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HABILITATION THESIS

FROM CLINIC GENETICS AND CYTOGENETICS TO MODERN METHODS IN MEDICAL GENETICS

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SUMMARY

ABSTRACT

This work is structured in 4 sections: the overview of my professional activity, the scientific activity, the future directions for my activity and the bibliography.

In first section I presented in a concise manner all my professional activity. I started with academic activity where I focused on the steps in development of academic career. However, I pointed also my activity in production of books and chapters of books. In second part of this section I presented my medical activity from the medical inter to senior physician in medical genetics. The end of section was reserved to details concerning my scientific activity represented by an important number of papers in scientific journals classified ISI (32 papers) or BDI (56 papers). These scientific papers were in correlation with my major field of medical interests: cytogenetics, reproductive medicine, prenatal diagnosis or genetics of rare diseases.

In the second part I presented in details the scientific activity. In first part I present some data that represent the background of my research activity in the field of chromosomal abnormalities and diseases. In the beginning of 1st chapter I present some works about chromosomal syndromes: Down, Patau, Triplo X and Edwards. The epidemiologic, clinic and cytogenetic study on Down syndrome represented a premier for Romanian medical literature and showed a good concordance between clinical genetic examination and results of chromosomal analyse, a high prevalence of major congenital anomalies, being the first Romanian study that calculated the incidence of Down's syndrome - 1.3‰ (1/739) in newborns in Iaşi County. In Patau's syndrome study we have a significant cohort of 14 cases and we concluded that Patau syndrome is a severe polymorphic chromosomal disease, but in our cohort specific clinical triad was not very frequent. The most severe element was holoprosencephaly spectrum, which it associated with increased in utero and perinatal mortality. The rettrospective study of X trisomy emphasizes the difficulty of establishing a genotype - phenotype correlations, with prepubertal diagnosis based on growth retardation, uncharacteristic facial dysmorphism and mild mental retardation, while main postpubertal aspects were ovarian dysgenesis and mild mental retardation. Another study concerning a rare chromosomal disorder concerned Edwards syndrome, when are presented 19 cases with trisomy 18. The clinical features were variable, but the most suggestive were: IUGR, typical craniofacial dysmorphism, heart defects, prominent calcaneus, overriding fingers and preterm birth. Early prenatal diagnosis of trisomy 18 is important for appropriate management decisions regarding neonatal care and invasive procedures. Also, another study about chromosomal disorders made a synthesis of cases confirmed in our laboratory during two calendaristic years (2015-2016). Our study showed the importance of use of different chromosomal analysis for confirmation of etiology of congenital anomalies. In 44.71% cases we discovered a chromosomal abnormality using a sequential strategy of genetic testing, first option remaining karyotype. In cases of a microdeletion, confirmation was apported by FISH or MLPA test. Finally, array-CGH confirmed small genomic imbalances, but its use is not routinely.

In second part of chromosomal pathology I present three studies about specific chromosomal anomalies. First is presented a collaborative study concerning ring chromosome (a very rare disorders). All our 6 cases have *de novo* ring chromosomes, presenting a different phenotype according to the loss of genetic material and genetic instability. The karyotype represents the main analysis for detection of ring chromosomes, but other molecular techniques are necessary for complete characterization. The second anomaly is also a rare chromosomal unbalanced abnormality: inverted duplication deletion of 8p chromosome, where the diagnosis was confirmed by array-CGH. In the end of this chapter we present also a report case, concerning a pseudodicentric 14;18 chromosome with two additional CNVs (attested by array-CGH).

The 2nd part of scientific activity was reserved for multifactorial disorders. The study on etiology of cardiac malformations showed a high frequency of chromosomal abnormalities among cases with cardiac malformation and mental retardation and proved the benefits of cytogenetic testing in plurimalformative syndromes with congenital heart disease. When "standard" chromosome analysis shows a normal karyotype, molecular cytogenetic techniques are useful to detect submicroscopic chromosomal abnormalities. In a study concerning patients with intellectual disability (ID) we applied MLPA test to detect subtelomeric abnormalities. We found 7.5% cases with such anomalies, higher than the average value reported in the literature, which illustrates the efficiency of using two subtelomeric screening kits. The follow-up kit allows confirmation of the abnormality and estimation of its size, which established the clinical significance of subtelomeric anomalies. The combined use of karyotype and MLPA kits for the screening of the most frequent submicroscopic anomalies represents an efficient strategy for establishing the etiologic diagnosis in ID patients, particularly when microarrays are unavailable as a first line approach. Another study concerning a multifactorial disorder was about hearing loss. We found 217 patients with pathogenic/likely pathogenic variants, 141 being confirmed by MLPA. We identified two new variants in *GBJ2* gene: c.109G>A and c.100A>T, but the most common variants were c.35delG, c.101T>C, c.313_326del14 and c.71G>A. All of the patients had been confirmed with Sanger Sequencing, proving that MLPA can be a cost-effective diagnosis method, useful for every patient with hearing impairment.

In the 3th part of this section I presented results of study in reproductive medicine. First I present studies that showed the importance of cytogenetic analysis for couples with reproductive disorders (RD). The results of a retrospective study on 266 couples with RD are consistent with the literature, emphasizing the importance of chromosomal analysis for etiologic diagnosis, management and genetic counseling for such couples. Another retrospective study concerning amenorrhea confirmed that sex chromosome abnormalities represent a major etiologic factor in this disorder. The majority of patients presented ovarian dysgenesis and hypergonadotropic hypogonadism. In this context, we emphasize the importance of cytogenetic investigations, especially the karyotype, for confirmation of diagnosis in all patients with amenorrhea.

After that I presented the studies about prenatal medicine. The most important study regarded noninvasive prenatal diagnostic method based on the comparison of the methylation status of maternal versus foetal DNA, a possible alternative to current invasive prenatal diagnostic methods. Our results indicated a good sensibility and specificity, but the number of cases (only 10) was not enough for pertinent conclusions. Other studies concerned application of conventional FISH method in prenatal diagnosis. The first such study attested the usefulness of FISH technique in prenatal diagnosis with 16.30% of cases identified by this method. The prenatal diagnosis by FISH technique ensures rapid results, allowing the choice of reproductive options in pathological cases. Other study using FISH technique highlights the limits of this in prenatal diagnosis of gonosomal anomalies, with main limitations related to impossibility of correct quantifying of mosaic gonosomal anomalies. A multicenter study was focused on polyploidy, being discussed 39 cases. Early diagnosis of polyploid pregnacies would provide an opportunity to early termination of an affected pregnancy. Chromosomal analyze continues to be the gold-standard in the detection of polyploidies, but molecular approaches may provide additional valuable information for the management of the case. In the final of chapter we analyzed the management of increased nuchal translucency, and indicated the need of a good ultrasound examination at 20 to 24 weeks to evaluate the prognostic of such pregnancies.

The final part of scientific activity concerns six rare diseases, a sort of disorders important for medical genetics. The study of cases with velo-cardio-facial syndrome revealed 6 patients with 22q11.2 microdeletion, but these patients are only the "tip of the iceberg". The clinical spectrum of DiGeorge syndrome is highly variable and diagnosis requests application

of molecular cytogenetic techniques in selected cases with evocative phenotype, using sensitive clinical score. The case with Limb Body Wall Complex detected prenatally and investigated by pathologic examination confirmed the importance of collaboration between obstetricians, geneticists and pathologists in elucidation of some anomalies of prenatal development. Pallister-Killian syndrome is a rare syndrome and our cohort of five cases represents an important number that allowed us to identify some particular features (marked excess of hair on the forehead and ears, a particular eye disorder, repeated episodes of infection and autonomic dysfunction, complex sensory impairments, and mild early myoclonic jerks). The use of MLPA and array CGH are reliable methods of diagnosis and could be applied as the first intention diagnostic tests. Gorham-Stout disease is a multifactorial disorder with massive osteolysis that have a poor prognosis. The bad prognosis factors are osteolytic lesions of the spine and pleura. Holt-Oram syndrome is a pleiotropic disorder, in which the main anatomic regions implicated are the heart and the upper limbs. Because of variable expressivity the diagnosis is sometimes delayed. An early diagnosis is necessary for preventing complications in patients and for identifying other family members at risk. Osteogenesis imperfecta is a rare condition characterized by an important genetic heterogeneity. In these conditions, screen for mutations of genes involved in OI pathogenesis imposes whole exome sequencing as the first intention technique. Computational analysis of the pathogenic variant with dedicated programs is absolutely necessary to establish the classification of the mutant variant, especially in prenatal genetic counselling.

The future directions of professional development will imply mainly the prenatal diagnosis, malformative pathology, reproductive disorders, different fields of genomics, oncogenetics and rare disease researches, in some fields being already applied programmes of research.

Section IV includes a number of 628 references used for the preparation of this thesis and the elaboration of all papers included here.

REZUMAT

Această lucrare este structurată în 4 secțiuni: sumarul întregii mele activități profesionale, principalele domenii științifice de interes, posibilele direcții de dezvoltare a activității mele și bibliografia.

În prima secțiune am prezentat într-o fromă concisă întreaga mea activitate profesională. Am început cu activitatea academică, focusându-mă pe etapele de dezvoltare a carierei academice. De asemenea, legat de această activitate am marcat și importantă activitate de editare de cărți sau participarea la redactarea de capitole de carte. În a doua parte a acestei secțiuni am prezentat activitatea mea medicală de la stagiar la amedic primar de genetică medicală. Sfârșitul acestei secțiuni l-am rezervat prezentării activității științifice, reprezentate de un număr important de articole publicate în reviste cotate ISI (32 articole) sau BDI (56 articole). Aceste articole științifice au fost corelate cu principalele domenii de interes medical: citogenetica, medicina reproducerii, diagnosticul prenatal sau genetica bolilor rare.

În prima parte a celei de-a doua secțiuni prezintă principalele mele realizări în domeniul anomaliilor și bolilor cromosomice. La începutul sunt prezentate informații despre sindroame cromosomice: Down, Patau, Triplo X și Edwards. Studiul epidemiologic, clinic și cytogenetic al sindromului Down reprezintă o premieră pentru literatura de specialitate din România și indică o bună concordanță între examinarea genetică clinică și rezultatele analizei cromosomice, o prevalență crescută a anomaliilor congenitale, fiind primul studiu românesc care a calculat incidența sindromului - 1.3‰ (1/739) de nou-născuți în județul Iaşi. Studiul despre sindrmul Patau a cuprins o cohortă semnificativă de 14 cazuri, indicând că această boală este o maladie cromosomică polimorfică severă, dar că în cazul lotului nostru triada clinică caracteristică nu a fost atât de frecventă. Cel mai sever element a fost spectrul de holoprosencefalie, care a fost asociat cu o mortalitate crescută prenatală și perinatală. Studiul retrospectiv despre trisomia X a confirmat dificultățile de idnetificare a corelațiilor genotip-fenotip, prepubertar diagnosticul fiind bazat pe retardul de creștere, dismorfia facală necaracteristică și retardul mental moderat, în timp la persoanele adulte se asociază disgenezia ovariană și dizabilitatea mentală moderată. Un alt studiu ce a vizat o boală cromosomică rară a fost cel despre sindromul Edwards, în care au fost sintetizate datele a 19 cazuri cu trisomie 18. Aspectul clinic a fost variabil, cele mai sugestive particularități fiind: RCIU, dismorfia craniofacială tipică, anomaliile cardiace, calcaneul proeminent, degetele încălecate și prematuritatea. Diagnosticul prenatal precoce al trisomiei 18 permite luarea unor decizii legate de îngrijirea neonatală și aplicarea de proceduri invazive. De asemenea, un alt studiu despre patologia cromosomică a vizat sinteza cazurilor confirmate în laboratorul nostru pe parcursul a doi ani calendaristici (2015-2016). Acest studiu a indicat importanța utilizării a diferite metode de analiză cromosomică pentru confirmarea etiologiei anomaliilor congenitale. În 44.71% din cazuri am găsit o anomalie cromosomică prin utilizarea unei strategii secvențiale de testare genetică, ce a început cu cariotipul. În cazurile cu microdelеții, confirmarea a fost realizată prin teste FISH sau MLPA. Prin metoda array-CGH au fost confirmate modificările genomice de mici dimensiuni, dar utilizarea acestei tehnici nu poate fi aplicată încă de rutină.

În continuare am prezentat trei studii ce au vizat anomalii cromosomice particulare. Primul studiu a fost rezultatul unei colaborări multicentrice asupra cromosomilor inelari (anomalie cromosomică extrem de rară). Toate cele șase cazuri au fost *de novo,* prezentând fenotipuri diferite în raport cu materialul genetic absent și instabilitatea genetică indusă de anomalie. Cariotipul reprezintă principala metodă de detecție a cromosomilor inelari, dar metodele moleculare sunt utile pentru caracterizarea completă. O a doua anomalie cromosomică neechilibrată rară a fost deleție duplicație inversată a cromosomului 8, a cărui diagnostic a fost confirmat prin array-CGH. La sfârșitul acestui capitol este prezentat un cromosom pseudodicentric 14;18 ale cărui două CNVs adiționale au fost atestate prin array-CGH.

Cea de-a doua parte a secțiunii II prezintă bolile multifactoriale. Studiul etiologiei malformațiilor cardiace a indicat o frecvență crescută a anomaliilor cromosomice la pacienții cu malformații cardiace și retard mental, atestând beneficiile testării citogenetice în sindroamele plurimalformative cu anomalii congenitale cardiace. În situațiile în care cariotipul este normal, tehnicile citogenetice moleculare permit detecția anomaliilor cromosomice submicroscopice. Studiul despre dizabilitatea intelectuală a confirmat utilitatea metodei MLPA pentru identificarea anomaliilor subtelomerice, permițând găsirea a 7.5% anomalii, valoare superioară celei citată în literatură, ceea ce confirmă eficiența utilizării celor două kit-uri de screening subtelomeric. Utilizarea kit-ului de follow-up a permis identificarea dimensiunii modificării cromosomice, aspect important pentru stabilirea semnificației clinice a anomaliilor subtelomerice. Utilizarea combinată a cariotipului și MLPA pentru detecția anomaliilor submicroscopice frecvente reprezintă o strategie eficientă pentru confirmarea diagnosticului etiologic al pacienților cu diazbilitate intelectuală, mai ales dacă array-CGH nu poate aplicată. Un alt studiu legat de patologia multifactorială a vizat hipoacuzia. Astfel, am găsit 217 pacienți cu variante patogenice/probabil patogenice, 141 fiind confirmate prin MLPA. Am identificat două variante noi în gena *GBJ2*: c.109G>A and c.100A>T, dar cele mai frecvente variante au fost: c.35delG, c.101T>C, c.313_326del14 și c.71G>A. La toți pacienții cu modificări genetice acestea au fost confirmate prin secvențiere Sanger, ceea ce atestă că tehnica MLPA este o metodă performantă și ieftină ce poate fi utilizată cu succes pentru diagnosticul dificultăților auditive.

În a treia parte a secțiunii II au fost prezentate rezultatele studiilor de medicină reproductivă. Întâi, am prezentat studii ce confirmă importanța analizelor citogenetice în cazul cuplurilor cu tulburări de reproducere (RD). Rezultatele studiului retrospectiv asupra 266 cupluri cu RD au fost concordante cu datele de literatură, confirmând importanța analizei cromosomice pentru diagnosticul etiologic, managementul și sfatul genetic al unor astfel de cupluri. Un alt studiu retrospective a vizat amenoreea confirmând că anomaliile gonosomilor reprezintă factorul etiologic major. Majoritatea pacientelor au avut disgenezie ovariană și hipogonadism hipergonadotropic. În acest context, am certificat importanța analizelor citogenetice, în special cariotipul, pentru confirmarea diagnosticului la toate pacientele cu amenoree.

În a doua parte a acestui capitol am prezentat date de medicină prenatală. Cel mai important studiu a vizat diagnosticul prenatal noninvaziv, bazat pe compararea statusului metilării ADN fetal și matern, care ar putea fi o metodă alternativă a metodelor curente invazive de diagnostic prenatal. Rezultatele obținute de noi au indicat o bună sensibilitate și specificitate, dar numărul de cazuri (doar 10) a fost insuficient pentru a furniza concluzii pertinente. Alte studii au făcut referire la aplicarea metodei FISH în diagnosticul prenatal. Primul din aceste studii a atestat utilitatea tehnicii FISH în diagnosticul prenatal indicând existența a 16,30% cazuri confirmate. Diagnosticul prenatal prin tehnica FISH permite obținerea de rezultate rapide, ceea ce reprezintă o alternativă optimă în cazurile patologice. Un alt studiu a confirmat impedimentele utilizării tehnicii FISH pentru diagnosticul prenatal al anomaliilor gonosomale, principala limitare fiind legată de imposibilitatea cuantificării cazurilor cu mozaicuri gonosomale. Un studiu multicentric a fost axat pe poliploidie, fiind discutate 39 cazuri. Diagnosticul precoce al sarcinilor cu poliploidie oferă oportunitatea unui avort terapeutic precoce. Analiza cromosomică continuă să fie metoda standard de detecție a poliploidiilor, dar metodele moleculare furnizează informații utile pentru managementul acestor cazuri. În finalul acestui capitol am prezentat managementul translucenței nucale crescute, studiul nostru confirmând utilitatea unei examinări ecografice performante 20-24 SA pentru evaluarea prognosticului sarcinii.

În ultima parte a secțiunii II au fost prezentate informații despre șase boli rare, tip de patologie ce este importantă pentru genetica medicală. Studiul cazurilor cu sindrom velo-cardio-facial a indicat existența a 6 pacienți cu microdeleție 22q11.2, dar aceste cazuri reprezintă doar vârful icebergului. Spectrul clinic al sindromului DiGeorge este extrem de variabil, iar confirmarea diagnosticului necesită aplicarea de tehnici citogenetice moleculare la cazurile cu

fenotip evocator selectate pe baza unor algoritmi clinici. Cazul cu Limb Body Wall Complex, detectat prenatal și investigatat anatomopatologic, confirmă importanța colaborării dintre obstetricieni, geneticieni și anatomopatologi în elucidarea unor anomalii de dezvoltare prenatală. Sindromul Pallister-Killian este o boală rară, iar cohorta noastră de cinci cazuri reprezintă un număr important ce a permis identificarea unor trăsături particulare (exces de păr pe frunte și urechi, anomalii oculare, episoade repetate de infecție și disfuncție autonomă, anomalii senzoriale complexe și mișcări mioclonice). MLPA și array CGH s-au dovedit a fi metode adecvate pentru diagnosticul acestei boli și ar trebui să reprezinte tehnicile de primă intenție pentru confirmarea supoziției clinice. Boala Gorham-Stout este o maladie multifactorială caracterizată prin osteoliză masivă și prognostic negativ. Principalele elemente de prognostic negativ sunt: leziunile osteolitice ale coloanei vertebrale și pleurei. Sindromul Holt-Oram este o boală pleiotropă, în care principalele regiuni anatomice modificate sunt cordul și membrele superioare. Din cauza expresivității variabile diagnosticul este deseori întârziat. Realizarea unui diagnostic precoce permite prevenirea complicațiilor și identificarea altor membri afectați ai familiei. Osteogenesis imperfecta (OI) este o boală rară caracterizată prin eterogenitate genetică. În aceste condiții, căutarea mutațiilor genelor implicate în patogenia OI impune utilizarea secvențierii întregului exom ca metodă de primă intenție. Analiza computerizată a variantelor patogenice prin folosirea de programe speciale este obligatorie pentru clasificarea mutației, în special pentru oferirea sfatului genetic prenatal.

