Karyotype-phenotype associations suggest that phenotype is significantly influenced by the expression and number of proteins encoded by X chromosome genes, therefore, the presence of haploinsufficiency, additional epigenetic processes or the presence of mosaicism have major influence on the final phenotype. Although the best-known karyotype is the classical 45,X, which is associated with the most prominent features, it should not be forgotten that a wide spectrum of other karyotypes shows certain phenotypic features and co-morbidities, and some macroscopic features may even be present in a greater proportion than expected.

In TS caused by mosaicism or other chromosomal abnormalities, phenotypic features and co-morbidities may be present in different proportions, which may require different screening strategies depending on the karyotype. In the long-term follow-up of TS patients, the transition from pediatric care to adult care requires special attention, as most observational studies show that a significant proportion of patients disappear from medical care mostly during this transition period. For the worsening of some co-morbidities incomplete or irregular follow-up or the lack of diagnostic or screening tests may be responsible. It is important that the role of high-prevalence diseases and the importance of follow-up should be emphasized to these patients, because follow-up is challenging due to the diverse spectrum of the possible co-morbidities. The most critical may be the management of cardiovascular disorders, as hypertension, tachycardia, congenital anomalies like mitral valve prolapse or coarctation of the aorta. These can lead to serious adverse events if not properly managed, which can contribute to increased mortality. In addition to cardiovascular abnormalities, it is important to highlight the close association between Turner syndrome and the presence of autoimmunity, which can manifest in numerous endocrine and metabolic disorders. In a large number of cases hypothyroidism or Hashimoto thyroiditis, diabetes, obesity, celiac disease or metabolic syndrome may be present, this complex effect can have a very negative impact on the general condition, as well as osteoporosis due to estrogen deficiency caused by POI.

Timely onset of estrogen replacement has a positive effect not only on puberty and the development of secondary sexual characteristics, but it also plays an important role in the development of the uterus. The role of mental health avoiding neuropsychiatric disorders has

also an outstanding importance in patients with Turner syndrome, as they may have higher prevalence of anxiety, depression or learning problems compared to the general population. A multidisciplinary approach is essential to address the many challenges faced by the disease, ensuring that patients receive effective care. TS patients are usually characterized by primary or secondary amenorrhoea and infertility due to POI, but this dominance may differ in case of karyotypic differences and mosaicism.

Late diagnosis or inadequate care may result in a negative change in the quality of life of TS patients, not to mention the fact that they have basically unfavorable reproductive chances at reproductive age. Although spontaneous puberty occurs in only 5-10% of patients, the development of assisted reproductive techniques (ART), and oocyte donation have offered a new approach. Irrespective of the method of conception, a multidisciplinary team is required from the preconception period followed by proper cardiologic, endocrine, genetic, pediatric and obstetric care throughout the pregnancy. Considering that TS-associated pregnancies are classified as high-risk, their care is advised to be managed in a tertiary centre with centralized obstetric and neonatal care, because increased incidence of miscarriage, preeclampsia, preterm birth, stillbirth and fetal growth retardation is known. Although the chances of spontaneous conception are lower than in the general population, we already have a growing knowledge of the fertility characteristics and outcomes of TS patients, but there are still many unanswered questions that need to be answered to understand the differences in the mechanisms that cause the syndrome. These include the understanding of intrauterine growth restriction and the long-term outcome of pregnancies regarding offspring karyotype.

Objectives

1.The aim of our study was to establish a comprehensive database of patients with Turner syndrome treated in the pediatric and gynecologic endocrinology care of our tertiary care center.

2. In order to individualize the follow-up strategy, we examined associations between age at diagnosis and specific karyotypes (Iso(Xq),Ring(X)), or karyotype subgroups (classical, non-classical, mosaic, non-mosaic), as well as phenotypic characteristics of Turner syndrome and co-morbidities.

3. The other aim of our study was to analyze pregnancy characteristics and outcomes of TS patients enrolled in our cohort with short and long-term neonatal outcomes.

Methods

This dissertation was performed based on a retrospective data analysis (ethical approval: DE RKEB/IKEB 5953-2022). Data of 75 patients diagnosed and treated with Turner syndrome at the Department of Obstetrics and Gynecology, University of Debrecen, between January 1, 2009, and January 1, 2023, were included in the data analysis using our medical databases. Inpatient and outpatient data, imaging tests and diagnostic results, collected from local (Med Solution, UDMed) and national (EESZT-Elektronikus Egészségügyi Szolgáltatási Tér) electronic medical databases in gynecology, pediatrics, neonatology, endocrinology, cardiology, internal medicine, urology, psychiatry, otorhinolaryngology, ophthalmology, neurology, dermatology and genetics were organized.