Direcţiile viitoare de dezvoltare profesională vor viza în principal diagnosticul prenatal, patologia malformativă, tulburările reproductive, domeniile genomicii, oncogeneticii şi patologiei bolilor rare, pentru unele direcţii fiind deja implicat în programe de cercetare.

Secțiunea IV include 628 indicații bibliografice, utilizate la pregătirea acestei teze și la elaborarea tututror articolelor ce au stat la baza acestei lucrări.

PREFACE

Professional development and personal achievement are intricate processes, often influenced by family, education and society. My own professional development has been strongly influenced by family and the academic environment where I've been continuously active since graduating from university.

My family provided the ideal setting and unconditional support to constantly improve my general and medical knowledge. Practically my upbringing contributed strongly to whom I am today, having developed and matured in a liberal open-minded environment. Through an extensive personal library, my parents introduced me to and developed my desire for reading from a very young age, having access to varied literature as well as general knowledge and medical books. Furthermore, my parents' friends, in their majority medical professionals from the University of Medicine Iasi, contributed themselves to my decision to embrace the same profession. Afterwards, during the six years of medical training, my development was augmented by the knowledge gained from a number of distinguished medical teaching staff from the University of Medicine Iasi. These factors contributed to my decision to continue my career in the academic teaching area, a step that I took successfully two years after graduation, when I applied for and gained the position of Assistant Lecturer at the Medical Genetics Department.

From then on, I began the second stage of my professional development, finalised with this competence dissertation. The teaching team from the Medical Genetics Department have put their trust in me, supported and helped me firstly gain the essential knowledge of this wonderful branch of science, and subsequently does thorough research work on genetic diseases.

During the first part of my academic career, the research projects were directed towards finalising the objectives for the PhD dissertation. This was overseen by the distinguished Professor Mircea Covic, the founder of the Medical Genetics School in Iasi and materialised with the title of *Doctor of Medical Sciences* awarded to me by the Ministry of Education in 2004.

Gaining the Doctorate was a great impetus to continue my research work and achieve professional accomplishment. Consequently, in time, I applied for and I was successively appointed to the positions of Lecturer, Research Fellow and Professor, whilst also becoming a Consultant in Medical Genetics. In time I have been involved in 27 research grants (completed or ongoing) either as coordinator or as a member of the research team.

My research work has materialised in a number of published materials with 25 medical books (as author or co-author), 32 ISI articles, 56 BDI articles, 7 unrated articles, 66 articles published in extenso in tomes of science journals and 136 summarised papers of which 79 were published in the supplement issues of some ISI rated journals.

The main areas of my research work focused on chromosomal diseases, congenital anomalies, prenatal diagnosis, reproductive dysfunctions and rare diseases, but the published materials also concentrated on summarising the medical literature for current issues. I have summarised all these aspects in the five chapters of this study where I explain the core of my research work from the time I finalised my PhD dissertation to present day. I have ended this study by outlining the main routes I wish to direct the medical research towards; however this step is not entirely up to me, but also to the way I will succeed in attracting eminent PhD students and stimulate my younger colleagues from the Genetics Department.

Finally, I would like to thank all those who contributed to my continuous development as a human being and as a professor. Thank you to my parents for their continuous help and support throughout my life. Thank you to my wife and my daughter for being by my side, for their support and for accepting my limited involvement (due to time restraints) at home. Thank

you to my colleagues from the Iași Genetic Center who supplied me with the evidence necessary to confirm the genetic diagnosis. I would also like to thank my colleagues from the Medical Genetics Department and especially the distinguished professors Mircea Covic and Ortansa Stoica who were my mentors and advisors in the many difficult situations I encountered during my career.

Iasi, 13th January 2022

Prof. Univ. Dr. Eusebiu Vlad Gorduza

I. OVERVIEW OF PROFESSIONAL, ACADEMIC AND SCIENTIFIC CONTRIBUTIONS

The medical career was my first and only one option in the end of High School. This choice was influenced by the training obtained during the High School period, but also by the familial entourage where my both parents sustained me in direction of medical studies. The six years as student in a prestigious medical school allowed me the skills to understand the human being and how should I act to improve the health of patients. The period of medical studies ended, in 1992, with a diploma thesis at the interface between the clinical and paraclinical field, which aimed at the study of superoxydysmutase in various human pathologies.

ACADEMIC ACTIVITY

My academic evolution encouraged by Prof. Dr. Mircea Covic, the coordinator of the discipline and my mentor in the university activity, started in second semester of academic year 1993-1994, after passed the exam for junior teaching assistant in the discipline of Medical Genetics.

After that I followed by exams all steps of academic hierarchy from junior teaching assistant to professor:

- 1994 – 1998 junior teaching assistant at discipline of Medical Genetics;
- 1998 – 2003 teaching assistant at discipline of Medical Genetics;
- 2003 – 2008 lecturer at discipline of Medical Genetics;
- 2008 – 2014 associate professor at discipline of Medical Genetics;
- From 2014 to present professor at discipline of Medical Genetics;

In 1996, I was admitted as a doctoral student, starting my studies under the coordination of Prof. Dr. Mircea Covic. My doctoral thesis had the title: ”Contributions to study of actual problems of chromosomal pathology”. These chromosomal studies concerned all the cases with chromosomal pathology identified in Medical Genetics Center between 1966 and 2003. The main directions of studies were:

a. highlighting the clinical, epidemiological and cytogenetic particularities of chromosomal syndromes: Down, Turner, Klinefelter,

b. ordering and processing clinical and cytogenetic data of patients with chromosomal diseases investigated in Iaşi Genetic Center and Cytogenetics Laboratory of the University of Medicine and Pharmacy "Gr T. Popa";

c. Correlation of clinical data with the results of the cytogenetics investigation (karyotype and X chromatin test) in patients with gonadal dysgenesis (ovarian or testicular);

d. Establishing connections between clinical and cytogenetic data, including polymorphic disease as trisomy X, mixed gonadal dysgenesis or some plurimalformatives chromosomal syndromes;

e. Highlighting quality of X chromatin test as screening analysis in patients with gonadal dysgenesis or intersex condition;

f. Outlining the advantages of using FISH technique in the diagnosis of gonadal dysgenesis syndromes plurimalformative and, highlighting the important role of clinical examination performance for DNA probes selection;

g. Customize genetic counseling in Down Syndrome by correlation with cytogenetic results and maternal age at conception.

The public defense of the Doctoral Thesis was held on November 21, 2003, acknowledging me as a Doctor in Medical Science by the Order of the Ministry of Education and Research in November 5, 2004.

I completed my didactic training activity by attending the Postgraduation Course of Psycho-Pedagogy, organized by Faculty of Pedagogy for "Alexandru Ioan Cuza" University from Iași, in 1998.

In addition, I acquired foreign language competence (French, level B2+) in 2010 (Certificate no 970/7.10.2010) granted by the French Department from Center for Foreign Languages and Continuous Education, Faculty of Letters, "Alexandru Ioan Cuza" University from Iași.

From the start from of my activity in Medical Genetics Department, I have carried out teaching activities – practical lessons and courses – with different categories of students from "Grigore T. Popa" University of Medicine and Pharmacy from Iași:

- Students of Medicine Faculty, Romanian and French Section;
- Students of Nurse College of Iași, Botoșani and Bacău;
- Students of Dentistry Faculty, Romanian and French Section;
- Students of Nutrition College;
- Master students;
- Doctoral students.

I participated as speaker at a broad range of postgraduation academic lectures organized by "Grigore T. Popa" University of Medicine and Pharmacy from Iași. Different types of lectures I presented in postgraduation academic lectures organized by Medical College of Iași County and I participated as invited speaker at national and international scientific congresses and conferences organized by scientific organization. Thus, I had plenary interventions at congresses of Medical Genetics, Pediatrics, Obstetrics-Gynecology, Neonatology, Endocrinology, Pediatrics Surgery etc.

I have coordinated the residents within the Medical Genetics residence program since 2000. From 2007, I have nominated as coordinator of Medical Genetics residence program in the "Grigore T. Popa" University of Medicine and Pharmacy from Iași. I had practical activities and courses with the residents of other specialties (Pediatrics, Neonatology, Obstetrics-Gynecology, Endocrinology, Pathology, Laboratory, Orthodontics, etc.).

In addition to "traditional" teaching activities, I was involved in different projects focused in professional development of different categories of medical personal (medicines, medical students, nurses, etc.). Thus, I participated in the following social projects:

- "Burse doctorale pentru cresterea competitivitatiii in domeniul medical si farmaceutic". Proiect POSDRU/88/1.5/S/58965. Manager de Proiect Conf. univ. dr. Dragos Pieptu
- "Stagii de pregătire practică pentru integrarea rapidă pe piaţa muncii a studenţilor specializaţi în medicina dentară". Proiect POSDRU/90/2.1/S/63942. Manager de Proiect Prof. dr. Norina Forna
- "Îmbunătățirea nivelului de competențe al profesioniștilor din sectorul medicalFormare profesionala a personalului medical in genetica medicala PROGEN". Proiect POCU/91/4/8/107623. Manager de Proiect: Prof. Dr. Ileana Constantinescu, UMF București. Coordonator Științific Regiunea Nord Est: Prof. Dr. Eusebiu Vlad Gorduza. Valoare proiect 11.702.201,54 lei . Perioadă de implementare 2 ani (7.12.2017-7.12.2019) – proiect cu termen de finalizare amânat pentru 1.12.2021.
- „Imbunatatirea competentelor profesionale ale personalului medical implicat in realizarea actului medical din specialități relevante pentru managementul multidisciplinar al bolilor genetice rare (PROGENERARE)". Proiect POCU/91/4/8/01.09.2016. Manager de proiect: Prof. Dr. Mihai Ioana, UMF Craiova.

Expert elaborare ghiduri practice regiunea Nord Est: Prof. Dr. Eusebiu Vlad Gorduza. Perioadă de implementare 3 ani (26.02.2018 – 25.02.2021).

My activity in ”Grigore T. Popa” University of Medicine and Pharmacy from Iași was not limited only to didactic field. Thus, I was involved in different other fields of academic life. From the beginning I participated at the organization of Admission Exam in University and in the last years I was the President of Admission Committee of Medicine Faculty or Vice-president of General Committee of University. In the last ten years I was involved also in organization of National Residency Exam of young doctors, acting at different levels from surveillance to Vice-president of General Committee of University.

I was involved in the organization of final exam of students in the university and I participated in the last 15 years in the Committee of Examination of Bachelor´s Thesis. In addition, I was member of different commission at doctoral admission or doctoral study guidance.

I am member of different scientific organizations:

- Romanian Medical College;
- Medical Genetics Romanian Society;
- European Society of Human Genetics;
- European Cytogenetics Society;
- European Society of Human Reproduction;
- European Society for Clinical Investigations.

My teaching experience was concretized by publications of 12 scientific books:

1. Gorduza V.M., Tarabasanu-Mihaila C., Athanasiu A., Gorduza E.V.,, “Coloranţi organici cu aplicaţii neconvenţionale”, Editura UNI-PRESS, Bucuresti, 2000, ISBN:973-98967-1-5.
2. Gorduza V.M., Tofan L., Şuteu D., Gorduza E.V., “Biomateriale– Biotehnologii – Biocontrol”, Editura CERMI, Iasi, 2002, ISBN:973-8188-12-1
3. Gorduza E.V., Stoica O.F., “Elemente de Genetică umană”, Editura TIMPUL, Iaşi, 2003, ISBN 973-612-059-7.
4. Gorduza E.V., Buhuşi M.C., Rusu C., “Genetică umană – manual de lucrări practice”, Ed. Kolos Group Iaşi, 2003, ISBN 973-86194-2-4.
5. Gorduza E.V., „Compendiu de genetică umană şi medicală”, Ed. Tehnopress, Iaşi, 2007, ISBN 978-973-702-492-3
6. Gorduza E.V., „Notions de base de la genetique humaine”, Ed. „Gr. T. Popa” UMF Iaşi, 2011, ISBN 978-606-544-045-6
7. Gorduza E.V., „Notions de base de la genetique medicale”, Ed. „Gr. T. Popa” UMF Iaşi, 2011, ISBN 978-606-544-054-8
8. Gorduza E.V., „Exercices et testes de genetique medicale”, Ed. Tehnopress Iaşi, 2015, ISBN 978-606-687-234-8
9. Socolov R., Socolov D., Gorduza E.V., „Actualităţi în patogenia şi diagnosticul infertilităţii”, Ed. „Gr. T. Popa” UMF Iaşi, 2016, ISBN 978-606-544-404-1
10. Covic M., Ştefănescu D., Sandovici I., Gorduza E.V., „Genetică Medicală”, Ed. Polirom Iaşi, 2017, ISBN 978-973-466-526-6
11. Covic A., Gorduza E.V., Covic M., ”Medicina genomică și bolile comune ale adultului”, Ed. Polirom Iași, 2021, ISBN: 978-973-46-8332-1

12. Caba L., Gorduza E.V., „Exerciții și teste de genetică medicală”, Ed. Tehnopres Iași, 2022, ISBN 978-606-687-476-2

Also, I wrote 24 chapters in 14 scientific books:

1. Covic M., Stoica O., Sandovici I., Gorduza E.V., “Rolul factorilor genetici în producerea bolilor” în “Genetică Medicală” Covic M., Ştefănescu D., Sandovici I. (editori), Ed. Polirom, Iaşi 2004, ISBN:973-681-334-7, pp. 271-293;
2. Covic M., Dimofte I., Gorduza E.V., “Bolile cromozomiale” în “Genetică Medicală” Covic M., Ştefănescu D., Sandovici I. (editori), Ed. Polirom, Iaşi 2004, ISBN:973-681-334-7, pp. 331-353
3. Gorduza E.V., Butnariu L., „Cauzele genetice ale hipostaturii”, în „Tulburări de creştere staturală şi ponderală” Găleşanu C. (editor), Ed. „Gr. T.Popa” Iaşi, 2007, ISBN 978-973-7682-11-6, pp. 181-248
4. Gorduza E.V., Mustaţă M., Militaru M., Puiu M., „Boli cromozomiale” în „Esenţialul în 101 boli genetice rare” Puiu M. (editor), Ed. Orizonturi Universitare, Timişoara, 2007, ISBN: 978-973-638-327-4, pp. 27-64
5. Covic M., Bembea M., Rusu C., Skrypnyk C., Dehelean L.A., Gorduza E.V., „Sindroame cu microdeleţii” în „Esenţialul în 101 boli genetice rare” Puiu M. (editor), Ed. Orizonturi Universitare, Timişoara, 2007, ISBN: 978-973-638-327-4, pp. 65-90
6. Rusu C., Borţun C.M., Mihailov D., Rădulescu A., Bembea M., Boia E., Severin E., Csep K., Gorduza E.V., Ştefănescu D., „Sindroame diverse” în „Esenţialul în 101 boli genetice rare” Puiu M. (editor), Ed. Orizonturi Universitare, Timişoara, 2007, ISBN: 978-973-638-327-4, pp. 91-166
7. Gorduza E.V., “Sindromul Turner”, în “Mic ghid de diagnostic in bolile rare” Puiu M., Skrypnyk C. (editor) Ed. Victor Babes, Timisoara 2009, ISBN 978-606-92022-0-3, p.11-12
8. Gorduza E.V., “Sindromul Triplo X”, în “Mic ghid de diagnostic in bolile rare” Puiu M., Skrypnyk C. (editor) Ed. Victor Babes, Timisoara 2009, ISBN 978-606-92022-0-3, p. 13.
9. Gorduza E.V., “Sindromul Klinefelter”, în “Mic ghid de diagnostic in bolile rare” Puiu M., Skrypnyk C. (editor) Ed. Victor Babes, Timisoara 2009, ISBN 978-606-92022-0-3, p. 14
10. Covic M., Gorduza E.V., Popovici C., “Structura şi organizarea celulară a ADN” în “Genetică Medicală” Covic M., Ştefănescu D., Sandovici I. (editori), ediţia a II-a revăzută şi actualizată, Ed. Polirom, Iaşi 2011, ISBN:978-973-46-1960-3, pp. 23-56
11. Popovici C., Covic M., Gorduza E.V., Sandovici I., Puiu M., „Variabilitatea genetică” în “Genetică Medicală” Covic M., Ştefănescu D., Sandovici I. (editori), ediţia a II-a revăzută şi actualizată, Ed. Polirom, Iaşi 2011, ISBN:978-973-46-1960-3, pp. 241-296
12. Covic M., Bembea M., Popovici C., Gorduza E.V., „Consultul şi sfatul genetic” – în “Genetică Medicală” Covic M., Ştefănescu D., Sandovici I. (editori), ediţia a II-a revăzută şi actualizată, Ed. Polirom, Iaşi 2011, ISBN:978-973-46-1960-3, pp. 345-382
13. Gorduza E.V., Popovici C., Skrypnyck C., Covic M., Belengeanu V., Stoicănescu D., Pânzaru M., Butnariu L., “Bolile cromozomiale” în “Genetică Medicală” Covic M., Ştefănescu D., Sandovici I. (editori), ediţia a II-a revăzută şi actualizată, Ed. Polirom, Iaşi 2011, ISBN:978-973-46-1960-3, pp. 384-416

14. Militaru M., Dumitriu S., Puiu M., Gorduza E.V., „Boli cromozomiale" în „Alerta medicală în bolile genetice rare", Puiu M. (coordonator) Ed. „Victor Babeş", Timişoara, 2011, ISBN 606-8054-39-X, pp. 21-48
15. Covic M., Bembea M., Rusu C., Skrypnyk C., Panzaru M., Jurcă C., Boia M., Popa C.A., Csep K., Gorduza E.V., „Sindroame cu microdeleţii" în „Alerta medicală în bolile genetice rare", Puiu M. (coordonator) Ed. „Victor Babeş", Timişoara, 2011, ISBN 606-8054-39-X, pp 49-90:
16. Rusu C., Borţun C.M., Mihailov D., David L.V., Bembea M., Kozma K., Boia E., Severin E., Csep K., Gorduza E.V., Boia M., Puiu M., „Sindroame diverse" în „Alerta medicală în bolile genetice rare", Puiu M. (coordonator) Ed. „Victor Babeş", Timişoara, 2011, ISBN 606-8054-39-X, pp. 91-152
17. Mititiuc I., Dominte A., Gorduza E.V., Segall L., „Bolile genetice renale", în „Nefrologie. Principii teoretice şi practice" Covic A. (editor), Ed. Demiurg Iaşi, 2011, ISBN 978-973-46-1960-3pp 453-476
18. Caba L., Gorduza E.V., „Implicaţii practice ale progreselor geneticii în bolile inflamatorii intestinale", în „Bolile inflamatori intestinale" Stanciu C., Trifan A., Sporea I. (editori) Ed. Gr. T. Popa Iaşi, 2014, ISBN 978-606-544-220-7, pp. 46-61
19. Stoica O., Gorduza E.V., Astărăstoae V., „Terapia bolilor genetice", în „Terapeutica medicală" Ungureanu G., Covic A., (editori) Ed. Polirom Iaşi, 2014, ISBN 978-973-46-4804-7pp. 830-844
20. Caba L., Gorduza E.V., „Genetica în tulburările funcţionale digestive" în „Tulburări funcţionale digestive" Drug V. (ed.) Ed. Prouniversitaria, 2015, ISBN 978-606-26-0420-2pp. 219-233
21. Gorduza EV, Pânzaru MC., Pădureţ A., „Gene implicate în morfogeneza cardiacă", în „Anatomia chirurgicală a sistemului cardiovascular", Tinică Gr., Furnică C., (eds.) Ed. „Gr. T. Popa" UMF Iaşi, 2017pp. 277-288
22. Gorduza E.V., Socolov D.G., Socolov R.V., "Prenatal Biochemical and Ultrasound Markers in Chromosomal Anomalies" in „From the Embryo to the Neonate", Tudorache Șt. (ed.) IntechOpen, 2018, pp 365 – 396
23. Gorduza E. V., Caba L., "Sfat genetic", în p"Obstetrică", Socolov D., Grigore M., Nemescu D., Socolov R., Ed. "Gr. T. Popa" Iași, 2020, ISBN 978-606-544-709-7, p. 464-481
24. Butnariu L.I., Gorduza E.V., "Malformațiile și anomaliile de dezvoltare și reactive ale cavității orale", în "Patologia cavității bucale" (eds.) Ed. "Gr. T. Popa" Iași, 2020 ISBN 978-606-544-691-5, pp. 9-33

MEDICAL ACTIVITY

I have graduated the Faculty of General Medicine of the "Grigore T. Popa" University of Medicine and Pharmacy from Iasi in 1992.

In the end of 1992 I started my medical career, being employed in "Saint Spiridon" Clinic Hospital as medical intern. During one year I made the main stages in internal medicine, surgery, obstetrics-gynecology and pediatric. In the end of 1993 I sustained the national residency competition and I choose specialty of general medicine.

However, my aspirations were for a university teaching career, taking as an example my mother who was a professor at the Polytechnic University of Iasi. Thus, I obtained, in 1994, by exam a position junior assistant in the discipline of Medical Genetics. At that moment, the specialty of medical genetics was not recognized by Minister of Health, and in these conditions,

based on obtaining the position of junior assistant, I choose laboratory medicine. With the recognition of specialty of medical genetics (in 1995) I changed one more time the specialty and I chose this specialty that is concordant with my teaching activities.

In 2000, I sustained the first exam in medical genetics specialty from Romania and I was confirmed, in the same year, by Minister of Health as specialist physician in Medical Genetics. Based on this Diploma I received a clinical integration and I was employed as specialist physician in Medical Genetics in 2003 in Prenatal Diagnostic Department of "Cuza Vodă" Obstetrics and Gynecology Hospital from Iasi.

In 2005, I sustained another exam and I become senior physician of medical genetics, being confirmed by Minister of Health.

Since 2003 I was appointed as a Chief of the Prenatal Diagnostic – Medical Genetics Department of "Cuza Vodă" Obstetrics and Gynecology Hospital from Iasi.

SCIENTIFIC ACTIVITY

The activity of a teacher in a medical university has three components that are intricated: teaching, medical and research. In fact, the research cannot be separated from the medical activity and all scientific activity reflects the information derivate from medical activity.

At the begin of my scientific activity I published under the supervision of Prof. Dr. Mircea Covic in different journals, classified as B and B+ by the Romanian authority of Education and Research. Also, I published papers in conference and congress proceedings.

The researches made during the preparation of PhD thesis offered me the premises to develop my research skills and opened me the opportunities to know and investigate the cytogenetic field. Thus, my researches were focused on different chromosomal syndromes (Down, Turner, Klinefelter) gonosomal dysgenesia syndromes (Turner, Klinefelter, triplo X) syndromes with multiple congenital anomalies (Prader-Willi, Williams, DiGiorge, etc.). To ameliorate the diagnosis of such syndromes I tried to improve cytogenetic techniques (Barr test, karyotype) and I used new methods of investigation (like FISH method). Based on my expertise in chromosomal disorders, I participated as a head of team from "Grigore T. Popa" University of Medicine and Pharmacy from Iași in the national scientific project type "partnership". This project, entitled „Implementation of an algorithm of diagnosis based on complex analyze of genomic profile of patients with congenital and development anomalies" (Project PN-II-PT-PCCA-2013-4-2240) took place during the period 2013-2017. In this project were applied different genetic analyses (karyotype, FISH, MLPA, array-CGH) with the purpose to allow a complete diagnosis of patients with multiple congenital anomalies selected in genetic centers from Iași, Bucharest and Timișoara. My research activity focused on cytogenetic and my expertise in this field were concretized by publication of a lot of scientific papers. The most representative of them are synthetized in the following list:

- **Gorduza E.V.,** Covic M., Stoica O., Voloşciuc M., Angheloni T., Butnariu L., Braha E., Studii clinice, epidemiologice și citogenetice pe un lot de 221 pacienţi cu sindrom Down, Rev. Med. Chir. Soc. Med. Nat. Iaşi, 2007, 111 (2), 363-372.
- **Gorduza E.V.,** Braha E., Grămescu M., Voloşciuc M., Bujoran C., Bartsch O., Covic M., Corelaţii clinico-citogenetice în disgenezia gonadică mixtă, Rev. Rom. Endocrinol. Metab., 2008, VII (2):61-66
- Butnariu L., Covic M., Onofriescu M., Grămescu M., Bujoran C., Caba L., **Gorduza E.V.,** Chromosomal evaluation in couples with reproductive disorders--retrospective study of a selected group of 266 couples, Rev Med Chir Soc Med Nat Iasi. 2010 Oct-Dec;114(4):1107-13

- Butnariu L., Covic M., Ivanov I., Bujoran C., Gramescu M., **Gorduza E.V.,** Clinical and cytogenetic correlation in primary and secondary amenorrhea: retrospective study on 531 patients, Rev. Rom. Med. Labor., 2011, 19 (2/4) 149-160
- Pânzaru M., Rusu C., Volosciuc M., Braha E., Butnariu L., Gramescu M., Popescu R., Caba L., Bujoran C., Ivanov I., Macovei M., Sireteanu A., Covic M., **Gorduza E.V.,** Benefits of cytogenetic testing in diagnosis of plurimalformative syndromes with congenital heart defects, Rev. Rom. Med. Labor., 2012, 20 (3/4) 265-272
- Caba L., Rusu C., Plăiaşu V., Gug G., Grămescu M., Bujoran C., Ochiană D., Voloşciuc M., Popescu R., Braha E., Pânzaru M., Butnariu L., Sireteanu A., Covic M., **Gorduza E.V.,** Ring autosomes: some unexpected findings, Balkan J. Med. Genet., 2012, 15 (2), 35-46
- Sireteanu A., Voloşciuc M., Grămescu M., **Gorduza E.V.,** Vulpoi C., Frunză I., Rusu C., Dicentric chromosome 14;18 plus two additional CNVS in a girl with microform holoprosencephaly and turner stigmata, BJMG 2013, 16 (2), 67-72
- Caba L., Rusu C., Butnariu L., Pânzaru M., Braha E., Voloşciuc M., Popescu R., Grămescu M., Bujoran C., Martiniuc V., Covic M., **Gorduza E.V.,** Phenotypic variability in Patau syndrome, Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2013, 117 (2), 321-327
- Butnariu L., Rusu C., Caba L., Pânzaru M., Braha E., Grămescu M., Popescu R., Bujoran C., **Gorduza E.V.,** Genotype - phenotype correlation in trisomy X: A retrospective study of a selected group of 36 patients and review of literature, Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2013, 117 (3), 714-721
- Pânzaru M., Caba L., Rusu C., Butnariu L., Braha E., Popescu R., Grămescu M., Popa S., Resmeriţă I., Bujoran C., Martiniuc V., **Gorduza E.V.,** Phenotypic variability in Edwards syndrome: synopsis of 19 cases with trisomy 18, Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2017, 121 (1), 172-177
- Butnariu L., Grămescu M., Caba L., Pânzaru M., Braha E., Popescu R., Popa S., Rusu C., Cardos G., Zeleniuc M., Martiniuc V., Plăiaşu V., Diaconu C., **Gorduza E.V.**, Using of multiple chromosomal and molecular analyses to elucidate the etiology of plurimalformative syndromes, Rev Med Chir, 2017, 121 (3): 581-593
- Arghir A, Popescu R, Resmerita I, Budisteanu M, Butnariu LI, **Gorduza EV,** Gramescu M, Panzaru MC, Papuc SM, Sireteanu A, Tutulan-Cunita A, Rusu C, Pallister–Killian Syndrome versus Trisomy 12p—A Clinical Study of 5 New Cases and a Literature Review, Genes 2021, 12, 811.
- Gavril EC, Luca AC, Curpan AS, Popescu R, Resmerita I, Panzaru MC, Butnariu LI, **Gorduza EV,** Gramescu M, Rusu C, Wolf-Hirschhorn Syndrome: Clinical and Genetic Study of 7 New Cases, and Mini Review. Children 2021, 8, 751.
- Popescu R, Grămescu M, Caba L, Pânzaru M-C, Butnariu L, Braha E, Popa S, Rusu C, Cardos G, Zeleniuc M, Martiniuc V, Gug C, Păduraru L, Stamatin M, Diaconu CC, **Gorduza EV.,** A Case of Inherited t(4;10)(q26;q26.2) Chromosomal Translocation Elucidated by Multiple Chromosomal and Molecular Analyses. Case Report and Review of the Literature. Genes. 2021; 12(12):1957.

My second major field of research is the prenatal diagnosis of genetic diseases. These investigations were facilitated by my clinical integration in ”Cuza Vodă” Obstetrics and Gynecology Hospital. The researches were focused on the prenatal discovery of chromosomal disorders (trisomies 21, 13, 18, monosomy X, triploidy) but also on the improving of prenatal screening and diagnosis. In the field of prenatal screening I applied and I won a intern grant of ”Grigore T. Popa” University of Medicine and Pharmacy from Iași (Grant intern

28210/16.12.2011). The title of this research grant was: Quantification of methylated fetal DNA – a new approach in noninvasive prenatal diagnosis of trisomy 21. The scientific results of this grant were promising, but unfortunately the implementation in practical medicine was impossible. The scientific activity in the field of prenatal screening and diagnosis was published in different journal (BDI and ISI) and most representative of them are synthetized in the following list:

- **Gorduza E.V.,** Onofriescu M., Martiniuc V, Grigore M., Mihălceanu E., Iliev G., Importanţa tehnicii FISH în diagnosticul prenatal al aneuploidiilor, Rev. Med. Chir. Soc. Med. Nat. Iaşi, 2007, 111 (4) 990-996
- Caba L., Panzaru M., Vicol M.C., Braha E.E., Popescu R., Cozaru G.C., Socolov D.G., **Gorduza E.V.,** Diagnostic prenatal invaziv versus noninvaziv: controverse și dileme etice (Invasive versus non-invasive prenatal diagnosis: controversy and ethical dilemmas), Rev. Rom. Bioet. (Rom. J. Bioethics) 2012, 10 (3), 29-37,
- **Gorduza E.V.,** Popescu R., Caba L., Ivanov I., Martiniuc V., Nedelea F., Militaru M., Socolov D.G., Prenatal diagnosis of 21 trisomy by quantification of methylated foetal DNA in maternal blood: study on 10 pregnancies, Rev. Rom. Med. Labor., 2013, 21 (3/4) 275-284
- Braha E., Martiniuc V., Pânzaru M., Caba L., Butnariu L., Onofriescu M., Socolov D., Grigore M., Nemescu D., Mihălceanu E., Iliev G., **Gorduza E.V.,** Prenatal diagnosis of gonosomal anomalies: Limitations of the FISH method and genetic counselling difficulties in 15 cases, Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2013, 117 (2), 450-456
- Grigore M., Vulpoi C., Preda C., Martiniuc V., Vasiliu I., **Gorduza E.V.,** Using HDlive technology to diagnose Turner syndrome in the first trimester of pregnancy: clinical cases presentation and literature review, Acta Endocrinologica, 2015, 11(1): 93-98,
- Socolov D., Mihălceanu E., Popovici D., **Gorduza E.V.,** Balan R., Martiniuc V., Socolov R., Prenatal diagnosis of triploidy in second trimester of pregnancy: a series of 4 cases over an eleven-year period (Diagnosticul prenatal al triploidiei în trimestrul al II-lea de sarcină: o serie de patru cazuri depistate în unsprezece ani), Rom Rev Laborat Med., 2015, 11 (2) 213-220
- Socolov R.V., Andreescu N.I., Haliciu A.M., **Gorduza E.V.,** Dumitrache F., Balan R.A., Puiu M., Dobrescu M.A., Socolov D.G., Intrapartum diagnostic of Roberts syndrome–case presentation, Rom J Morphol Embryol, 2015, 56 (2): 585-588,
- Socolov D., Socolov R., **Gorduza E.V.,** Butureanu T., Stanculescu R., Carauleanu A., Pavaleanu I., Increased nuchal translucency in fetuses with a normal karyotype—diagnosis and management An observational study, Medicine (Baltimore) 2017, 96:29, e7521
- Gug C., Burada F., Ioana M., Riza A.L., Moldovan M., Mozos I., Ratiu A., Martiniuc V., **Gorduza E.V.**, Polyploidy in First and Second Trimester Pregnancies in Romania - a Retrospective Study, Clin. Lab. 2020, 66:517-527
- Gug C, Mozos I, Ratiu A, Tudor A, **Gorduza EV,** Caba L, Gug M, Cojocariu C, Furau C, Furau G, Vaida MA, Stoicanescu D. Genetic Counseling and Management: The First Study to Report NIPT Findings in a Romanian Population. Medicina. 2022; 58(1):79.

However, because the majority of the genetic disorders are rare diseases my scientific interest focused also on this problematic. These researches are very difficult because the number of patients is reduced, the obtention of representative data is limited and the published papers usually concerned a single case. The most representative papers in this field are presented in the following list:

- Socolov D., Terinte C., **Gorduza E.V.,** Socolov R., Puiu J.M., Limb Body Wall Complex – case presentation and literature review, Rom. J. Leg. Med., 2009, XVII (2), 133-138
- Pânzaru M., Rusu C., Voloşciuc M., Braha E., Butnariu L., Ivanov I., Grămescu M., Popescu R., Caba L., Sireteanu A., Macovei M., Covic M., **Gorduza E.V.,** Optimizarea strategiei de diagnostic genetic în sindromul velo-cardio-facial, Rev. Med. Chir. Soc. Med. Nat. Iaşi, 2011, 115(3) 756-761
- Spiridon M.R., Petris A.O., **Gorduza E.V.,** Petras A.S., Popescu R., Caba L., Holt-Oram Syndrome With Multiple Cardiac Abnormalities, Cardiol Res. 2018;9(5):324-329
- Gug C., Caba L., Mozos I., Stoian D., Atasie D., Gug M., **Gorduza E.V.,** Rare splicing mutation in COL1A1 gene identified by whole exomes sequencing in a patient with Osteogenesis imperfecta type I followed by prenatal diagnosis: a case report and review of the literature, Gene, 2020, PII: S0378-1119(20)30234-1
- Gug, C., **Gorduza, E. V.,** Lăcătuşu, A., Vaida, M. A., Bîrsăşteanu, F., Puiu, M., Stoicănescu, D., CHARGE syndrome associated with de novo (I1460Rfs*15) frameshift mutation of CHD7 gene in a patient with arteria lusoria and horseshoe kidney. Exp Ther Med 2020, 20: 479-485.
  Arghir A, Popescu R, Resmerita I, Budisteanu M, Butnariu LI, **Gorduza EV,** Gramescu M, Panzaru MC, Papuc SM, Sireteanu A, Tutulan-Cunita A, Rusu C, Pallister–Killian Syndrome versus Trisomy 12p—A Clinical Study of 5 New Cases and a Literature Review, Genes 2021, 12, 811.
- Momanu A, Caba L, Gorduza NC, Arhire OE, Popa AD, Ianole V, **Gorduza EV,** Gorham-Stout Disease with Multiple Bone Involvement—Challenging Diagnosis of a Rare Disease and Literature Review. Medicina 2021, 57, 681.
- Florea L, Caba L, **Gorduza EV,** Bardet-Biedl Syndrome—Multiple Kaleidoscope Images: Insight into Mechanisms of Genotype-Phenotype Correlations. Genes 2021, 12, 1353.
- Gavril EC, Luca AC, Curpan AS, Popescu R, Resmerita I, Panzaru MC, Butnariu LI, **Gorduza EV,** Gramescu M, Rusu C, Wolf-Hirschhorn Syndrome: Clinical and Genetic Study of 7 New Cases, and Mini Review. Children 2021, 8, 751.
- Butnariu LI, Țarcă E, Cojocaru E, Rusu C, Moisă ȘM, Leon Constantin M-M, **Gorduza EV,** Trandafir LM. Genetic Modifying Factors of Cystic Fibrosis Phenotype: A Challenge for Modern Medicine. J Clin Med. 2021; 10(24):5821.

Research activity is impossible to make alone and for this reason I participated during my entire scientific activity in different research teams that won national or international grants. The list of this research projects is presented above:

1. Compuşi antioxidanţi vegetali şi succedanee de sinteză - Grant CNCSIS 2001-2003 (306/A-24/2001, 384/A-24/2002, 320/A-24/2003) Director grant: Prof. Dr. Ing. Valeria-Marta Gorduza. Reprezentant din partea UMF Iaşi: şef lucr. dr. Eusebiu Vlad Gorduza. Valoare grant 42.400.000 + 50.000.000 + 67.500.000 = 159.900.000 lei vechi (15.990 RON)

2. Nanomateriale inteligente fotosensibile aplicabile în monitorizarea proceselor biotehnologice, biocontrol şi teste citogenetice - Grant CNCSIS 2004-2006 (514/A-25/2004, 514/A-30/2005, 514/A-39/2006) Director grant: Prof. Dr. Ing. Valeria-Marta Gorduza. Reprezentant din partea UMF Iaşi: şef lucr. dr. Eusebiu Vlad Gorduza. Valoare 150.000.000 + 200.000.000 + 250.000.000 = 600.000.000 (60.000 RON).