All patients diagnosed and followed up during the period were included, using anonymous data. In addition, we also assessed the short- and long-term obstetric and neonatal outcomes of TS patients who were pregnant during this period, and we analyzed the outcomes of a total of 10 pregnancies. Newborns were divided by gestational age: extreme preterm babies were born before 28 weeks' gestation, preterm babies were born between 28 weeks and 36 weeks and 6 days' gestation, and term neonates were born between 37 weeks and 38 weeks and 6 days' gestation. During pregnancy, all patients underwent routine prenatal care according to national guidelines, with additional steps with multidisciplinary surveillance specific for Turner syndrome.

While one patient had ART by oocyte donation, three patients had spontaneous conception, amniocentesis was performed after second trimester genetic counseling to determine the karyotype of the fetus. In every case the diagnosis was confirmed by karyotyping using peripheral blood lymphocytes. All patients with TS were screened for co-morbidities according to local protocols following the recommendations of the International Turner Syndrome Consensus Group published in 2017.

Fourty-five patient-specific variables were evaluated and categorized (phenotypic characteristics especially TS specific features, growth failure, presence of gynecologic, cardiologic, endocrine, gastroenterologic diseases, diseases affecting the sensory organs, mental health or kidneys). Baseline characteristics and TS-specific phenotypic features, vital parameters were recorded during the physical examination at diagnosis. After confirmation of TS, echocardiography, abdominal ultrasound, audiometry, ophthalmologic examination, and routine blood tests (TSH, thyroid function by fT4 test, thyroid autoimmunity screening by anti-TPO test, diabetes screening by HbA1c test, and celiac disease screening by anti-tTG test) were

performed. As recommended by international guidelines, testing was repeated every 1-5 years, depending on the disease being tested and the baseline results. Genital organs were evaluated by gynecologic examination, by ultrasonography abnormal findings were described as streak gonads, underdeveloped/hypoplastic uterus or thin endometrial layer. Psychiatric evaluation was performed only if symptoms of mental disorder were present. Obesity was defined as a BMI >30 kg/m2. If body height was below the 3 percentiles of the age-specific growth rate, recombinant growth hormone (rGH) treatment was recommended. If initial karyotyping occurred before birth, karyotyping was performed later on from peripheral lymphocytes. Fetal cytogenetic analysis during prenatal care was performed by amniocentesis and confirmed by postnatal lymphocyte examination from peripheral blood. One patient underwent in vitro fertilisation in a foreign hospital in Europe, where the patient's sister's oocyte was fertilized and implanted after preliminary cytogenetic analysis.

The samples were evaluated at the Cytogenetics Laboratory, Department of Obstetrics and Gynecology, University of Debrecen. Prenatal diagnostics was achieved on long-term cell cultures: in addition to fetal karyotyping, the test was available for further cytogenetic or molecular analysis provided the detection of mosaic cases.

In case of urgency due to advanced age of gestation, both chorionic villus and amniotic fluid samples were analyzed, firstly for a quick diagnosis and to obtain long-term assessment of cell cultures. After a short cell culture (72 h), further analysis was performed on 50-100 cells, and minor structural rearrangements were examined by fluorescence in situ hybridization (FISH), which was also appropriate for microdeletion detections. In adult cases cytogenetic analysis was performed by peripheral lymphocyte examination, similar to the postnatal evaluation. At least 30 lymphocytes were used for karyotyping, and in case of inconclusive results or suspected mosaicism, FISH was performed to confirm the results. All karyotyping was performed using G-banding technique, Giemsa staining was used to improve the staining of each band, and samples were analyzed after protein digestion with trypsin under a light microscope at 1000x magnification. In one case of a pregnant patient (later referred to as patient 1), the placenta and the umbilical cord were examined by routine histology. In this case, during caesarean section a histological sample was taken from the maternal ovaries with the patient's consent, and routine histological evaluation was performed by hematoxylin and eosin (H&E) staining with X-chromosome centromere-specific FISH.

FISH was performed using MetaSystems CEP X Orange Probe according to the local protocol of the Department of Pathology, University of Debrecen. Formalin-fixed paraffin-embedded slides were pretreated with a mixture corresponding to the FISH probe, dried and

covered with a cover glass. After denaturation at 75 °C for 2 min, the slides were incubated overnight at 37 °C in a wet cabin and post-hybridization washes were performed while maintaining pH and temperature. Post-staining was carried out with 10 microliters of 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Slides were stored at -20 °C in the dark until evaluation. Hybridization signals were estimated using a fluorescence laser microscope equipped with excitation and emission filters of different wavelengths, which visualized the red hybridization signal of the blue nucleus and the X chromosome centromeric region. Fluorescence in situ hybridization microscopic evaluation was performed on 50 ovarian cells (80% stromal cells and 20% follicular cells).

The incidence of each karyotype in the cohort and the age distribution of diagnosis were determined. To assess the role of each karyotype, comparisons were made between those with the classical 45,X karyotype and other karyotypes; between all mosaic and non-mosaic karyotypes; and between Xq isochromosome or X ring chromosome as chromosomal abnormalities. We hypothesized that differences in TS specific features and co-morbidities would be seen in early pre-menarche and late post-menarche diagnoses, so we compared these two groups.