3. Platforma interdisciplinară de medicină moleculară, grant platformă CNCSIS 2006-2008 (nr. contract 32/06.06.2006), Coordonator proiect: Prof. dr. Eugen Carasevici. Membru şef lucr. dr. Eusebiu Vlad Gorduza. Valoare contract: 3.780.000 RON,
4. Sisteme mezomorfe cromogen-polimerice organizate in arhitecturi supramoleculare pentru suprafete inteligente cu proprietati controlabile (CLICOPOL). Proiect Nr. 1855, contract nr. 71092 / 18.09.2007 – CLICOPOL. Coordonator proiect: - Institutul National de Cercetare-Dezvoltare pentru Chimie-Petrochimie – ICECHIM. Partener 2 (subcontract UTI 6157P/14.09.2007) Universitatea Tehnica Gheorghe Asachi Iasi, Responsabil tema: Prof.dr.ing. Valeria-Marta Gorduza. Membru - Sef lucr.dr. Eusebiu-Vlad Gorduza – UMF Iasi - aplicatii biomedicale. Valoare contract 2.000.000 RON/ 2007 – 2010 (34 luni)
5. Analiza FISH a eritroblastelor nucleate fetale din sângele matern Proiect 1196/2007 - Programul PNCDI -2 –Idei. Perioadă 2007-2009. Director de proiect: Şef lucr. dr. Nemescu Dragoş. Membru Sef lucr.dr. Eusebiu-Vlad Gorduza
6. Corelarea aspectelor clinice, genetice şi epigenetice în înţelegerea etiologiei bolilor genomice Prader-Willi şi Angelman: model de abordare multidisciplinară a bolilor rare în România, PWA-Clin-Epi-Gen. Proiect 42-113/2008 - Programul PNCDI -2. Desfăşurare: 2008-2011. Director de proiect: Conf. Dr. Maria Puiu, UMF Timişoara. Valoare proiect 300.000 lei. Membru Sef lucr.dr. Eusebiu-Vlad Gorduza
7. Cuantificarea ADN-ului fetal metilat – o nouă abordare în diagnosticul prenatal neinvaziv al trisomiei 21. Grant intern Universitatea de Medicină şi Farmacie „Grigore T. Popa" Iaşi 28210/16.12.2011. Coordonator de proiect: Conf. Dr. Eusebiu Vlad Gorduza. Perioada: 16.12.2011 – 15.12.2012. Valoare proiect 21.500 lei
8. Réseau partenaire francophone pour la validation clinique des protocoles diagnostiques et thérapeutiques de la médecine fœtale dans la région de l'Europe orientale. Proiect Agence Universitaire de la Francophonie 2012. Manager de proiect: Şef lucr. Demetra Socolov. Membru Conf. Dr. Eusebiu Vlad Gorduza. Perioadă: 01.10.2012 - 01.10.2014. Valoare proiect 20.000 euro
9. Implementarea unui algoritm de diagnostic bazat pe analiza complexă a profilului genomic pentru pacienții cu anormalități congenitale și de dezvoltare. Proiect de tip Partnerships PN-II-PT-PCCA-2013-4-2240. Coordonator: Institutul de Virusologie "Ștefan S. Nicolau" din București. Partner 2 Universitatea de Medicină şi Farmacie "Grigore T . Popa" Iaşi – Director: Conf. Dr. Eusebiu Vlad Gorduza. Valoare proiect: 1.250.000 lei
10. Promoters of Advanced oncogenetics open online training and multimedia raise awareness on multidisciplinary assessment of patients and their families at risk of hereditary of familial cancer (HOPE). Proiect Erasmus+ - Contract 2018-1-RO01-KA202-049189. Coordonator: Universitatea de Medicină şi Farmacie "Grigore T . Popa" Iaşi. Membru: Prof. dr. Eusebiu Vlad Gorduza. Perioadă de implementare 2018-2020
11. Initier et developer une collaboration scientifique et medicale dans le domaine des anomalies vasculaires. Proiect Internațional WB1-FNRS (Belgique) – Academia Română, reprezentată de Institutul de Chimie Macromoleculară "Petru Poni" Iași. Coordonator proiect : Cerc. Grd. I Mariana Pinteală. Membru Prof. dr. Eusebiu Vlad Gorduza. Perioadă de implementare 2019-2021

My scientific researches were published in national and international journal indexed in Clarivate Web of Science Core (former Thompson) or another international database. I have also disseminated my scientific results at local, national, and international congresses,

conferences, seminars or workshops. My scientific activity can be resumed in the following numbers:

- **95 papers published in extenso**:
  - 32 in ISI journals
  - 56 in BDI journals
  - 7 in non-indexed journals
- **136 papers published in abstract**:
  - 79 in ISI journals
  - 57 in non-indexed journals
- **148 papers published in proceedings of different scientific meetings**:
  - 66 in extenso
  - 82 in abstract

My works were cited by 302 papers or books.
My scientific activity is recognized at international level by a Hirsch Index of 7.

In conclusion, to overview my activity, I think that is important to highlight some hallmarks:

- Academic activity covers more than 25 years, period when I accessed all the steps of academic hierarchy.
- During this period, I had teaching activity (laboratory or courses) with different categories of students from Medicine Faculty, Stomatology Faculty, Pharmacy Faculty.
- My academic activity imposed to write books or chapters of books.
- My medical activity was focused to the reproductive medicine in connection with my clinical integration (in the last two decades) in ”Cuza Vodă” Obstetrics and Gynecology Hospital Iasi.
- My research activity focused different fields of genetics, the most important being the cytogenetics, and medicine of reproduction. This activity was supported by my presence in more than 10 projects of research at local, national or international level.
- The research activity allowed important data that was used for publication of scientific papers in ISI or BDI journals. Per total I published 32 papers in ISI journals and 56 papers in BDI journals.
- My scientific activity was recognized by more than 300 citations in different journals and by a Hirsch Index of 7.

II. SCIENTIFIC ACTIVITY

II.1. CHROMOSOMAL ANOMALIES AND DISEASES

The researches concerning chromosomal anomalies and chromosomal diseases were published in the following papers
Sireteanu A., Covic M., **Gorduza E. V.**, Hibridizarea genomică comparativă pe microreţele: consideraţii tehnice şi aplicaţii, Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2012, 116 (2), 545-551
Gorduza E.V., Covic M., Stoica O., Voloşciuc M., Angheloni T., Butnariu L., Braha E., *Studii clinice, epidemiologice şi citogenetice pe un lot de 221 pacienţi cu sindrom Down,* Rev. Med. Chir. Soc. Med. Nat. Iaşi, 2007, 111 (2), 363-372
Socolov D., Iliev Gh., Scripcaru D., **Gorduza E.V.,** Socolov R., Puiu M., *Trisomy 13 with cyclopia and proboscis – a case presentation,* J. Pediatr., 2009, XII (45-46), 16-18
Caba L., Rusu C., Butnariu L., Pânzaru M., Braha E., Voloşciuc M., Popescu R., Grămescu M., Bujoran C., Martiniuc V., Covic M., **Gorduza E.V.,** *Phenotypic variability in Patau syndrome,* Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2013, 117 (2), 321-327
Butnariu L., Rusu C., Caba L., Pânzaru M., Braha E., Grămescu M., Popescu R., Bujoran C., **Gorduza E.V.**, *Genotype - phenotype correlation in trisomy X: A retrospective study of a selected group of 36 patients and review of literature",* Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2013, 117 (3), 714-721
Pânzaru M., Caba L., Rusu C., Butnariu L., Braha E., Popescu R., Grămescu M., Popa S., Resmeriţă I., Bujoran C., Martiniuc V., **Gorduza E.V.,** *Phenotypic variability in Edwards syndrome: synopsis of 19 cases with trisomy 18,* Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2017, 121 (1), 172-177
Butnariu L., Grămescu M., Caba L., Pânzaru M., Braha E., Popescu R., Popa S., Rusu C., Cardos G., Zeleniuc M., Martiniuc V., Plăiaşu V., Diaconu C., **Gorduza E.V.,** *Using of multiple chromosomal and molecular analyses to elucidate the etiology of plurimalformative syndromes,* Rev Med Chir, 2017, 121 (3): 581-593
Gorduza E.V., Bujoran C., Pădurariu L., Grămescu M., Ivanov I., Martiniuc V., Caba L., Popescu R., Stamatin M., Covic M., *Presentation of a new case with a mosaic ring chromosome 13: 46,XY,r(13)(p11.2-q34)/45,XY,-13 and a review of literature,* Rom J Rare Dis, 2010, 1 (1), 17-23
Caba L., Rusu C., Plăiaşu V., Gug G., Grămescu M., Bujoran C., Ochiană D., Voloşciuc M., Popescu R., Braha E., Pânzaru M., Butnariu L., Sireteanu A., Covic M., **Gorduza E.V.**, *Ring autosomes: some unexpected findings,* Balkan J. Med. Genet., 2012, 15 (2), 35-46
Sireteanu A., Braha E., Popescu R., Gramescu M., **Gorduza E.V.**, Rusu C. *Inverted duplication deletion of 8p: characterization by standard cytogenetic and SNP array analyses,* Rev. Med. Chir. Soc. Med. Nat., Iaşi, 2013, 117 (3), 731-734
Sireteanu A., Voloşciuc M., Grămescu M., **Gorduza E.V.,** Vulpoi C., Frunză I., Rusu C., *Dicentric chromosome 14;18 plus two additional CNVS in a girl with microform holoprosencephaly and Turner stigmata,* BJMG 2013, 16 (2), 67-72
Popescu R, Grămescu M, Caba L, Pânzaru M-C, Butnariu L, Braha E, Popa S, Rusu C, Cardos G, Zeleniuc M, Martiniuc V, Gug C, Păduraru L, Stamatin M, Diaconu CC, **Gorduza EV.,** *A Case of Inherited t(4;10)(q26;q26.2) Chromosomal Translocation Elucidated by Multiple Chromosomal and Molecular Analyses. Case Report and Review of the Literature.* Genes. 2021; 12(12):1957. https://www.mdpi.com/2073-4425/12/12/1957, https://doi.org/10.3390/genes12121957, IF – 4,096
Gorduza V.M., **Gorduza E.V.,** Simionescu C.I., Galiţă L., *DNA micro arrays with fluorescent markers applied in molecular genetics and nanobiotechnology,* Memoriile Sectiilor Ştiinţifice ale Academiei Române, 2007, seria IV, tom XXX 113-139 (http://www.academiaromana-is.ro/Reviste/mem_content2007.html, http://www.academiaromana-is.ro/Reviste/mem_sc_st_2007/mss_series_IV_tome_XXX_2007_p113.pdf

II.1.1. INTRODUCTION

Chromosome identification became possible by introducing of chromosome banding techniques that highlight bands patterns characteristic for each chromosome. Association of molecular techniques with cytogenetic techniques opened a new era in cytogenetics, initated by FISH and continued by aCGH.

Chromosomal anomalies are an important component of hereditary pathology, because they have a high frequency and produce major phenotypic and reproductive consequences. They produce about 100 chromosomal syndromes affecting 2% of the pregnancies of women older than 35 years, and 50% of miscarriages in the first weeks of pregnancy. The most common chromosomal abnormalities are trisomy 21 affecting 1/750 neonates, while monosomy X and trisomy 16 are the most common causes of miscarriage.

Chromosomal anomalies are the result of genomic or chromosomal mutations. The genomic mutation rate is high, about 10^{-2}/cell division, but the frequency is low, because most are not perpetuating and are incompatible with either survival or reproduction.

Unbalanced chromosomal abnormalities produces chromosomal diseases, identifiable by improving the cytogenetic diagnostic methods. „Classical" chromosomal diseases syndromes represented by complete aneuploidies (monosomy X and trisomies 21, 18, 13, 8, XXX, XXY, XYY) or large partial aneuploidy (deletions 4p, 5p, X Y, etc.) are detectable by conventional cytogenetic techniques. „Modern" chromosomal diseases represented by plurimalformative syndromes, produced by microdeletions, microduplications or different types of unbalanced chromosomal rearrangements was identified by the combination of classic cytogenetic techniques with molecular technology. In these diseases the affected chromosomal segment is less of 3-5 Mb and the detection of anomaly is possible only by FISH or array-CGH.

II.1.2. APPLICATIONS OF DNA MICROARRAYS

The micro-arrays consist of thousands of different probes (each specific for a DNA sequence), non-labelled, attached to a glass slide or a silicon chip, in small dots with precise coordinates, arranged in orderly rows, in the form of a network of high density (DNA chip). In order to achieve the analysis the genomic DNA is extracted from the patient (lymphocytes, fibroblasts and other tissues) and a reference sample is taken (from the same cell line like the patient but provided from one or more normal individuals). The two samples of DNA are fragmented (by ultrasound or by digestion with a restriction enzyme) labelled with different fluorochrome (Cy3 - green for patient, Cy5 - red for marker), mixed in equal amounts, denatured and co-hybridized on microarray [351, 583]. After washing, the microarray is scanned and the images are analyzed using a program that quantifies the intensities of red and green fluorochromes emission for each spot on the network and calculate their ratio. If the intensities of the two fluorochromes are equal in one spot (compared Cy3/Cy5 = 1 or log2 = 0) the region of the patient's DNA is interpreted as normal. If there is a deletion, marker DNA preferentially hybridized and report Cy3/Cy5 will be below par (ie. 1: 2, log2 = -1). If there is a duplication, the patient's DNA will hybridize preferentially and the Cy3/Cy5 report will be greater than one (ie. 3: 2, log2 = 0.58.) (Figure II.1.) [351].

In practice there can be used different types of microarrays: "targeted" microarrays, whole-genome microarrays or a combination these types. Targeted microarrays contain specific fragments of the genome where microdeletions / microduplications involved in the production of plurimalformative syndromes occur frequently. This type of microarray is used for diagnostic purposes or for screening [497, 581]. Whole genome microarray ("tiling path") analyzes the whole genome with a resolution that depends on the distance between the probes; this type of microarray identifies 5% more anomalies than a targeted microarray [26]. The whole genome microarray with very high density is used in research and leads to the definition

of new syndromes and characterization of the breakpoints in different chromosomal abnormalities or gene mutations, but has not got clinical applicability due to the identification of many CNV (Copy Number Variations - DNA segments with lengths of more than 1 kb, which are present in the genome of different individuals in a variable number of copies, and do not result from the insertion or deletion of transposable elements), which is difficult to interpret [148].

The probes can be a BAC (Bacterial Artificial Chromosome), cDNA, oligonucleotide or SNP. Initially BAC probes containing DNA isolated from large clones, but they were gradually dropped because their production was laborious, and the resolution was limited. Oligonucleotide probes, obtained by chemical synthesis provide a high resolution due to their small size. Oligonucleotide probes containing SNPs allow both the identification of genomic imbalances type deletion / duplication and anomalies without changing the number of copy, such as loss of heterozygosity and uniparental disomy [132].

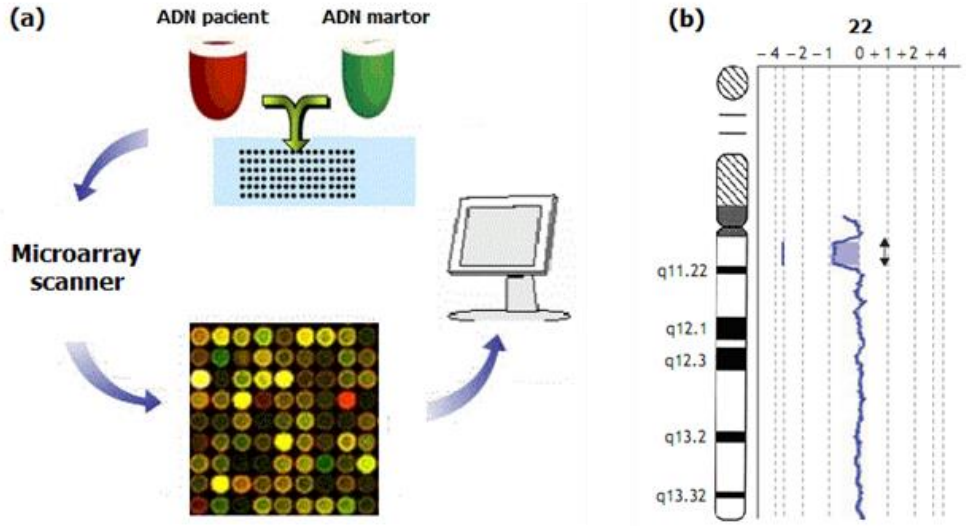


Figure II.1. Principle of aCGH technique.

(A) The DNA from the patient sample and the reference are marked with different fluorochromes and cohybridizate on a microarray containing genomic DNA fragments fixed on a glass slide. After scanning fluorescent signals are captured and digitally analyzed based on the intensity, resulting a graphical representation. (B) Example of aCGH: 22q11.21 deletion. The horizontal axis indicates the log ratio of fluorescence (negative values - deletions; positive - duplications). The vertical axis indicates the locus on chromosome.

By applying aCGH technique in normal individuals are identified numerous genomic rearrangements, impossible to detect by other methods, such CNV [607]. The term is neutral in relation to the pathogenicity and frequency of appearence, but in cases where the frequency in the population is over 1% copy number polymorphism is used [620]. The clinical relevance of CNV is uncertain, because the identification of such variations is not necessarily associated with a disease.

Clinical testing by aCGH, regardless of the type of probes or microarray used requires the following algorithm: if a known anomaly is detected, it will be validated by other methods and reported. Furthermore, the parents will be tested in order to identify the genitor from which the CNV was inherited. The method will be applied to future pregnancies to determine the presence / absence of pathological change in the offspring. If the detected CNV is not associated with a particular disease, the result obtained is compared with the information from the Database of Genomic Variants (DGV), in order to see if the change has been found in healthy individuals. If the anomaly is not found in the DGV or the number of reported cases is small, both parents will be tested. If one of them shows the CNV, it is likely to be a benign variant. If the variation is de novo, investigation of the region where CNV was identified will be continued to identify the genes located there [148].

The aCGH method has many advantages:

- It allows global detection, with high resolution of genomic imbalances.
- It does not require cell division and can be analysed with biological samples that do not contain living cells.
- It can detect chromosomal mosaics in peripheral blood, impossible to detect by karyotype, because nucleated cells of blood have been analyzed
- Method requires a very small amount of biologic product – only a few micrograms of genomic DNA.
- Provides definitive results in a short time and is suitable for automation [28].

aCGH disadvantages include:

- Inability to detect balanced chromosomal abnormalities and polyploidy [233, 507].
- The costs per analysis are much higher than those for classical karyotype.
- If a CNV is identified, it is necessary to validate the genomic rearrangements by FISH or qPCR techniques that allow a target-specific detection of DNA sequences [607].

The applications of aCGH include mental retardation, multiple congenital anomalies, prenatal diagnosis, cancer, discovery of new syndromes, identification of candidate genes and toxicology.