Statistical analysis was performed using IBM SPSS Statistics for Windows version 25.0 software (IBM Corp., Armonk, NY). For continuous variables, Komogorov-Smirnov test was used to check the normality of distribution, and Levene test was used to determine the equality of variances. For parametric variables, independent samples t-test was used to compare the equality of means, and Pearson's correlation was used to determine the degree of correlation. For non-parametric variables, Spearman's correlation was used to determine the degree of correlation and Mann-Whitney U-test to compare means. Nominal variables were compared using the Chi-square test or Fisher's exact test. Logistic regression was used to determine odds ratios and the strength of association for binary outcomes. A p-value < 0.05 was considered statistically significant. Results were presented as mean ± SD, median, or odds ratio (OR) and 95% confidence interval (CI).

Results

In our study, the data of 75 patients were analyzed, with the most important inclusion criteria being available karyotyping results, which also led to the diagnosis of TS. Most patients were diagnosed in childhood or adolescence: 44 (58%) were diagnosed under the age of 12 years, 26 (35%) between the ages of 12 and 18 years, and only 5 (7%) were diagnosed over the age of 18 years. The average age at diagnosis was 9.6 years. Although the age at diagnosis

varied between 0 and 22 years, there were 2 peaks in the data where 8-8 patients (10.7-10.7%) were diagnosed. One peak was seen shortly after birth at the age of 1 year, and the other peak was at the time of expected first menstrual period at age 12 years. Since the manifestation and follow-up of several co-morbidities depends on the individual follow-up period, results were analyzed using the median follow-up period, which in our case was 14 years. In our population, the median age at diagnosis was 10 years, and the median age at the time of our study was 24 years.

Cytogenetic analysis of the 75 cases detected 13 different karyotypes. Six registered karyotypes were classified as non-mosaic, while 7 karyotypes were classified as mosaic subgroups. Forty-seven patients (62.7%) belonged to one of the 6 non-mosaic types, while 28 cases (37.3%) belonged to one of the 7 different mosaic karyotype subgroups. The most common karyotype was the classic 45,X, but there was also a high proportion of 45,X/46,XX mosaic karyotypes. Other less frequent variations were also present: 7-7 cases (9.3-9.3%) had an Xq isochromosome [i(Xq)] and an X ring chromosome [r(X)]. In 66.7% of all cases numeric variation was seen, in a further 13.3% structural variation was observed, and in the remaining 20% a combination of numeric and structural variation was found.

The incidence of short stature and pterygium colli were just above the significance level: comparing mosaic and non-mosaic cases, the incidence of short stature was 89% vs. 71% (OR 0.30, p=0.055) in the two groups, while pterygium colli occurred in 29% vs. 10% (OR 0.28, p=0.06). Hypertrichosis was only found in the non-mosaic group (27.7% vs. 0%). In terms of co-morbidities, mitral valve prolapse was diagnosed four times more frequently in the mosaic group compared to the non-mosaic group (21.4% vs. 6.4%, OR: 4.0). Compared with the results observed for all other karyotypes, the 45,X/46,XX mosaic karyotype (n=12) also had a higher prevalence of hypertension, which was found to be significant (3/9 cases, 33.3% vs. 4/66 cases, 6.1%, OR 7.75, 95% CI 1.39-43.08, p=0.019). Comparing the results of patients with other karyotypes and the classic 45,X karyotype, at least a tendency of differences, but in most cases significant differences were observed: the prevalence of short stature was 91% vs. 75% (OR 3.56, 95% CI 0.89-14.17, p=0.072), hypertrichosis was found in 28% vs. 7% prevalence (OR 4.9, 95% CI 1.23-19.72, p=0.02), while pterygium colli was reported in 34% vs. 12% (OR 3.65, 59% CI 1.13-11.74, p=0.03). Abnormal pelvic ultrasound findings, including the presence of streak gonads, were recorded in 60% vs 40% (OR 2.25 59% CI 0.89-5.68, p=0.086), but in that case the difference was not significant. Otherwise, there was no significant difference in the prevalence of co-morbidities between the two groups. Although there was no significant difference in clinical findings, there was a statistically significant difference in age and in height

and weight between the two groups evaluated, with an average delay of 0.7 years between diagnosis in patients with karyotype 45,X.

We also compared Xq isochromosome with other karyotypes and found a positive correlation with celiac disease: its prevalence was 28% vs. 3%, which difference was found to be statistically significant (OR 13.2, 95% CI 1.5-114.5, p=0.019). However, when comparing the karyotype containing the Xq isochromosome with the 45,X karyotype, no significant phenotypic difference was found.

More frequent karyotype was the X ring chromosome [r(X)], which was compared with the other karyotypes, but no significant difference was found in the parameters assessed. The group carrying the X ring chromosome had a lower incidence of short stature (57% vs. 85%), with a tendency of correlation (OR 0.23, 95% CI 0.045-1.186, p=0.079).

Comparing baseline height and weight values in cases diagnosed at age <12 years with those diagnosed at age ≥12 years, clear differences were seen. There was a tendency in BMI values, with higher BMI values at late diagnosis (23.4 vs. 26.2 kg/m2, p=0.066). Diagnosis at younger age showed a higher prevalence of specific TS features: short stature in 91% vs. 71% (OR 4.09), use of GH therapy was seen in 88% vs. 61% (OR 4.93), while underdeveloped breasts and widely spaced nipples were seen in 77% vs. 42% (OR 4.71), low hairline or flat face in 71% vs. 42% (OR 3.30), low-set ears in 64% vs. 39% (OR 2.52). Hypertension was less frequent in the early diagnosis group (2% vs 19%, OR 0.10), but mitral valve prolapse was more frequently reported (18% vs 3%, OR 6.67). There was a lower prevalence of hepatosplenomegaly in the early diagnosis group (4% vs 32%, OR 0.10) and in the three cases where epilepsy was diagnosed, the diagnosis was made before the age of 12 years (7% vs 0%). Next to the general phenotypic characteristics and co-morbidities of our patient we studied other parameters like obstetric-gynecologic and fertility data were specifically focused on. Spontaneous menarche was observed in twelve patients (16% of the patients), of these three patients experienced spontaneous pregnancy and one patient underwent in vitro fertilization by oocyte donation.

Ten pregnancies were identified, of which seven were live births (7/10, 70%), two ended with spontaneous abortion in the first trimester (2/10, 20%) and one was an arteficial abortion (1/10, 10%) in four TS patients (4/75, 5.3%). Regarding the karyotype of the patients, two cases had a classic 45,X and the other two had a 45,X/46,XX mosaic karyotype. One patient was diagnosed with Turner syndrome at <12 years of age, three patients at ≥12 years of age, two of them around the expected date of menarche and the fourth patient at late puberty.

All patients had short stature; three patients received GH therapy after diagnosis due to a height below the 5 percentiles of the age-specific height. No TS-related phenotypic feature was recorded in case of Patient 1 except short stature, but in the other cases, low-set ears, flat face, pterygium colli and hypertrichosis were observed. Maternal co-morbidities were mostly expressed such as hypertension, mitral valve prolapse, tachycardia, but no severe structural heart disease was observed. Hypothyroidism, benign frontal haemangioma and benign breast tumor were also present as other patient-specific abnormalities, all of them were already preconceptionally known and adequately followed up. Antenatal care was performed according to the national guidelines, with a multidisciplinary approach including obstetric, genetic, endocrine and cardiologic evaluations.

In cases where spontaneous pregnancy occurred, amniocentesis was performed in the second trimester after genetic counselling to determine the fetal karyotype. In one patient, in vitro fertilisation was performed using donor oocyte from the patient's sister, and pre-implantation genetic testing (PGT-A) revealed 46,XY karyotypes.

Patient 1 had a previous obstetric history of two pregnancies, both of them was conceived spontaneously, one was a term delivery and one arteficial abortion. Antenatal care in the third trimester was performed due to intrauterine growth restriction and oligohydramnios at the Department of Obstetrics and Gynecology, University of Debrecen. At 37 weeks of gestation, an elective caesarean section was performed, where histological examination of placental and umbilical cord samples and histological examination of maternal ovarian biopsy with fluorescence in situ hybridization (FISH) were made. Histological examination confirmed a physiologic placental and umbilical cord structure, while the maternal ovarian sample had a rich stromal structure (50% stromal, 50% normal ovarian tissue) and some sporadic primordial follicles were identified. FISH test confirmed the presence of mosaicism in ovarian cells. 13.2% of the stromal cells showed 1 hybridization signal and 6.8% showed 2 hybridization signals in X-chromosome centromere-specific FISH. Due to the low number of oocytes and the morphological difficulty to identify them, the sample was not adequate for the analysis of oocytes.

In case of Patient 2, an elective caesarean section was performed at 39 weeks of gestation, conceived by IVF with oocyte donation, the indication of surgery was unstable lie of the fetus. No fetal distress, intrauterine growth restriction, or oligohydramnios were described during pregnancy, and the neonatal birth weight was appropriate for gestational age.

In case of Patient 3 two pregnancies are known, both children have the classic 45,X karyotype, which were already detected during cytogenetic analysis during pregnancy. Her first

pregnancy underwent a caesarean section at 38 weeks of gestation due to intrauterine growth restriction and fetal distress. During her second pregnancy, an extremely preterm delivery occurred: the patient was referred at 24 weeks gestation due to lack of fetal movement to the Department of Obstetrics and Gynecology, University of Debrecen. In the early phase of observation severe IUGR and oligohydramnios were noted. The patient received steroid prophylaxis, but shortly after admission cardiotocogram showed severe fetal cardiac abnormalities, and an emergency caesarean section was performed. The operation resulted in the birth of a 300 g, extremely preterm baby, observation was continued at the neonatal intensive care unit (NICU). Thanks to the neonatal care general condition of the neonate showed a great improvement and he was discharged a few months after birth.