An important application of aCGH technology is to identify chromosomal abomalies in patients with idiopathic mental retardation (MR), with or without facial dysmorphia or multiple congenital anomalies (ACM). Chromosomal anomalies are the most common cause of RM and subtelomeric rearrangements are responsible for 5-7% of cases [99]. Identifying imbalanced chromosome anomalies in subtelomeric chromosomal by G banding is difficult because this type of banding does not highlight the chromosomal ends. On the other hand, the FISH technique requires obtaining good metaphasic preparation and evaluation of all subtelomeric regions; this is very expensive and cannot be done in a single analysis. aCGH can identify a large number of rearrangements, it can measure the size of the chromosomal segment, can diagnose complex rearrangements and can prove a deletion or duplication of an adjacent segment [29]. The use of aCGH method in cases of RM allowed identification of new duplications and deletions and established the prevalence of chromosomal mosaics. Thus, application of high-resolution aCGH in unselected patients allowed the detection of pathogenic anomalies in 19% of the cases, a value higher than corresponding to chromosomal analysis (~ 3.7%) and FISH (~ 9 5%) [233, 505, 575]. In addition, aCGH detected mosaics in 8-10% of individuals with RM and facial dysmorphia from unselected groups [28].

The application of aCGH in prenatal diagnosis eliminates some of the inconveniences of fetal chromosomal analysis (long period - 2-3 weeks - for obtaining the final result, the potential failure of culture, low quality of chromosomal preparations and low number of chromosomal bands). The method uses genomic DNA from fetal cells and can be applied directly or on cultured cells. The sensitivity of method is higher than that of the karyotype and allows detection of imbalanced chromosomal anomalies (excepting polyploidy) including subtelomeric rearrangements [484]. For prenatal diagnosis only targeted microarrays are used, containing genomic clones and subtelomeric regions, frequently involved in syndromes with microdeletions / microduplications [456]. Given that most duplications and deletions associated with genomic disorders arise de novo and seem to have the same frequency regardless of maternal age, aCGH could replace cytogenetic exploration in most cases of prenatal diagnosis [526]. Because aCGH can not detect balanced translocations and polyploidy, when fetal ultrasound shows congenital anomalies and aCGH is normal, it is useful also to apply classic cytogenetic techniques [340]. aCGH has applicability for the study of abortion products when is impossible to make a karyotype or fetal cells have a cryptic chromosomal abnormality [70].

In cancer cells, aCGH allows identification of amplification of a chromosomal segment, of deletions in tumor growth suppressor genes, of chromosomal deletions or rearrangements involving proto-oncogenes that provides new information on genes involved in cancer. The high resolution and sensitivity of aCGH allows the evaluation of genomic imbalances and improves cancer diagnosis, tumor classification schemes, deciphers and monitors the mechanism for the development and progress of neoplasia [312, 314, 491]. However, while in various forms of cancer complex chromosomal translocations are present, the application of aCGH is limited due to the inability to detect balanced translocations [507].

The implementation of aCGH has elucidated some plurimalformative syndromes caused by chromosomal microdeletions or microduplications. By use of aCGH on a large batch of patients the description of new syndromes by "reverse dysmorfology" was facilitated when the discovery of cytogenetic anomalies preceded the corresponding phenotype definition. After analyzing the results and defining a specific chromosomal region, the phenotypes are compared to determine the essential clinical features, which can be subsequently identified in other patients with the same anomaly [620].

The measurements of gene expression levels upon exposure to a chemical can be used both to provide information about the mechanism of action of the toxicant and to form a sort of genetic signature for the identification of toxic products. The development of gene arrays has allowed this technology to become a standard tool in molecular toxicology and ecotoxicology [218, 594]. The possibility of analyzing the effect of chemicals and environmental stressors on a large number of genes has led to the development of the toxicogenomics. Proponents of toxicogenomics aim to apply both mRNA and protein expression technology to study chemical effects in biological systems [405]. It is of interest to evaluate the effects of nephrotoxicants, genotoxic chemicals [389], and hepatotoxicants on gene expression. The gene expression data can provide meaningful information on the physical location of the toxicity and the dose-dependent changes [424]. Gene expression profiling has been used to show that the specific genes are repressed or induced upon exposure to a toxic stress that varies depending on the cell type and the type of toxicants to which the cells were exposed [16]. Thus new up-regulated genes were found encoding proinflammatory cytokines, antioxidants, acute-phase proteins, complements, chemokines, and cell adhesion molecules [159]. Transcript profiling using microarrays showed clear differences between the left and median lobes of liver, both at low doses and at doses that cause hepatotoxicity [255].

Gene expression profiles represent the primary level of integration between environmental factors and the genome, providing the basis for protein synthesis. The analysis of gene expression changes is a powerful tool both to diagnose major stressors in natural populations and to analyze the mechanisms of such stress responses [450].

II.1.3. DOWN'S SYNDROME

II.1.3.1. BACKGROUND

Down's syndrome (DS), produced by trisomy 21 has an incidence of 1:650 – 1:1.000 newborns and a *sex ratio* ♂:♀ = 3: 2 [20, 250, 284].

The etiology of trisomy 21 is unknonw, but the main incriminated factor is advanced maternal age. The risk for a child with homogenous trisomy 21 grows from 1/1.400 at age of 20 years to 1/25 after age of 45 years [20, 72, 380, 498, 618].

The symptomatology is variable and the features presented in all patients are learning difficulties and neonatal muscular hypotonia [157, 227, 299].

In newborns**,** trisomy 21 is sugerated by: growth retardation, muscular hypotonia and typical cranio-facial dysmorphism, associated with visceral malformations [19, 157, 216, 284, 560].

In childhood and adulthood, the following features are added: learning difficulties, short stature and Alzheimer-like dementia with precocious start [19, 299, 454].

Cytogenetic analysis**,** essential for etiologic diagnostic, could reveal: homogenous free *trisomy 21*, *trisomy 21 by Robertsonian translocation*, *trisomy 21 mosaicism* or partial 21 *trisomy* [239, 528].

II.1.3.2. COHORT AND METHODS

The patients with DS were selected from the archives of the Genetics Center Iaşi (CPG) and the karyotype register of the Cytogenetic Laboratory of "Grigore T. Popa" University of Medicine and Pharmacy Iaşi. In period 1985-1999, CPG Iaşi reviewed all congenital anomalies and genetic diseases in children at the age of 0-1 years, born in Iaşi County. The number of births was 169,148 and 3,128 (18,492‰) children presented congenital anomalies. In Iaşi town there were 53,625 children born of whom 1,444 (26,927‰) children presented congenital anomalies. In this period 221 children were examinated with a clinical diagnosis of DS: 126 children with only clinical diagnosis (*cohort A*) and 95 children with cytogenetic confirmation of diagnosis (*cohort B*).

Methodes used for investigation were: *clinical, epidemiological and cytogenetic*. Clinical aspects investigated were dysmorphisms characteristic for DS and major congenital anomalies [19, 20, 45, 166, 178, 284, 439, 560]. Epidemiologic analyse was focused on incidence of DS in newborns, number of cases of SD depending the maternal age and the mortality by DS. Cytogenetic analyses were made by G banding and 32 metaphases were analysed in each case. The study was focused on the identification of types of chromosomal anomalies and the frequency of each type.

II.1.3.3. RESULTS

We analised 22 clinical signs for each patient with DS (except one patient in each cohort) and compared these with other studies (table II.1.).

Hall sugested thad DS must be suposed in presence of minimum 6 from a list of 10 clinical signs. With the exception of pelvian dysplasia all other signs were searched in our study. The search of these signs was very important in cohort A, because these patients did not benefit from cytogenetic investigation. More than 2/3 of patients had 5 or more signs, and this was an argument that attested the quality of clinic diagnostic. Using ***t-independent test*** we compared the clinical features in our cohort with data from literature and we found higher values for brachicephaly and up slanting palpebral fissures, but lower values for articular hyperflexibility, muscular hypotonia, Brushfield spots, anomalies of palate, clinodactily and wide I interdigital space in feet ($P<0.05$). Explanations for these discordances could be***:*** *difficulties to find these signs in newborns, incomplete clinical examination* or *particularities of our cohorts* [20, 216].

We found *major congenital anomalies* in 82 patients (37.1%). Cardiac congenital anomalies were found in 76 patients that reflect their high frequency in DS. In 40 cases, the cardiac anomaly was confirmed by echocardiography or pathologic examination. We found 26 isolated cardiovascular anomalies, 9 complex cardiovascular anomalies and 5 cardiac anomalies in patients with multiple congenital anomalies. The most frequent cardiac anomalies were: atrial septal defect - ASD (13 cases) ventricular septal defect - VSD (12 cases) and common atrio-ventricular canal - CAVC (11 cases). The severity of cardiac anomalies was confirmed by the death of 25 of our patients in their first year of life. Digestive congenital anomalies were found in 9 patients (5 with isolated anomalies and 4 with multiple congenital anomalies) and were severe causing the death of 7 of 9 patients.

We calculated the incidence of DS in Iaşi city and county, for each calendar year, for five years and for all period, and the obtained data indicated higher global incidence of DS in

Iaşi County compared with Iaşi city. Incidence of DS in Iaşi county varied between 0.859‰-2.006‰, with an average of **1,306‰ (1/769 newborns)** and a median values of 1.091‰. In Iaşi city, incidence of DS varied between 0.765‰-2.212‰ (average 1.286‰; median value 1.437‰). In Iaşi County, but excluding Iaşi city, incidence of DS varied between 0.558‰-2.310‰ (average 1.315‰; median value 1.428‰).

Table II.1. Frequency of dysmorphic features in DS

Results	**Children with SD from Iaşi County**						**Data from literature**	
	Cohort A		**Cohort B**		**Total**		**[20]**	**[216]**
	Nr.	**%**	**Nr.**	**%**	**Nr.**	**%**	**%**	**%**
Newborns								
Muscular hypotonia	60	48	46	48.93	106	48.40	100	80
Articular hyperflexibility	43	34.4	40	42.55	83	37.89	75	80
Flat facies	87	69.6	65	69.14	152	69.40		90
Up slanting palpebral fissures	120	96	89	94.68	209	95.43	80	80
Dysplasic ears	112	89.6	76	80.85	188	85.84		60
Folded skin on neck	91	72.8	68	72.34	159	72.60		80
Simian crease	56	44.8	47	50	103	47.03		45
Clinodactily	36	28.8	33	35.10	69	31.50		60
Femural hyperabduction	14	11.2	13	13.82	27	12.32		70
Other signs								
Brachycephaly	107	85.6	79	84.04	186	84.93	75	
Round face	116	92.8	84	94.68	200	91.32		
Epicantus	93	74.4	63	67.02	156	71.23	60	
Hypertelorism	68	54.4	48	51.06	116	52.96		
Brushfield spots	26	20.8	18	19.14	44	20.09	55	
Open mouth	91	71.8	61	64.89	152	69.40	60	
Macroglosia	77	61.6	50	53.19	127	57.99	55	
Anomalies of palate	23	18.4	14	14.89	37	16.89	75	
Anomalies of nose	85	68	81	86.17	166	75.79		
Short neck	21	16.8	22	23.40	43	19.63	60	
Short hands	73	58.4	70	74.46	143	65.29	65	
Short fingers	91	72.8	72	76.59	163	74.42		
Wide I interdigital space in feet	31	24.8	39	41.48	70	31.96	70	

Sex ratio for our cohort was ♂:♀ = 1.302, having 125 male patients and 96 female patients.

The relationship between DS and maternal age is presented in table II.2. In total, 37% of our patients with DS were born by women older than 35 years. Because 62.91% of patients were born by mothers younger than 35 years it is obviously necessary to have prenatal screening and diagnosis to improve the prevention.

Incidence of DS in newborns depends on maternal age, quality of census of cases and ending by therapeutic abortion of pregnancies with foetus with trisomy 21 [91]. In our cohort, the last parameters are not relevant, because the prenatal diagnosis of DS was not applied. The quality of census was good and the accuracy of clinical diagnosis was confirmed by high number of signs discovered in our patients and by a low number of cytogenetic unconfirmed cases (only 6 cases – 5.94%).

Table II.2. Relationship between number of cases with Down syndrome and maternal age

Maternal age	Cohort A (clinic)		Cohort B (cytogenetic)		Total	
	No. of cases	%	No. of cases	%	No. of cases	%
<19 years	6	4.76	3	3.15	9	4.07
20-24 years	24	19.04	32	33.68	56	25.33
25-29 years	23	18.25	22	23.15	45	20.36
30-34 years	17	13.49	12	12.63	29	13.12
35-39 years	31	24.60	12	12.63	43	19.45
> 40 years	25	19.84	14	14.73	39	17.64
Total	126	100	95	100	221	100

As the Medical Statistics Department of Iaşi County does not have information concerning the indicator *number of births/year of fertile age/ calendar year*, it was impossible to directly cuantify the influence of maternal age under incidence of DS. Thus, to standardise the results we utilised the index *BINSMA (Birth Index Inversely Standardised for Maternal Age)* defined by relation II.1. [91].

$$BINSMA = \frac{1}{n}\sum\frac{b_i}{R_i} \qquad \text{relation II.1.}$$

where: n is number of birhs in temporal period; b_i – number of children with DS born by mothers with age i, and R_i – incidence of disease at maternal age i.

We calculated index R_i (table II.3.), using as reference the results of an epidemiologic study of DS, made by Staples in Australia in period 1960-1989 [528].

Table II.3. Comparative presentation between our results and results of Staples et al. [528]

1 - <19 years; 2 – 20-24 years; 3 – 25-29 years; 4 – 30-34 years; 5- 35-40 years; 6 - >40 years

	Maternal age					
	1	2	3	4	5	6
Number of cases with SD in our cohort	9	56	45	29	43	39
Number of cases with SD in our cohort after correction (b_i)	8,46	52,64	42,3	27,26	40,42	36,66
Number of cases with SD in Australia	28	139	158	139	146	122
Number of births in Australia	52.880	195.889	209.527	101.714	36.987	9.076
Incidence of SD in Australia (R_i)	0,5295	0,7095	0,754	1,366	3,947	13,442
b_i/R_i	15,977	74,193	56,1	19,956	10,24	2,272

Because our cohort contained DS patients with cytogenetic confirmation and without cytogenetic confirmation, we used a correction factor correlated with the number of DS unconfirmed cytogenetic. Using relation II.1. and data of table II.2. (reported to total number of 169,148 birth), we obtained a ***corrected value of incidence of SD at birth – 1.056‰.***

Analysing our data we found that 65 de patients with DS died in the first year of life (29.41% of cases). The death cause was established in 40 patients and was represented by: cardiac congenital anomalies (12 cases) digestive congenital anomalies (2 cases) multiple congenital anomalies (5 cases), pneumonia usually in the context of congenital anomalies (21 cases). A high number of deaths by unknown causes (25 cases) or by pneumonia (21 cases) indicated the poor economic and health standards in Iaşi County.

The patients of cohort B are represented by cases of DS where the karyotype (table II.4.) is applied. In 95 out of 101 cases investigated (94.05%) the clinical suposition was correct, the patients had a form of trisomy 21. In the majority of cases we found a homogenous free trisomy 21.

Table II.4. Results of cytogenetic investigation in patients with confirmed diagnosis of Down's syndrome

	Sex: Masculine	Sex: Feminine	Total (%)
Homogenous trisomy 21	53	34	87 (91,57)
Mosaicism trisomy 21	2	1	3 (3,15)
Trisomy 21 by Robertsonian translocation	1	4	5 (5,26)
Total	56	39	95

II.1.3.4. DISCUSSION

Torfs and Christianson [560] identified cardiac congenital anomalies in 55.9% of cases with DS, value similar to that of Stoll et al. - 50% [537], Khoury and Erikson - 50% [284], and Wells et al. – 48% [599], but higher than of Fabia and Drolette – 28.8% [161], or Kallen et al. – 25.6% [276]. ***Our value was 34.38%, finding 76 cardiac congenital anomalies in 221 cases with DS***, but our data could be underestimated in the absence of a complete cardiological investigation.

Torfs and Christianson [560] found duodenal atresia/stenosis in 4.8% cases, similar with data by Stoll et al. – 4.3% [537], but higher than of Fabia and Drolette – 2.6% [161], or Kallen et al. – 2.2% in swedish cohort and 3.4% in italian cohort [276]. *We found* ***duodenal atresia/stenosis in 2.71% cases, a value similar to that from the literature.***

The prevalence of anorectal anomalies was 12.8‰ in Torfs and Christianson's study [560], a value higher than data found by Fabia and Drolette – 7‰ [161], or Kallen et al. – 9‰ [276], but similar with data by Zlotogora [626], Urioste and Martinez-Frias [569] or Torfs and Curry [561]. ***In our cohort we identitied two cases with anorectal anomalies - 9‰ – that is comparabile with data by Kallen et al.*** [276]**.**

We identified only one case with Hirschprung disease – ***4.45‰ –*** values similar to those from Kallen et al. - 5,0‰ [276], or Fabia and Drolette – 3.7‰ [161], but lower than found by Torfs and Christianson [560] – 13.82‰ or Stoll et al. – 8.8‰ [537].

Incidence of DS in newborns depends on maternal age, quality of cension and ending by therapeutic abortion of pregnancies with foetus with trisomy 21 [91]. Global incidence of SD in Iaşi county, between 1985-1999, was 1.306‰, similar to data from Lau et al. [317] - 1.28‰, Lopez et al. [341] 1.20‰, but higher than incidence found by Ghosh et al. [188] 0.9‰ newborns or Hoshi et al. [241] who identified in Japan 1.299 cases of DS at 2,232,694 newborns (incidence 0.58‰).

For our cohort, using *BINSMA* index, ***the corrected value of incidence of DS at birth is 1.056‰.*** This value is concordant with data from Carothers et al., who meta-analysed 47 studies concerning DS and found a 0.94‰ (DS=0.1476) median value for *BINSMA* [91].

In our cohort, 37.09% of patients with DS were born by mothers older than 35 years. This data is similar with that identified by Lau et al. [317] – 41.89% of patients born by mothers older than 35 years or Hoshi et al. [241] – 41.11% of cases with DS resulted from pregnancies in women older than 35 years.

General infantile mortality in Iaşi County was 27.55‰ in period 1985-1999. The mortality by DS for the same period was 266.44‰. This data is similar to that reported in Latin America by Castilla et al., that identified a 25.2 ‰ mortality by SD and a 264‰ general infantil mortality [94] between 1988-1992. From 1985 to1999, we identified 65 cases of DS who died in the first year of life (29.41% of total). In 53.84% of total the cause of death was acute pneumonia (15.38%), congenital anomalies (16.91%) or unknown (38.46%). This value is much higher than identified in South America (21%) [94], Italy (16%) [356]. Canada (11%) [24], England (10%) [364], Denmark (7%) [367]), Eire (4%) [224]. We consider that this could be associated with poor economic and health conditions in our region. In addition, the value of 46.15% for mortality by cardiac anomalies was higher than found in South America (34%) [94], Denmark (28%) [367], Canada (33%) [24], Italy (37%) [356] or England (40%) [364]. Thus, it is obviously a ***necessity to offer neonatal cardiological examination to all children with DS and an improved cardio-vascular surgical program in Romania.***

An important aspect concerning our cohort is a good confirmation of clinical suspicion for DS – 94.23% - that attests the quality of clinical trial. The unconfirmed cytogenetic cases could be explained by errors of diagnosis, weak somatic mosaics or small unbalanced anomalies of chromosome 21. We found a homogenous free trisomy 21 in 91.57% of confirmed cases, aspect similar to the value of 95% cited by Sherman et al. [504] or Yon et al. [618].