Patient 4 had a total of five known pregnancies, of which two were first-trimester spontaneous abortions, one preterm and two early-term deliveries. The indication for an emergency caesarean section at 36 weeks' gestation was severe fetal heart rate abnormality seen on cardiotocogram with known severe IUGR and oligohydramnios.

Expect the 1st pregnancy of Patient 3, third trimester antenatal care of each pregnancy was performed at the Department of Obstetrics and Gynecology, University of Debrecen. During the observation period, routine ultrasound, Doppler ultrasound, non-stress test and biophysical profile were performed. Different severity of IUGR and oligohydramnios were described in all pregnancies, fetal distress was observed at a certain point of observation in almost all cases, and caesarean section was performed in all cases. In case of Patient 3, during her 2nd pregnancy, an emergency caesarean section was performed due to severe IUGR and fetal heart rate abnormalities. All spontaneously conceived pregnancies showed other signs of placental insufficiency, such as oligohydramnios and fetal distress on cardiotocogram or Doppler ultrasound with IUGR. Caesarean section was performed in every case with fetal prophylactic or emergency indications. No maternal complications occurred during pregnancy, in peripartum or the puerperium.

Regarding neonatal outcomes, seven neonates were born (four girls and three boys), one case was known to have a long-term follow-up due to somatomental retardation. Prenatal and preimplantation cytogenetic testing confirmed 46,XX or 46,XY karyotype in five offspring and 45,X karyotype in two offspring. No severe congenital cardiovascular or other abnormalities were observed pre- or postnatally and the further neurological development of the offspring was favorable except in one case. Specific neonatal and neurologic complications occurred only in the case of the offspring born from the 2nd pregnancy of Patient 3, during which an extremely preterm neonate was born with an emergency caesarean section. Long-term complications

described can be explained by the nature of the complications of extremely preterm birth, although it is obviously difficult to separate from the abnormalities caused by preterm birth. The girl, who is currently 6 years old, is receiving ongoing follow-up for somatomental retardation and is known to have hypothyroidism, ophthalmologic disorders and short stature. Also, in case of Patient 3, the 14-year-old child born from her 1st pregnancy, is known to have short stature and strabism, which are considered to be TS-associated abnormalities.

Discussion

Turner syndrome is one of the most common non-heritable and life-compatible genetic disorders. Its negative impact on the whole genome, due to partial or complete loss of the second sex chromosome, can affect not only mental health or cognitive function, but also cardiovascular development, growth and metabolism, all of which contribute to the phenotypic features and complications that are characteristic of TS.

There is an established association between a variation in the SHOX gene located in the short arm Xp22.3 pseudoautosomal region of the X chromosome and short stature with other disease-specific signs. Due to chromosomal instability caused by genome-wide DNA hypomethylation and histone modification have been identified, such as mutations in the ZFYVE9 gene, which may be responsible for the increased incidence of aortic aneurysm and aortic dilatation, in the CNR1 gene and obesity, or in the IGFBP3 gene, which is important for its identified role in growth. Both international and our new findings highlight the importance of distinction between karyotypes with respect to mosaicism. In mosaicism genetic or epigenetic abnormalities affect only a certain percentage of cells, which results in a reduced prevalence of specific phenotypic features and co-morbidities. The genetic background of not all TS-specific features or co-morbidities is known.

In our research, we have tried to find correlations between the genetic background and some of the characteristics recorded. The originality of our study arises from the very small number of studies on TS patients investigating karyotype-phenotype associations in the international literature and the lack of a database of Hungarian patients, although the previous sparse multicentre studies have shown significant regional differences.

In our cohort, we found significant differences in the distribution of age at diagnosis of TS regarding childhood, adolescent and adult diagnoses. The peak ages at diagnosis were mostly observed at age 1 year and at the expected time of menarche, usually between 11 and 12 years of age. Strong phenotypic features are seen in the majority of the most typical cases, which may help early diagnosis, but even if these phenotypic features are absent, the absence

of first menstruation usually leads to further reproductive tests. This may explain the increasing number of diagnoses around the average age of menarche. In our study, we found clear differences according to age at diagnosis: earlier diagnosis was associated with a more pronounced expression of phenotypic features, which is probably the reason for earlier recognition of the disease. The positive impact on future care should be highlighted, as early detection and proper follow-up can prevent the onset of specific diseases or optimize medical care for existing diseases, leading to further improvements in quality of life.

Suboptimal care of young and adult female patients has been shown to result increased morbidity and mortality; however, TS alone is associated with increased mortality. Among our patients, karyotype was not associated with early diagnosis, a stronger association between the presence of phenotypic features and early diagnosis was found. Nevertheless, further questions arise when examining disease-associations by age at diagnosis. Age-specific manifestation of some diseases was seen only 2% of patients with early diagnosis had hypertension, compared with 20% of patients with late diagnosis. By the end of our follow-up period, the early diagnosis group was also significantly younger than the late diagnosis group (20.1±7.1 vs 32.2±9.2 years), which could explain a higher prevalence of hypertension in the late diagnosis group. However, whichever group is analyzed, they have a higher prevalence of hypertension than hypertension prevalence in the general populations of the same age, which suggests the causal role of TS.

While the prevalence of mitral valve prolapse was higher in the early-diagnosed group (18% vs. 3%), the presence of hepatosplenomegaly was more frequent in the late-diagnosed group (32% vs. 4%). In our study, mitral valve prolapse, and atrial septal defect were the two most common structural heart abnormalities, affecting 12%-12% of patients. The association between mitral valve prolapse and early diagnosis can be explained by the strong clinical manifestation in this group. However, it remains a question to why mitral valve prolapse is observed with higher incidence in the group of early diagnosed TS patients, in comparison to other cardiovascular abnormalities. For the increased prevalence of hepatosplenomegaly in the late-diagnosed group, it can be suggested that prolonged estrogen deficiency may play a role. Previous multicentric studies have shown a five-fold increased prevalence of elevated liver enzymes and cirrhosis in TS patients. In our data, we found it in 16% of the cohort, in most cases with the co-existence of steatosis. In comparison, it occurs in only 1-2% of the average US adult population. The prevalence of overweight in TS is also known (22% of our patients), but surprisingly, no significant difference in liver enzyme levels has been found in TS patients compared to normal weight patients. That is more likely that structural abnormalities in small nodules could be the basis of the abnormalities, leading to nodular regenerative hyperplasia

(NRH) or multiple focal nodular hyperplasia (FNH) and later to cirrhosis, which has been described in TS patients in former studies. Recent studies have demonstrated the positive effects of estrogen replacement on the liver in TS: results suggest that it reduces hepatic fat storage and inhibits insulin-induced signaling pathways in the liver. We can speculate that early-initiated estrogen replacement in patients with structural liver abnormalities in Turner syndrome improved liver function, and at the same time, in the late-diagnosis group, where we also found a higher incidence, estrogen replacement initiated later may lead to a significantly higher incidence of hepatosplenomegaly.

Not surprisingly, the prevalence of TS-associated phenotypic features and co-morbidities was higher in our patient population than in the general population. The distribution of karyotypes was also in agreement with the results of the larger cohorts: classic 45,X karyotype was the most common; in two thirds of the cases numeric variation was seen, in the remaining third mosaic karyotypes were observed. Comparing the patients with non-mosaic and mosaic karyotypes, some features such as short stature, pterygium colli, or hypertrichosis were significantly more frequent in the non-mosaic group. The higher prevalence of these TS-specific phenotypic features is due to the higher penetration which supports our hypothesis that patients with classic karyotype should be examined separately from other TS individuals. None of the comorbidities analyzed was found to be more frequent in the non-mosaic karyotype than in the mosaic karyotype when comparing 45,X karyotype with other karyotypes. When comparing 45,X/46,XX mosaic karyotype with other karyotypes, an increased risk of hypertension was observed (OR 7.75).

Although comparative analysis of different karyotypes was limited by the smaller number of cases, karyotypes containing the Xq isochromosome and the X-ring chromosome could be analyzed separately based on their number of cases. The only recorded significant difference was a higher incidence of celiac disease in the Xq isochromosome group (28% vs 3% in the group without the i(Xq) chromosome, OR: 13.2). The different number of gene copies on the Xq and Xp arms seems to be crucial in some karyotype-phenotype associations, therefore it seemed logical to investigate the possible differences in two subgroups: in the absence of Xq genes in the classical 45,X karyotype, and the increased presence of Xq genes in the 46,Xi(Xq) isochromosome. Based on our results, no significant difference was found in the prevalence of co-morbidities. Several teams have tried to establish a direct association between karyotype and phenotypic features or co-morbidities of Turner syndrome. As rare karyotypes are represented in relatively small numbers of cases even in large national analyses, it is not always possible to draw karyotype-specific epidemiological conclusions. For this reason, in these analyses rare

karyotypes are often pooled for statistical reasons, forming subgroups like "mosaic" or "Xq isochromosome-containing", as well as we also did in our study. On the one hand, this type of classification can help to categorise patients in risk groups, but it can also lead to conflicting results, as these groups do not include the same patients because of the different karyotype distribution. The diversity of results is further increased by the different prevalence of co-morbidities in different geographical areas and the characteristics of national screening strategies.