II.1.3.5. CONCLUSIONS

The frequency of characteristic signs for Down syndrome was similar in both cohorts of patients: cytogenetic confirmed and those without chromosomal examination.

In 37.1% of our patients we found major congenital anomalies, which influenced infantile morbidity and mortality. The main congenital anomalies were cardiac (34.4%) especiallly septal defects.

The incidence of Down's syndrome in Iaşi County in period 1985-1999, was 1.3‰ (1/739) in newborns, and s*ex ratio* ♂:♀ was 1.3, both concordant with other studies.

Approximately 1/3 Down's syndrome cases (37%) have mothers older than 35 years, which attests a direct correlation between the risk of Down syndrome and maternal age.

We found a high mortality in the first year of life (29.4% of cases) correlated with cardiac malformations and severe infections. Average mortality by Down's syndrome was 10 fold greater than global infantile mortality.

Cytogenetic analysis, made in 101 patients with Down's, confirmed in majority of cases the clinical supposition (94.05%) that attested the quality of clinical examination. Most patients have a homogenous free trisomy 21 (91.57%).

II.1.4. PATAU SYNDROME

II.1.4.1. BACKGROUND

Patau syndrome is caused by trisomy 13, the chromosomal etiology being identified by Patau et al. [422]. The 3rd of the "common trisomies", trisomy 13, is less frequent than trisomy 21 or 18, occurring in 1/12,000 live births. It is usually fatal in the 1st year of life, with only 8,6% survival rate after 1 year of life, because infants with trisomy 13 have numerous malformations, some of them incompatible with life [229]. Chromosome 13 disomy was detected in 0.12% of sperm cells [548]. Expression of severity of trisomy 13 is proven by the decrease of its incidence during pregnancy from 5.7% in abortion [62], to 0.004% in newborn deaths [238, 260] and 0.0002% in living neonates [259].

Patau syndrome has some cardinal signs like cleft lip/palate, microphthalmia/anophthalmia and postaxial polydactyly. Other signs are scalp defects, holoprosencephaly, congenital heart anomalies [93].

In most cases it is a free homogeneous trisomy, the extra chromosome having in 90% of cases a maternal origin [78, 267].

The prenatal detection is important for prophilaxy. This imposes ultrasound examination for detection of congenital anomalies and severe growth restriction, followed by chromosomal prenatal diagnosis.

II.1.4.2. MATERIAL AND METHODS

We made a retrospective clinical and cytogenetic study performed on a group of 14 cases of Patau syndrome, confirmed by chromosomal analysis between 2000 and 2012 at Iasi Medical Genetics Centre.

II.1.4.3. RESULTS

We identified three cases diagnosed postmortem (2 aborted and stillborn) and 11 cases diagnosed in the neonatal period. Most cases were female with sex ratio ♀: ♂ = 10:4.

The clinical elements that supported the clinical diagnosis of Patau syndrome (table II.5., figure II.2.) were: abnormal ears (11 cases), cardiac anomalies (10 cases), flattened nasal root (10 cases), ocular anomalies (8 cases), polydactyly (7 cases), cleft lip/palate (6 cases), scalp anomalies (6 cases). Suggestive triad - cleft lip/palate, microphthalmia/anophthalmia and postaxial polydactyly - was identified only in one case, two major signs was discovered in five cases, a single sign was present in 6 cases, while in two cases we found no cardinal sign.

In one case, prenatal diagnosis was confirmed by cytogenetic analysis and necroptic examination. An ultrasound scan (US) of foetus at age of 27 weeks of gestation revealed: female fetus with growth restriction and biometry for 25 weeks, microcephaly with semilobar holoprosencephaly, severe midline facial defects - cyclopia, absence of the nose, proboscis – and postaxial polydactyly at the right hand. A previous ultrasound scan was normal ant also triple test was normal. The chromosomal analysis after amniocentesis indicated a homogenous trisomy 13. The parents asked for termination of pregnancy. A 600 g female foetus was born and died immediately after delivery. The malformations found by US scan were confirmed at the necropsy.

Table II.5. The clinical features of patients with Patau syndrome

Clinical sign	C 1	C 2	C 3	C 4	C 5	C 6	C 7	C 8	C 9	C 10	C 11	C 12	C 13	C 14
HPE	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Microphtalmia/ anophtalmia	+	-	-	+	-	+	-	+	-	+	+	-	-	+
Cleft lip/ palate	-	+	+	-	-	-	+	-	-	+	+	+	-	-
Postaxial polydactyly	+	-	-	+	+	+	-	-	+	-	+	-	-	+
Cardiac malformations	-	+	+	+	+	+	+	+	-	+	+	+	-	-
Nose anomalies	-	+	-	-	+	+	+	+	-	+	+	+	+	+
Ear anomalies	+	+	-	-	+	+	+	+	-	+	+	+	+	+
Kidney malformations	-	-	-	+	-	-	-	-	-	-	-	+	-	-
Omphalocele	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Cutis aplasia	-	-	-	-	-	+	-	-	+	+	-	+	-	+
Severe deficiency of intellectual development	-	-	-	-	-	+	-	-	+	-	-	+	-	-
Age of diagnosis	sb	a b	a b	n	n	n	n	n	n	n	n	n	n	n

ab – abortion; sb – stilbirth; n –neonatal

Clinical diagnosis was confirmed by chromosomal analysis in all cases: a free homogeneous trisomy 13 (11/14 cases), a mosaic trisomy 13 (1/14), a Robertsonian translocation trisomy 13/14 - (1/14) and a homogeneous trisomy 13 associated with a complex abnormality 47,XX,+13/48,XX,del(2)(p11.2→pter),i(2p),+13[29]/[2] (1/14).

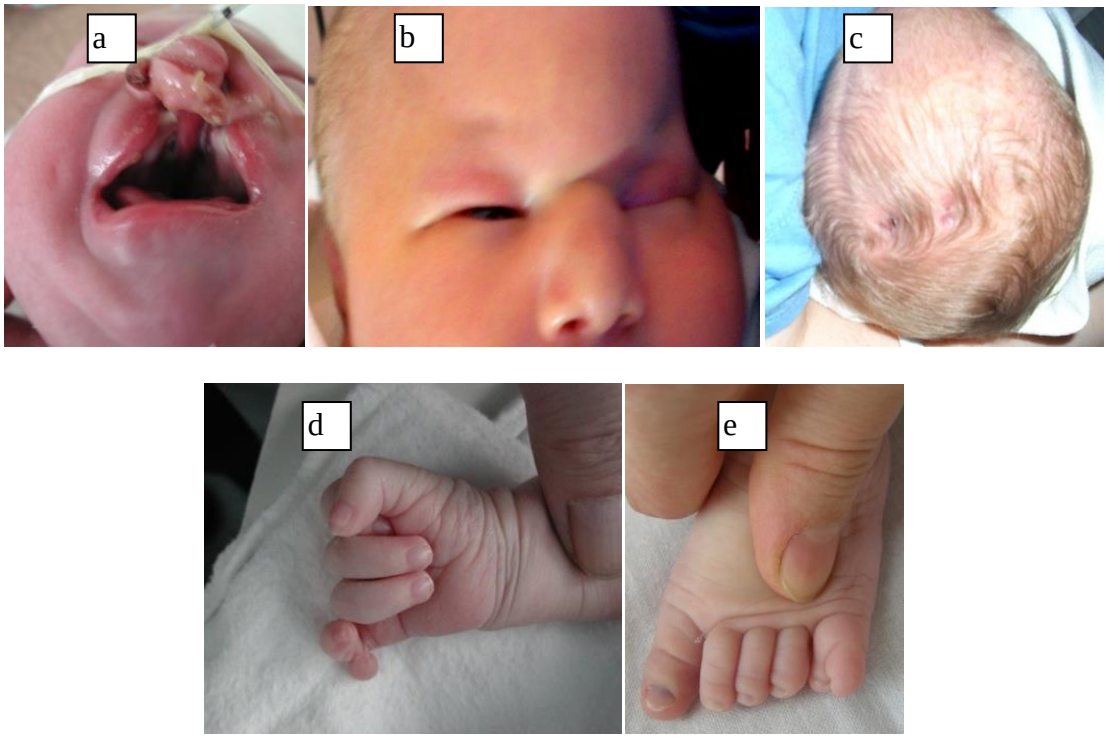


Figure II.2. Clinical signs of Patau syndrome:
a. cleft lip and palate; b. anophthalmia and microphtalmia, flattened nasal root; c. scalp defect; d. hand postaxial polydactyly; d. foot postaxial polydactyly

II.1.4.4. DISCUSSION

Patau syndrome is a rare chromosomal disorder that affects approximately 1/10.000 - 1/20.000 births. The disease is severe and, in most cases, produces a miscarriage or prenatal/perinatal death [93].

Clinical diagnosis is based on suggestive symptomatology, but is characterized by variable expressivity, which can complicate the diagnostic process. We tried to identify these variable elements based on a relatively large study group - 14 cases.

We found a sex ratio ♀: ♂ = 2.5, aspect which is discordant in relation to most studies of literature. Thus, Alberman et al. analysed 985 cases of trisomy 13 found mainly in male patients, while Huether et al. found a sex ratio ♀: ♂ = 0.9 (293 patients) and Bugge et al. found a sex ratio ♀: ♂ = 0.73 (82 cases) [13, 78, 250]. This discrepancy could be related to the small size of the study group, because similar results were cited by Moerman et al. who found a sex ratio ♀: ♂ = 2 on a sample of 12 cases [372].

The most serious malformations of Patau syndrome are the anomalies of the central nervous system, especially the holoprosencephaly (HPE). HPE can affect the brain (one cerebral hemisphere, unique cerebral ventricle, agenesis of the corpus callosum), ocular structures (microphthalmia/anophthalmia or cyclopia) and frontal bud (proboscis, hypotelorism, velo-palatine median cleft). In minor forms, the only clinical manifestation is single central maxillary incisor [116]. HPE is identified in 1/10.000-15.000 live newborns,

1/8.000 foetuses and 1/250 embryos, which indicates that most cases are spontaneously aborted [80, 172].

Although aetiology is complex, about two fifths of the cases have a chromosomal aetiology and 75% of them being trisomy 13 [80, 195, 406]. In our study, HPE was present in 4/14 cases (28.57%) that is consistent with the literature data. HPE in Patau syndrome is detected in 17-39% of cases [329, 421]. However, pathological examination of cases of trisomy 13 has shown a frequency of 67% for HPE aspect that could be correlated with lethality of this trisomy [520]. According to Cassidy, HPE presence is a negative prognostic factor in trisomy 13 [93]. We identified one such feature ourselves, because the four cases of HPE have been identified in the two abortions, one stillbirth and one patient who died in the neonatal period.

Microphthalmia affects about 14/100,000 newborns, while anophthalmia is four times more rare (3/100,000 neonates) [378]. This type of congenital anomaly is present in 60-70% of cases of Patau syndrome [172, 378].

In our study group these abnormalities were detected in 50% of cases, consistent with literature data point, microphthalmia being present in four cases and anophthalmia in three. Another anomaly was iris coloboma, detected in two patients, in one case being associated with microphthalmia.

Polydactyly is a common congenital anomaly detected in 1:1,000 - 1:2,000 births. The polydactyly in Patau syndrome is a key element of diagnosis, being found in 60-70% of cases [172]. In our study, polydactyly was present in 7 patients: hand polydactyly (in all) and foot polydactyly (in 3 patients). The bilateral form was more frequent than the unilateral one (5 cases versus 2 cases) [53].

Cardiac defects were present in 80% of patients with trisomy 13 [93, 172]. Usually is found an ASD or VSD. We have identified cardiac abnormalities in 71.42% of the cases (10/14) and the most common defect were ASD (6/14), patent ductus arteriosus (2/14), VSD (2/14), or dextrocardia (2/14).

Although, cardiac anomalies are frequent, they are not a cause of death. Musewe (1990) indicated that only 6% of patients with trisomy 13 had a potentially lethal cardiac anomaly in the first two years of life [382].

Cleft lip and palate is a common anomaly, detected in 1/700-1/1.000 newborns. In most cases (80%) the anomaly is unilateral (usually left) and may be associated in 28-37% with other congenital anomalies [172]. Cleft lip and palate is frequent in Patau syndrome and is found in 60-70% of cases [367, 536]. In our group the anomaly was found in 42.85% case - 6 patients. In four patients the cleft was bilateral and in two cases it was associated with holoprosencephaly.

Scalp defect affects the epidermis, dermis and subcutaneous tissue, and is located mostly at the vertex. The aetiology may be varied; trisomy 13 being one of the most common chromosomal causes [533]. Detection of the anomaly in 6 of our patients is also an argument for this statement.

Nasal abnormalities and **ear abnormalities** are common in Patau syndrome. We found such anomalies in 71.42% of cases (10/14), respectivelly in 78.57% of cases (11/14). The most serious anomaly was proboscis found in 3 cases.

Severe deficiency of intellectual development was identified in all patients after the neonatal period [93]. Such a deficit was found in all three infants in our study.

Prenatal diagnosis of trisomy 13 is relatively easy concordant with severe abnormalities central nervous system anomalies, cardiac defects, facial anomalies, growth restriction, holoprosencephaly and renal anomalies. The sensitivity of prenatal sonography for the detection of trisomy 13 is very high, most studies reporting sensitivities greater than 90% [321, 391, 559].

Clinical diagnosis of Patau syndrome should be completed with **chromosome analysis**. In our study, cytogenetic results showed 12 cases of free homogeneous trisomy 13, one case of mosaic trisomy 13 and 1 case of trisomy 13 by de novo Robertsonian translocation, issues consistent with the literature where the frequency of free homogeneous trisomy 13 is over 90% and a frequency of trisomy by Robertsonian translocation is 5-10% [133, 172, 200].

Given the absence of inherited chromosomal abnormalities we have estimated that the risk of recurrence is <1% in all 14 cases.

II.1.4.5. CONCLUSIONS

Patau syndrome is a severe polymorphic chromosomal disease. Although, the diagnosis is confirmed by a specific clinical triad - cleft lip and palate, microphthalmia/anophthalmia and postaxial polydactyly – we discovered such association in only one case, while two patients had none of these clinical elements. Holoprosencephaly spectrum is the most severe, being associated with increased in utero and perinatal mortality. Knowing phenotypic variability, the detection of atypical cases is important for a correct clinical management.

II.1.5. TRISOMY X

II.1.5.1. BACKGROUND

Trisomy X, which determines the triple X syndrome is a gonosomal aneuploidy characterized by the presence of an extra X chromosome in a female [544]. This syndrome has a frequency of 1/1.000 female births, but only 10% of cases are diagnosed. In 90% of cases, the extra X has a maternal origin, being associated with increased maternal age at conception [395, 410, 544].

Prepubertal patients have hypotonia, delayed development, uncharacteristic facial dysmorphism, IQ at the lower limit of normal, cognitive impairment or behaviour, anxiety. Postpubertal patients display a gonadal dysgenesis with: early menarche, irregular periods, early menopause, infertility or repeated miscarriages) [95, 182, 210, 235, 324, 410, 451, 544, 591].

Cytogenetic tests confirm the diagnosis of trisomy X. The X chromatin test evidences cells with one or two Barr bodies [214, 238, 387]. The chromosomal anomalies are homogeneous trisomy 47,XXX or a mosaic (in less of 10% of cases): 46,XX/47,XXX; 47,XXX/48,XXXX; 45,X/47,XXX; 45,X/46,XX/47,XXX [182, 451].

II.1.5.2. MATERIAL AND METHODS

We included in the study 36 patients who were diagnosed cytogenetically with homogeneous or mosaic trisomy X in period 1965-2012. This group represents 5.35% of 672 patients with ovarian dysgenesis.

Clinical evaluation of patients included anthropometric measurements, assessment of pubertal development and evidence of clinical signs suggestive for a gonadal dysgenesis.

Cytogenetic evaluation included the *X chromatin test* and the G banding *chromosome analysis*. *The X chromatin test* of buccal mucosa smear requests counting of >300 cells and is confirmed by presence of 2 Barr body [206]. *Chromosomal analysis* was based on a short-term culture of activated T- lymphocytes. Metaphases were examined by optical microscope and karyotyped >16 metaphases. In cases of detected cells with different number of chromosomes X, the number of cells analyzed was increased to 32, 64 or even 96.

II.1.5.3. RESULTS

Based on the analysis of registers of Cytogenetics Laboratory of the University of Medicine and Pharmacy "Gr.T. Popa" Iaşi, we identified 36 patients with trisomy X. In 25 patients (69.44%) the reference diagnosis was related to a gonadal dysgenesis. We note that the clinical suspicion of triple X syndrome existed only in 2 cases (5.55%). The age of patients

at diagnosis ranged from 1 month to 42 years. In prepubertal period were diagnosed 11 cases (30.55%), 12 other cases (33.33%) were diagnosed during puberty and 13 cases (36.11%) were diagnosed in the post pubertal period.

The X chromatin test was positive in all cases, revealed the presence of 1, 2 or 3 Barr bodies, and the results were compared with the chromosomal analysis results. In presence of cell line with X monosomy the percentage of X chromatin test was low (<10%). The karyotype was 47,XXX in 8 patients (22.22%), 46,XX/47,XXX in 6 cases (16.66%), 47,XXX/48,XXX in 2 patients (5.55%) and different gonosomal mosaics containing a cell line with trisomy X in 20 cases (55.55%).

For prepubertal patients the main clinical signs were: growth retardation (90.9%) and facial dysmorphism with anomaly of shaped ears (72.72%), thin lips (63.63%), hypertelorism (54.54%), epichantus (54.54%) and upslanted palpebral fissure (63.63%) with just only one patient having tall stature (9.09%). Moderate mental retardation was detected in only 3 patients (27.27%) with 46,XX/47,XXX karyotype. In a case with mosaic 47,XXX/48,XXXX a severe psychomotor retardation was detected. Behavioural disorders were identified in two patients with mosaicism 46,XX/47,XXX.

Other anomalies were: skeletal (45.45%), genitals (36.36%), cardiac (18.18%) and reno-urinary (9.09%). In pubertal and postpubertal cases the main clinical signs were ovarian dysgenesis: late menarche (24%), delayed puberty (28%), reduced development of breasts (40%), reduced pubic hair growth (36%), reduced axillary hair growth (40%), irregular menstrual cycles (24%), primary amenorrhea (24%) and secondary amenorrhea (20%). Facial dysmorphism was uncharacteristic being presented upslanted or down-slanted palpebral fissure abnormal ear shape and thin lips. Tall stature was present in 20% of cases, and growth retardation in 32% of cases. Psychiatric disorders were present in 4 patients (16%), one of them having a sexual identity disorders without cognitive impairment.

II.1.5.4. DISCUSSION

The frequencies of the different types of chromosome abnormalities detected in our study compared to other studies are shown in table II.6. The frequency of trisomy 47,XXX (22.22%) was similar to that detected by Robinson et al. - 18.03% [458], while the frequency of mosaic 45,X/47,XXX (25%) was similar to that detected by Sybert - 33.33% [540]. For other types of mosaic trisomy X, the frequency varied in all three studies.