Examining the results of other international cohorts, a strong karyotype-phenotype correlation between 45,X/46,XX and 45,X/46,Xi(Xq) mosaic karyotypes and hypothyroidism was described. Otherside, increased mortality was reported for karyotype 45, X and isochromosome Xq, while karyotype 45,X/46,XX had the lowest incidence of co-morbidities. However, in other studies, the Xq isochromosome has been shown to be a protective factor for cardiovascular disease (less bicuspid valve insufficiency, decreased aortic diameter index), and a lower prevalence of hearing loss and hypothyroidism in this karyotype. In 45,X/46,XY mosaicism lower incidence of short stature, hearing loss or hypothyroidism can be seen. The X-ring chromosome was found to have a high prevalence of metabolic syndrome (elevated HbA1c, GGT, hypertension), and a higher rate of short stature. Other studies have reported an increased risk of metabolic syndrome and an atherogenic profile associated with the classic karyotype 45,X. The Xq isochromosome was associated with an increased risk of diabetes mellitus compared to the 45,X karyotype.

In the light of the literature data, our results raise the possibility of some novel associations: an increased incidence of short stature, hypertrichosis and pterygium colli in the non-mosaic karyotype especially in case of 45,X karyotype, an increased risk of hypertension in case of 45,X/46,XX karyotype and an increased risk of celiac disease in case of Xq isochromosome. No matter how strong the penetrance of the classic Turner features, international evidence also supports the recommendation to follow a tailored treatment and follow-up strategy for TS patients, depending on the karyotype.

Although POI may be one of the leading abnormalities of TS, the possibility of spontaneous conception, even if at a lower rate, still exists. But it is more likely in cases of spontaneous menarche or mosaicism. The rate of spontaneous conception among our TS patients was 5.3%, which was similar to the results reported by Bernard and his group (5.6%) and in other studies, ranging from 1.3% to 5.6% based on the literature. Our data have shown that three of our patients had spontaneous menarche followed by spontaneous conception and irregular menstrual cycles. Two patients had a mosaic karyotype, while one case had a classic

45,X karyotype. Previous studies have reported a higher incidence of spontaneous abortion in TS pregnancies besides spontaneous conception. This may be due to an increased rate of fetal chromosomal abnormalities, but associated maternal autoimmune diseases or low estrogen levels may also affect obstetric outcome. In our cases, spontaneous abortion occurred in two out of nine spontaneous pregnancies (22.2%), similarly to the 20-25% reported in the general population and slightly lower than the incidence rate seen in the French cohort (30.8%). It is not certain that all first trimester abortions are well documented, since in the patients with spontaneous menarche cycles are mostly irregular. The small number of cases does not yet allow us to draw long-term conclusions. There have been several international studies on IVF pregnancies in TS patients, but their association with spontaneous conception and obstetric complications is poorly documented.

In the largest cohort of cases, Bernard and colleagues have fertility data from nearly 500 TS patients, and their study is a very important landmark in Turner syndrome and antenatal care. It is important to note, that the majority of studies do not mention relevant factors such as placental insufficiency, IUGR, fetal distress and early-term caesarean section, which all influence the antenatal care of TS patients and their pregnancy outcomes - this is one of the limitations that our research attempts to address.

Subnormal birth weight is a commonly reported TS-associated abnormality, but it's cause by placental insufficiency or chromosomal abnormality is not clearly established. To the best of our knowledge, no study has been performed yet to analyze pregnancies of TS patients conceived spontaneously and by ART, knowing the maternal and fetal karyotype, and performing histological and FISH analysis of maternal ovarian biopsy and fetal umbilical cord samples.

Considering that all pregnancies showed some level of placental insufficiency, our study focused on novel findings in other aspects: it is one of the first studies to report both maternal and offspring karyotype, and to report both short- and long-term outcomes of offspring. Based on our data, we can consider that in TS-associated spontaneously conceived pregnancies with a normal fetal karyotype, severe placental insufficiency can be observed, with prominent adverse events in the fetus. In Turner syndrome, the incidence of congenital heart defects may exceed 50%. Depending on the type and severity of the abnormality in the pregnant woman, life-threatening complications such as aortic aneurysms or aortic dissection due to dilatation can occur during pregnancy. Although there is a relatively high rate of these abnormalities, no serious cardiovascular complications were recorded in our patients, although in case of three out of four cases mild cardiovascular abnormality required close observation.

Intrauterine growth restriction was present in all spontaneously conceived pregnancies of our TS patients. In case of placental insufficiency, oligohydramnios and fetal distress were also seen and, as we have observed, their presence was the leading indication for caesarean section. It is well known that there is a strong correlation between gestational hypertension, placental insufficiency, fetal distress and preterm delivery, so it might be self-evident that hypertension as a common TS-associated comorbidity further contributes to catalyze the process. However, development of IUGR must have been associated with other causal factors in our patients, since in our cohort, except for essential hypertension in Patient 4, none of the cases had hypertension or preeclampsia.