Table II.6. Frequency of chromosome anomalies in homogeneous and mosaic trisomy X

Type of chromosomal abnormality	**Present study**	**Sybert [540]**	**Robinson et al. [458]**
Number of cases (%)	36	87	61
47,XXX	8 (22.22%)	-	11(18.03%)
46,XX/47,XXX	6 (16.66%)	-	2 (3.27%)
47,XXX/48,XXX	2 (5.55%)	-	-
45,X/46,XX/47,XXX	9 (25%)	68 (91.17%)	2 (3.27%)
45,X/47,XXX	9 (25%)	29 (33.33%)	-
45,X/47,XXX/48,XXX	1(2.77%)	-	-
45,X/46,XX/46,X,r(X)/47,XXX	1(2.77%)	-	-
45,XX,dic/45,X/46,XX/47,XXX	1(2.77%)	-	-

The characteristic phenotypic variability of trisomy X and reduced number of patients (36 cases) generated difficulties in genotype-phenotype correlations.

In prepubertal period the major clinical features were: growth retardation, mild craniofacial dysmorphia and mild mental retardation associated with behavioral disorders. In the post pubertal period the elements suggestive for trisomy X were ovarian dysgenesis sometimes associated with mild mental retardation and psychiatric disorders.

Clinical signs detected in patients with homogeneous and trisomy X mosaic was similar to those presented in the literature. The only discordant element concerned the stature. Bibliographic data indicates the tall stature in 80-89% of patients [201, 544]. At us from cases with X homogeneous trisomy, 7 had normal stature and one showed a slight short stature. In patients with 46,XX/47,XXX mosaic, five showed a normal stature and one had tall stature.

Severe psychomotor retardation is rare in trisomy X, but 5-10% of cases may have a borderline intelligence, but overall IQ is lower than the first-degree relatives [46, 47, 544]. Patients have issues with language and neuromotor development [46]. In our study a slight intellectual deficit was identified in 5 patients and behavioural disorder were diagnosed in 2 patients.

The most common heart defect detected were ASD and VSD, pulmonary stenosis and aortic coarctation [223, 585]. Only two of our patients had cardiac defects.

Data from the literature show that 10% of cases with trisomy X have a mosaic: 46,XX/47,XXX, 47,XXX/48,XXXX, 45,X/47,XXX or 45,X/46,XX/47,XXX [182, 591] and 3-4% of the patients diagnosed with Turner syndrome had a cell line with 47,XXX trisomy [540, 544].

In 20 patients (55.55%) with found a gonosomal mosaicism with trisomy X and monosomy X. All 9 patients diagnosed with Turner syndrome and karyotype 45,X/46,XX/47,XXX had short stature, normal intellect and absence of lymphedema in neonatal period.

In 5 patients identified postpubertal, the main clinical signs were delayed puberty, primary amenorrhea and premature ovarian failure. Sybert indicated that the average age of diagnosis in patients with trisomy X was higher than in Turner syndrome, most of the cases being detected in adulthood. In patients with Turner syndrome X trisomy cell line lymphedema and mental retardation were not detected and short stature was rare [540].

The spontaneous menarche in trisomy X mosaic was detected by Sybert in 70% of cases, compared with 11% of patients with monosomy 45,X and 34% for those with mosaic 45,X/46,XX. Functional cell death is earlier than in normal women, generating secondary amenorrhea. Often, the reproductive function is deficient, particularly correlated with primary sterility or increased frequency of spontaneous abortions [540].

In our study we identified two patients with pregnancy: one with mosaic 46,X,dic(X)/45,X/46,XX/47,XXX who had a stillborn baby with plurimalformative syndrome and one with 45,X/47,XXX mosaic whose pregnancy was terminated by miscarriage.

In patients with mosaic 45,X/47,XXX or 45,X/46,XX/47,XXX, we have not detected deficits of learning, language, visual or auditory perception, aspect revealed also by Sybert [540].

II.1.5.5. CONCLUSIONS

The results of our retrospective study, consistent with the literature, emphasize the difficulty of establishing a genotype - phenotype correlations for homogeneous and mosaic trisomy X.

Prepubertal, the diagnosis was established on the basis of growth retardation, uncharacteristic facial dysmorphism and mild mental retardation and, postpubertal, on by the presence of ovarian dysgenesis associated with mild mental retardation and psychiatric disorders.

II.1.6. EDWARDS SYNDROME

II.1.6.1. BACKGROUND

Edwards syndrome (ES) caused by trisomy 18, is the second most common autosomal trisomy syndrome after trisomy 21 [97]. The first reported cases were described in 1960 by Edwards *et al* and Smith *et a*l [149, 514]. ES pregnancies have a high risk of fetal loss and stilbirth, so overall prevalence (1/2,600) is higher than liveborn prevalence (1/10,000) [254]. The probability of survival to term increases with the increase of gestational age: 28% at 12 weeks, 35% at 18 weeks and 41% at 20 weeks [377]. The prevalence at birth is higher in females compared to males (♀:♂ = 6:4) due to higher rate of foetal loss in males [118].

The cytogenetic changes in ES are: homogeneous complete trisomy (94% of cases), mosaics (about 5% of cases) or partial 18q trisomy (about 1% of cases) [116]. The extra chromosome 18 has in majority of cases a maternal origin, being generated by a meiotic non-disjunction [79, 152, 173, 482].

ES is characterized by: prenatal growth deficiency, characteristic craniofacial features (dolichocephaly, short palpebral fissures, micrognathia, and dysplastic ears), distinctive hand posture (clenched fists with overriding fingers), nail hypoplasia, posterior prominence of calcaneus (rocker-bottom foot), short sternum and visceral major malformations (particularly cardiac) [270]. The phenotype is variable: more than 130 different anomalies have been reported in the literature, which may affect all organs and systems [88, 463].

II.1.6.2. MATERIAL AND METHODS

The clinical and cytogenetic retrospective study on a group of 19 cases of ES was performed between 2003 and 2015 in Iasi Medical Genetics Center and all cases were confirmed by chromosomal analyse.

II.1.6.3. RESULTS

In our group 11 patients were males and 8 females (♂:♀ = 1.37). The most frequent clinical features (table II.7.) were: intrauterine growth retardation (IUGR) (19 cases), cranial dysmorphism (figure II.3.) with micrognathia (19 cases), dysplastic ears (19 cases), dolicocephaly (18 cases), heart defects (18 cases), prominent calcaneus (17 cases) (figure II.4.) overriding fingers (13 cases) (figure II.5.) and preterm birth (13 cases).

An increased frequency of caesareans procedure was noticed (14 cases). The visceral congenital anomalies were represented by: cardiac (18/19 cases) gastrointestinal (8/19) renal (7/19) and central nervous system (5/19). The most common anomalies in descending order of frequency were: ventricular septal defect (12 cases) patent ductus arteriosus (11 cases), horseshoe kidney (4 cases), esophageal atresia (4 cases), hydrocephalus (4 cases) pentalogy of Fallot (3 cases) and Meckel diverticulum (3 cases).

The obstetrical history revealed IUGR (all cases) associated with polyhydramnios in 5 cases. Breech presentation was noticed in 8 cases and an advanced maternal age was found in 6 cases.

The prognosis was bad in majority of cases. From 16 patients with known period of survival 10 died in first month of life, 5 lived longer than 1 month and only one girl survived beyond the first year.

Clinical diagnosis was confirmed by chromosomal analysis in all cases: a complete and homogeneous 18 trisomy; 47,XX(XY),+18 (18/19 cases) and a partial 18 trisomy associated with a complex abnormality 46,X,Yqh+,1qh+,+18,der(13;18) (q12;p11.2) (1 case). The patient (C19) with complex abnormality (partial 18 trisomy and a very small partial 13 monosomy) had suggestive features of ES and diaphragmatic hernia.

Table II.7. The features of ES patients

Sign	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19
preterm birth	+	-	+	+	+	-	+	-	-	+	-	+	+	-	+	+	+	+	+
caesarean	+	+	+	+	+	-	+	+	-	+	-	+	+	-	+	+	+	+	-
breech presentation	+	+	+	+	-	+	-	-	-	-	-	-	+	-	+	+	-	-	-
IUGR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
polyhydramnios	-	-	-	-	+	+	+	-	-	-	-	-	+	+	-	-	-	-	-
dolichocephaly	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
micrognathia	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
dysplastic ears	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
overriding fingers	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	-	-	-
nail hypoplasia	+	+	-	-	+	+	+	-	+	+	-	-	+	-	+	+	-	-	-
prominent calcaneus	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
heart defects	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
kidney anomalies	-	-	+	+	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-
gastrointestinal defects	-	-	+	-	-	+	+	-	+	-	-	+	+	-	-	+	+	-	-
cleft lip/ palate	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
central nervous system anomalies	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-
diaphragmatic hernia	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
limb anomalies	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-	+	+	-

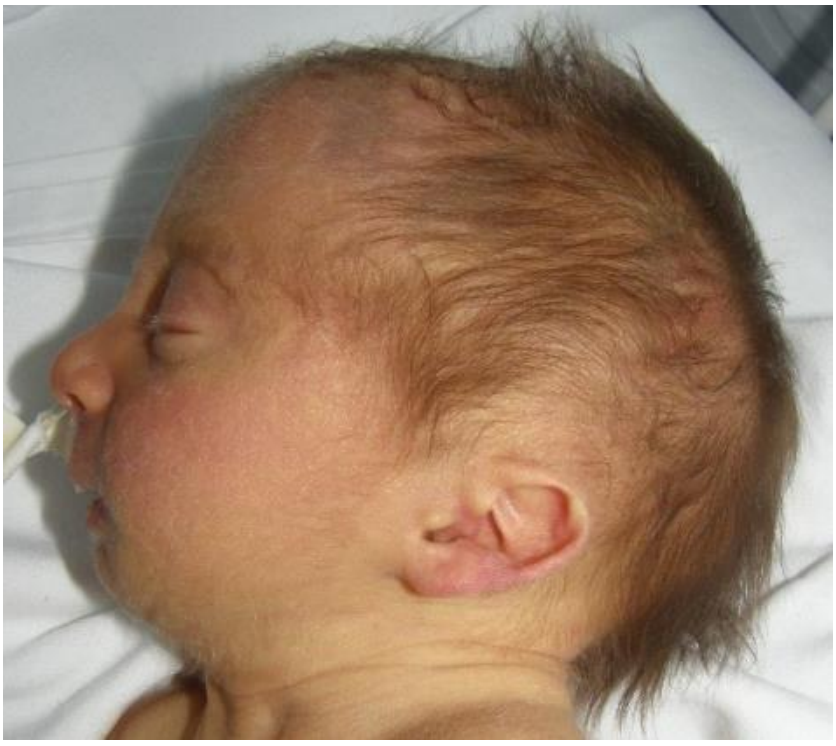


Figure II.3. Cranial dysmorphism

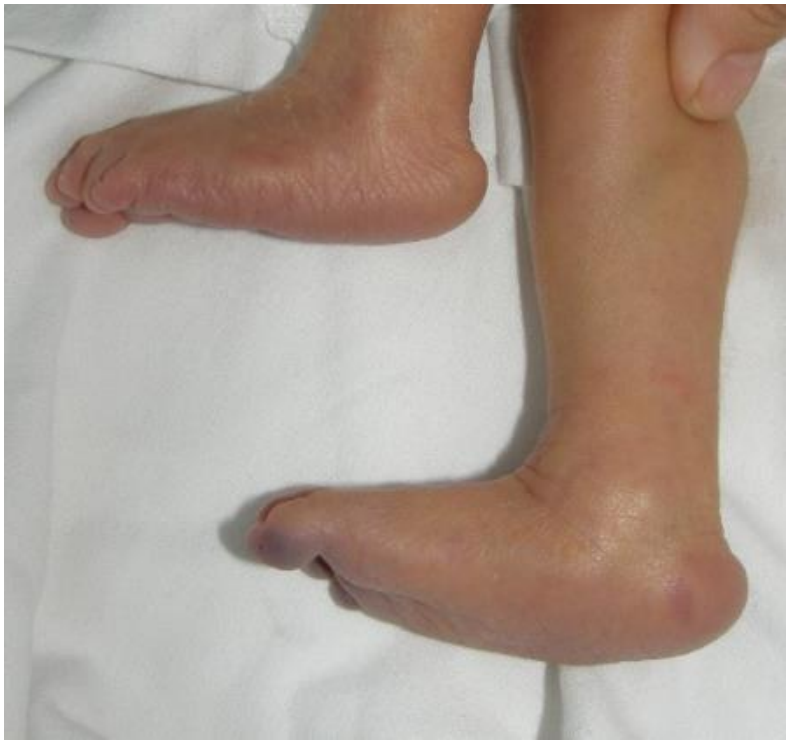


Figure II.4. Prominent calcaneus

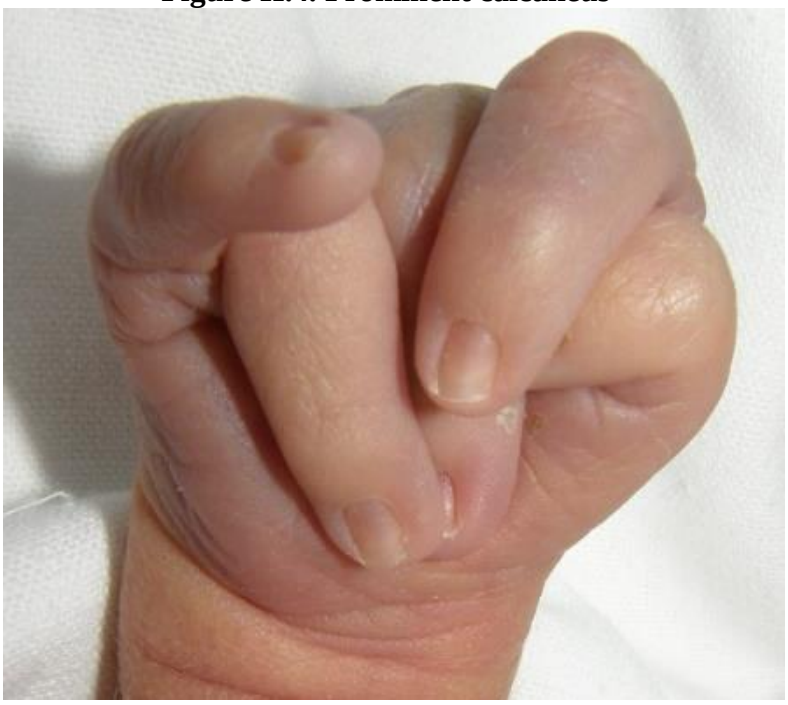


Figure II.5. Overriding fingers

II.1.6.4. DISCUSSION

ES is characterized by variable clinical manifestations, with involvement of multiple organs and systems. Some features overlapping with other syndromes can sometimes complicate the diagnosis. In order to facilitate accurate diagnosis, we tried to identify the most suggestive combinations of clinical features.

IUGR is one of the most frequent finding in ES; the mean birth weight is 1700-1800 g at a mean gestational age of 37 weeks [155]. In our study IUGR was present in all cases, consistent with literature data. The growth deficiency continues after birth for all major

anthropometric parameters – weight, height and cranial circumference – but with major implication on the weight [39]. Gastrointestinal anomalies and orofacial clefts may contribute to feeding problems [88].

The triad of craniofacial features: prominent occipital bone, low-set, dysplastic ears („faun ears") and micrognathia was highly suggestive for ES (18/19 cases). Rosa *et al* in a study on 50 patients reported as main craniofacial abnormalities: microretrognathia (76%), abnormalities of the ear helix/dysplastic ears (70%) and prominent occipital bone (52%) [462]. Cleft lip/palate was present in 3/19 of our cases and these results are similar to literature data, orofacial clefts being a less frequent (5-25%) anomaly in ES [39, 97].

Previous studies show that 80-100% of ES patients have congenital heart defects (CHD); the most common cardiac anomalies are ventricular and atrial septal defects (VSD, ASD), patent ductus arteriosus (PDA) and polyvalvular disease [88, 578]. We found a similar situation and we identified heart defects in 18/19 of cases. VSD (12/18) and PDA (11/18) were the most common cardiac anomalies. Other detected CHD were: ASD (4/18), polyvalvular disease (4/18), pentallogy of Fallot (3/18), coarctation of aorta (2/18), anomalous origin of coronary arteries (2/18), bicuspid aortic valve (2/18), single ventricle (1/18), dextrocardia (1/18). The complex heart defects were detected in 3/19 of cases, similar to literature data (10%) [88].

In our study, 17/19 of the patients had prominent calcaneus ("rocker bottom foot"). This abnormality has also been reported in the literature as occurring from 48 to 90% of the cases [234, 328]. Other features concerning the lower limb identified in our study were: syndactyly of the toes – 4 cases, short hallux – 3 cases, and clubfoot – 2 cases, aspects concordant with literature [39, 234, 328].

The most frequent abnormality of upper limbs in our study was clenched fists with overriding fingers: 13/19 of cases. In other reports the frequency was from 58 to 96% of the cases [242, 287, 328]. Many authors consider this feature one of the important diagnostic clues, and it is often detected sonographically in the prenatal period (especially in third trimester of pregnancy) [88, 97].

In the literature, a major malformation of limb occurs in 5-10% of patients, including radial aplasia and other preaxial limb defects [39, 97, 234]. We detected the radial aplasia in one case and agenesis of the thumb in other case. Nail hypoplasia is cited in the literature with a highly variable frequency (13-63%) [234, 328]. In our study this feature was detected in 10/19 of cases.

In the literature, the frequency of esophageal atresia/ tracheo-esophageal fistula among the patients with trisomy 18 ranges from 16 to 20% [328, 461]. In our study the frequency was similar 4/19. Omphalocele have been described in less than 10% of the cases, which is compatible with the rate in our study – 1/19 [39, 373]. Meckel diverticulum was detected in 3 cases in our study, similar to literature data (less than 25%) [39]. In our study the global frequency of gastrointestinal anomalies (42.10%) was higher than reported in the literature (less than 25%) [39].

Horseshoe kidney (HK) is a common congenital anomaly of the kidney and this anomaly was reported in 20-66% of patients [39, 40]. In our study, only 4 cases had this anomaly. Other renal anomalies found by us were: renal hypoplasia – 1 case, duplication of ureter – 1 case and renal cysts – 1 case.

Structural abnormalities of the central nervous system (CNS) have been reported in trisomy 18; the most frequent are cerebellar hypoplasia, agenesis of corpus callosum, hydrocephalus and myelomeningocele. These anomalies are present in about 5-25% of infants [39, 97]. Our study is consistent with literature: hydrocephalus (4 cases) agenesis of corpus callosum (1 case) and myelomeningocele (1 case).

Yamanaka et al reported a higher preterm birth frequency (35%) compared to general population. In our study the frequency of preterm birth was even higher 68.42%. An increased incidence (64%) of caesareans has been reported [234]. In our study 14/19 (73.68%) of patients were born by caesarean.