It is hypothesized that genetic abnormalities other than fetal aneuploidy influence the development of placental insufficiency. Due to hypomethylation of the pseudoautosomal region, including the PAR1 region, located in the terminal part of the short arm of the X-chromosome, the effect of X-chromosome inactivation is less pronounced, resulting a difference in the expression of genes in this region, including SHOX gene. Some studies have found that the prevalence of IUGR was significantly higher in patients lacking one of the gene copies, suggesting a role for this gene not only in postnatal growth but also in intrauterine growth. In addition to a broad spectrum of maternal comorbidities, placental insufficiency contributes to the condition of IUGR, which was observed in all cases of spontaneous pregnancy in our case series and was present regardless of fetal karyotype.

Interestingly, the only normal-weight neonate was oocyte donation by IVF-conceived. Future research may further investigate the genetic and immunological aspects of this process and may reveal a background of immunotolerance. However, no histologically clear discrepancy was identified in the examination of the umbilical cord and placenta in Patient 1. In case of Patient 1, a maternal ovarian biopsy was performed during the caesarean section, followed by histology and FISH. Results showed ovarian stromal cell mosaicism, in correlation with what had been previously diagnosed from peripheral lymphocytes in this patient. Histopathology also demonstrated an abnormal ovarian stromal structure with a small number of primordial follicles, supporting the definitive existence of POI, but even this structural abnormality did not prevent spontaneous conception. Literature data suggest that mosaicism in the ovaries may explain the altered reproductive potential of Turner syndrome patients with non-mosaic karyotypes, because the karyotype of the ovarian residual cells may differ from the karyotype of cells elsewhere in the body, which is termed “cryptic ovarian mosaicism”. Follicular atresia is only partially achieved, and individual ovarian motility may allow spontaneous conception even in the presence of classical Turner syndrome. Knowledge of

ovarian mosaicism can provide a number of insights for estimating fertility potential. Due to the development of premature follicular atresia, the retrieval and cryopreservation of oocytes or ovarian tissue may be an alternative option, but this option is not currently allowed in TS patients at the national level, although it is available on an experimental basis in other countries.

Based on our findings, the karyotype of spontaneously conceived fetuses in Patients 1 and 4 with mosaic karyotype and the karyotype of the IVF-conceived fetus in Patient 2 with non-mosaic karyotype were normal, however, in spontaneously conceived pregnancies of Patient 3 (45,X), both fetuses were diagnosed with 45,X karyotype from amniotic fluid sample taken during amniocentesis. Bernard et al. determined the karyotypes of 11 offspring from 17 neonates from spontaneous TS pregnancies, of which 2 TS karyotypes were identified. It is important to emphasize that both maternal and fetal karyotyping are important in predicting prognosis. However, our data also showed that the long-term outcome can be favorable in most cases despite the presence of maternal or fetal Turner syndrome or IUGR. Moreover, the circumstances of delivery, the degree of prematurity and birth weight seemed to play a role as important as the presence of the genetic disease itself in the long-term neurodevelopment of infants. The only case associated with severe long-term complications was the previously described 300 g extremely low birth weight extreme preterm baby born retarded at 24 weeks gestation.

Summary

Our research reports new findings in several aspects: a unique national analysis of a cohort of 75 patients based on nearly 50 criteria summarizes the characteristics of a Hungarian Turner syndrome patient population. We examined the role of age at diagnosis, the prevalence of classic TS-specific physical characteristics and co-morbidities, their association with karyotypes and the strength of associations. In the group diagnosed before the age of 12 years a higher rate of short stature and GH treatment use with a higher rate of breast atrophy, flat face or mitral valve prolapse was present, and a higher incidence of hypertension and hepatosplenomegaly was observed in those diagnosed at a later age. These findings may allow the use of targeted screening strategy for specific features and co-morbidities depending on the karyotype of TS and the time of diagnosis.

Regarding obstetric outcomes, our analysis of pregnant cases showed that placental insufficiency causing intrauterine growth restriction can occur even if the fetal karyotype is normal and there is no maternal co-morbidity before or around conception. Although the multidisciplinary team for patients with TS should include a cardiologist and an

endocrinologist, obstetricians should be especially alert to the possible involvement of fetal distress, which our results suggest being predominant. If extreme prematurity and peripartum complications can be avoided, the long-term outcome of the infants can be expected to be very favorable.

Publications

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List of publications related to the dissertation

1. **Vida, B.**, Török, O., Felszeghy, E. N., Orosz, M., Krasznai, Z. T., Tándor, Z., Jakab, A., Deli, T.: Associations of Karyotype and Age at Diagnosis with Physical Features and Comorbidities in Turner Syndrome: a Single-Site Experience.
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3. Szeőcs, D., **Vida, B.**, Petővári, G., Póliska, S., Janka, E. A., Sipos, A., Uray, K., Sebestyén, A., Krasznai, Z. T., Bai, P.: Cell-free ascites from ovarian cancer patients induces Warburg metabolism and cell proliferation through TGFbeta-ERK signalling.
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