We found a sex ratio ♂:♀ of 1.37. This finding is discordant with other studies that showed a sex ratio of prenatally diagnosed cases of 1:1, but for the live-born sex ratio ♂:♀ was between 0.63 and 0.56 [251, 394]. This discrepancy could be related to small size of our study group.

II.1.6.5. CONCLUSIONS

ES is a chromosomal syndrome with variable clinical features. The most suggestive clinical findings were: IUGR, typical craniofacial dysmorphism (dolicocephaly, dysplastic ears, micrognathia), heart defects, prominent calcaneus, overriding fingers and preterm birth. Gastrointestinal and renal anomalies are also common features. Early diagnosis is important for appropriate management decisions regarding neonatal care and invasive procedures.

II.1.7. PLURIMALFORMATIVE SYNDROMES

II.1.7.1. BACKGROUND

Congenital anomalies (CA) are structural or functional abnormalities that occur during intrauterine life [6]. They affect about 3% of all pregnancies and 10-20% of cases are identifiable syndromes [90]. Etiology is heterogeneous: 45% have genetic causes, 5% non-genetic and 50% of cases unknown causal factor [4, 69, 117]. Among the genetic causes we mention: unbalanced chromosomal abnormalities (6% of total CA), monogenic mutations (7.5% of isolated CA), multifactorial hereditary diseases (20-30%) [164, 218]

CA are a major public health problem because they are frequent and severe birth defects, representing an important cause of intrauterine death and infant morbidity and mortality [200, 357]. The EUROCAT Statistical Monitoring Report-2012, which aimed at pan-European analysis of births from the period 2003-2012, revealed that there is an ascending trend for chromosomal abnormalities due to the increase in the prevalence of Down and Edwards syndrome determined by the increase in maternal age in Europe [4].

For the detection of unbalanced chromosomal abnormalities involved in CA pathogenesis, chromosomal analysis is required. The cytogenetic techniques used are *conventional cytogenetic techniques* (karyotype) and *molecular cytogenetic techniques* (FISH, M-FISH, MLPA, QF-PCR, CGH, DNA microarrays). Depending on the number of abnormalities identified, there are pangenomic and targeted techniques. Pangenomic techniques (karyotype, M-FISH, DNA microarrays) can reveal several anomalies that may be of interest to all chromosomes. Target techniques (FISH) can reveal fewer anomalies that only interest certain chromosomes.

II.1.7.2. MATERIAL AND METHODS

Our study was based on conventional banding cytogenetic, FISH analyses and MLPA. In all cases, the chromosomal analysis was performed on a short time T lymphocyte culture. The chromosomes were G-banded using trypsin and Giemsa solution according to standard techniques. The FISH and MLPA methods were applied only in selected cases when a microdeletion syndrome was suspected. The results of conventional banding cytogenetic analysis and FISH were elaborated in accordance with the guidelines of the International System of Human Cytogenetic Nomenclature (ISCN) (2013) [499].

The study group consisted of 331 patients clinically investigated at the Genetics Pathology Center in Iaşi. Conventional and molecular cytogenetic diagnostic investigations were applied in the Cytogenetics Laboratory of the University of Medicine and Pharmacy

"Grigore" T. Popa "Iasi, from 1 January 2015 to 31 December 2016. The study group was subdivided into four subgroups depending on clinical suspicion: Subgroup A - patients with plurimalformative syndromes or with association of multiple congenital anomalies; Subgroup B - patients with craniofacial dysmorphism; Subgroup C - Patients with clinical suspicion of Down syndrome; Subgroup D - patients with clinical suspicion of Turner Syndrome

II.1.7.3. RESULTS

The four subgroups had the following composition:

- Subgroup A –108 cases, of which 54 males and 54 females;
- Subgroup B - 88 cases, of which 32 males and 56 females;
- Subgroup C - 96 cases, of which 40 males and 56 females;
- Subgroup D - 39 cases.

Investigations of the patients from subgroup A (pluriformative syndromes) and subgroup B (craniofacial dysmorphism) by conventional chromosomal analysis - G-banded karyotype – confirmed the presence of unbalanced numerical or structural chromosomal abnormalities in 28 cases (table II.8.).

In other 24 patients from the subgroup A or B, the clinical phenotype was suggestive for a plurimalformative syndrome: Williams syndrome (one case confirmed by using of 7q11.23 probe), Wolf-Hirschhorn syndrome (3 cases from 7 confirmed by FISH and MLPA), velocardiofacial syndrome (4 cases from 7 confirmed by using 22q11.2 FISH probes), Prader-Willi syndrome (3 cases confirmed by different methods – karyotype, FISH or MLPA), *cri du chat* syndrome (one case confirmed by karyotype and FISH), Miller-Dicker syndrome (one case confirmed by FISH with 17p13.3 probes). In these cases we applied FISH method using appropriate DNA probes from Metasystem®, Cytocell® and Kreatech®:

In the subgroup C we had 96 patients with clinical suspicion of Down syndrome. Six of these cases were not confirmed by karyotype (five girls and one boy). Most of the patients had free and homogeneous trisomy 21, with karyotype 47,XX,+21, in 47 of the 51 girls with Down syndrome, respectively 47,XY,+21 in 35 of the 39 boys with Down syndrome. We identified three cases of mosaic (47,XX,+21/46,XX two cases and 47,XY,+21/46,XY – one case). We discovered two cases of Down syndrome with robertsonian translocation: 46,XY,rob(14;21)(q10;q10). Excepting the typical chromosomal formulas, in subgroup of Down syndrome patients we found some particular karyotype: 46,XX,dic(14;22)(p11.2;p11.2),+21, 46,XY,ins(21;16)(q22.1;p12.1p13.13) and 47,XX,1qh+,16qh+,+der(21)del(21)(q11.2q22.1).

In clinical suspicion of Turner syndrome (subgroup D), 16 cases were confirmed. In six cases a homogenous anomaly was present: X monosomy (five cases) and an Xq isochromosome (one case). In the other ten cases we identified different types of mosaics with two or three cell lines. In two cases we discovered aneuploid mosaics: 45,X/46,XX and 45,X/47,XXX. In other cases we found mosaics that had an association between a complete X monosomy and a partial X monosomy produced by an unbalanced structural chromosomal anomaly (Xq isochromosome – 5 cases; X ring chromosome – 1 case; deletion on the long arm of X chromosome – 1 case; dicentric X chromosome – 1 case).

A particular case (83 in table II.8) was a male patient investigated at the age of 3 days due to the presence of a plurimalformative syndrome. Its birth occurred naturally, but prematurelly. Newborn anthropometric data showed intrauterine growth retardation: weight – 2100 g; length - 44 cm; head circumference - 29 cm; APGAR score- 5.

The clinical examination revealed a plurimalformative syndrome with craniofacial dysmorphism (microcephaly, dolicocephaly, broad and flat nasal root, micrognathia, dysplastic ears) associated with abnormalities in the hallux position and shape, micropenis and atrioventricular defect (figure II.6.).

Table II.8. Patients with plurimalformative syndromes and craniofacial dysmorphism with unbalanced chromosomal abnormalities

Crt.	No. case	Name	Age	Clinical suspicion	Result of karyotype	Molecular analysis
1.	9	TR	3 m	PMS	46,XX,add(1)(pter)	MLPA add 6p and del(1)(q36)
2.	20	CDN	7 y	MOS	46,XX,del(1)(p36.2→pter)	Array CGH in progress
3.	23	T ♂	nb	VCFS	46,XY	FISH del(22)(q11.2)
4.	25	LA	3 y	PMS	46,XY,del(11)(q23.1→qter)	MLPA del 11q Array CGH in progress
5.	29	PMC	3 y	VCFS	46,XY	FISH + MLPA del(22)(q11.2)
6.	37	HV	2 y	CFD	46,XY	MLPA del 18p şi dup 18q
7.	41	P ♂	nb	PMS	47,XY,+18	
8.	54	NL	6 w	PMS	46,XY,9qh+,inv(9)(p12q13)	
9.	56	PAP	5 y	CFD	46,XX,add(16)(p13.3)	Array CGH in progress
10.	60	ZA	nb	PMS	46,XX	MLPA del 10q
11.	61	MD	5 m	PMS	46,XY	MLPA del 10q
12.	64	HM	nb	PMS	47,XY,+21/48,XY,+21,+mar [77]/[3]	
13.	71	CE	4 y	PMS	46,XX,1qh+/46,XX,1qh+,t(2;9)(q37.3;q12) [30]/[8]	Array CGH in progress
14.	72	CM	3 m	CCS	46,XY,del(5)(p15.1→pter)	FISH + MLPA del(5)(p15.3)
15.	75	M ♀	nb	PMS	46,XX/47,XX,+18 [47]/[3]	FISH trisomy 18
16.	76	MAM	1 y	CFD	46,XX,der(9)inv(9)(p13.3q21.13),del(9)(p13.1p13.3)	Array CGH in progress
17.	82	C ♀	nb	PMS	47,XX,+18	
18.	83	B ♂	nb	PMS	46,X,Yqh+,1qh+,der(18)t(13;18)(q12;p11.2)	Confirmed by FISH, C and NOR banding
19.	100	SI	8 y	VCFS	46,XX	FISH + MLPA del(22)(q11.2)
20.	106	CO	5 y	CLS	46,XX,add(3)(p25)	MLPA dup 3q Array CGH in progress
21.	114	RRP	2 w	PMS	46,XY	MLPA dup 2p and del 1q
22.	121	A ♀	2 w	PMS	46,XX/47,XX,+20	
23.	123	CAM	17 y	PMS	46,X,del(X)(qter→p11.2)/45,X [29]/[3]	
24.	125	PGC ♀	21 y	MS	46,XY	
25.	126	PAM ♀	9 y	MS	46,XY	
26.	137	GE	nb	PWS	46,XX,del(15)(q11.2q12)	FISH + MLPA del(15)(q11-13)
27.	144	FŞ	5 y	WS	46,XX	FISH + MLPA del(7)(q11.23)
28.	148	LNA	nb	PWS	46,XY	MLPA –15q11-13 imprinting defect
29.	152	R ♂	nb	VCFS	46,XY	FISH + MLPA del(22)(q11.2)
30.	173	MSR	13 y	PMS	46,XX	MLPA del 2q
31.	181	BMI	14 y	PMS	48,XXXY	-
32.	278	ADI	nb	CFD	46,XX	MLPA – del 1p + dup 4q
33.	283	SS	6 m	CFD	46,XX,inv(9)(p12q13),add(10)(qter)	-
34.	284	BLA	6 m	PMS	46,XY,der(10)(10pter→10q26.2::4q26→4qter)	Array CGH in progress
35.	285	BMA*	32 y	-	46,XY	
36.	286	BAE**	28 y	-	46,XX,t(4;10)(q26;q26.2)	-
37.	296	CA	10 y	MDS	46,XY	FISH del 17p13.3 Array CGH in progress
38.	300	I	nb	WHS	46,XY,r(4)(p15.1q35)/45,XY,-4/46,XY,-4,+mar/47,XY, r(4)(p15.1q35),+mar/47,XY,der(4),+mar [53]/[6]/[3]/[2]/[2]	FISH + MLPA del 4p
39.	303	BDA	nb	PMS	46,XY[30]/45,X[5]	FISH – X monosomy
40.	308	IA	5 y	CFD	47,XXY	-
41.	318	BSD	nb	PS	47,XX,+13	-
42.	325	SDG	1 y	WHS	46,XY	FISH + MLPA del 4p
43.	326	GSM	nb	WHS	46,XX,del(4)(p16→pter)	FISH + MLPA del 4p

PMS – plurimalformative syndrome; DS – Down syndrome; MOS – Moebius syndrome; CFD – craniofacial dysmorphism; VCFS – velocardiofacial syndrome; CCS – cri du chat syndrome; CLS – Cornelia de Lange syndrome; MS – Morris syndrome; PWS – Prader-Willi syndrome; WS – Williams syndrome; WHS – Wolf-Hirschhorn syndrome; MDS – Miller-Dieker syndrome; PS – Patau syndrome; * -father of case 284; ** - mother of case 284

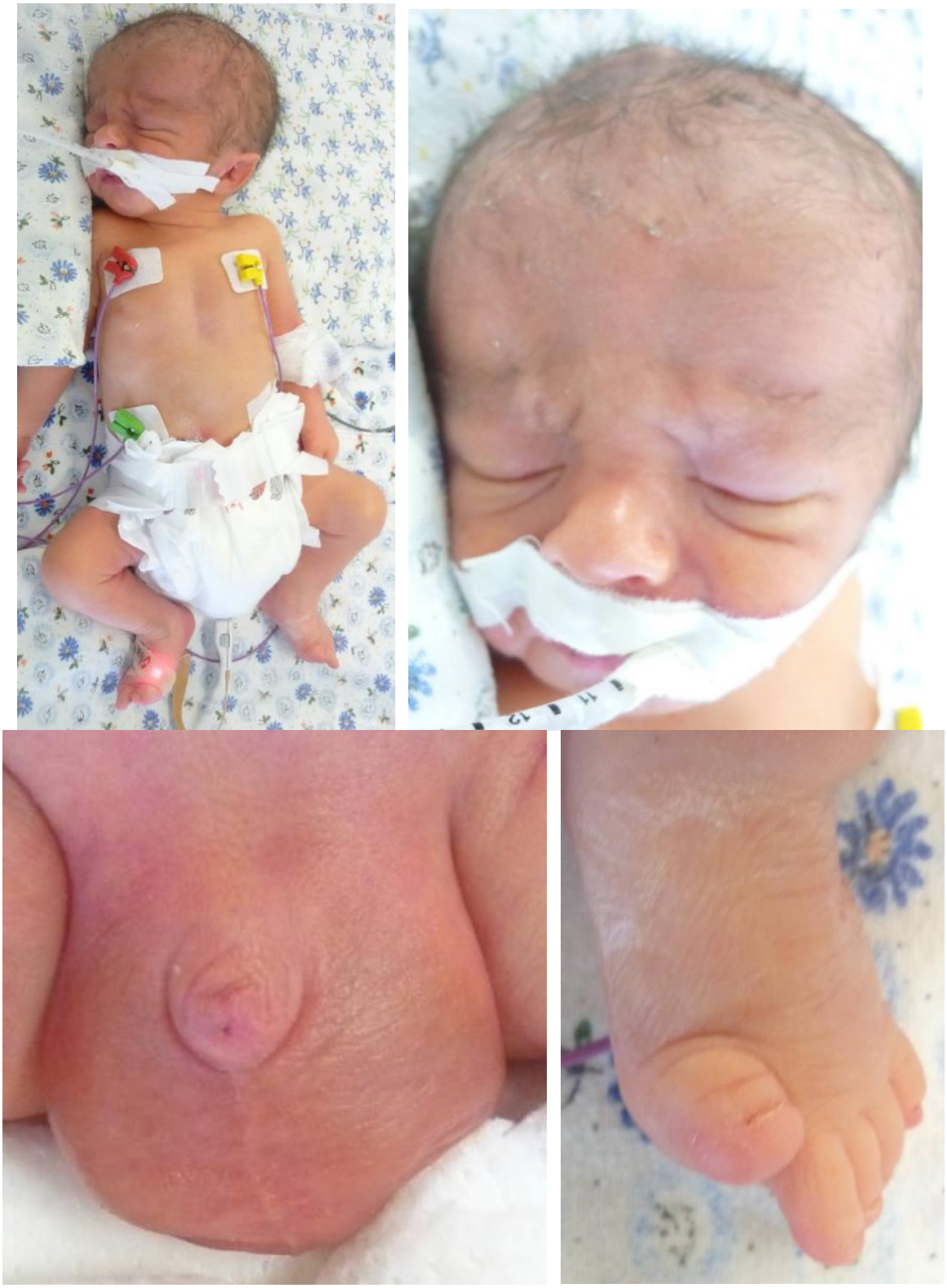


Figure II.6. Phenotype of case 83

The karyotype revealed the presence of a derivative chromosome, resulting from the union of segments from chromosomes 13 and 18 – chromosomal formula: 46,X,Yqh+,1qh+,der(18)t(13;18)(q12;p11.2) (figure II.7.). In order to verify whether the derivative chromosome was monocentric or dicentric, a C- banding technique was applied, which established that this abnormal chromosome had a single centromere and also we applied a FISH assay using Metasystem® probes corresponding to chromosomes 13, 18 and 21

(commonly used for the prenatal diagnosis of aneuploidies). Thus, we established that this derivative chromosome has a centromere derived from chromosome 18 (figure II.8.).

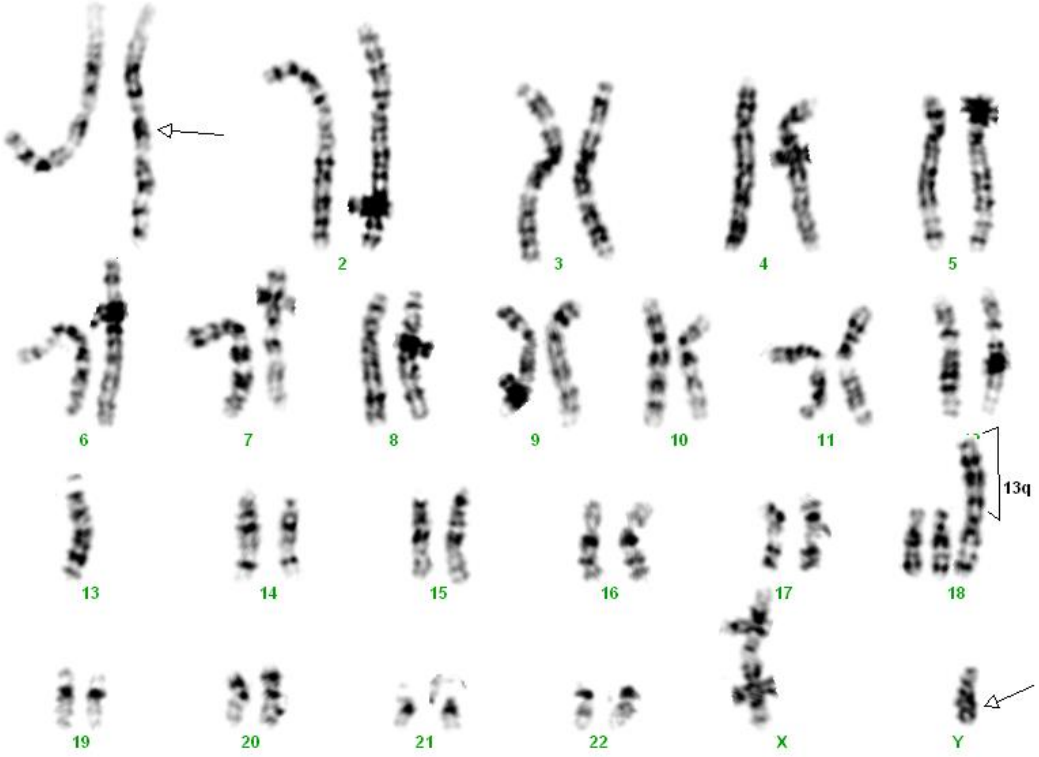


Figure II.7. G banding – karyotype 46,X,Yqh+,1qh+,der(18)t(13;18)(q12;p11.2)

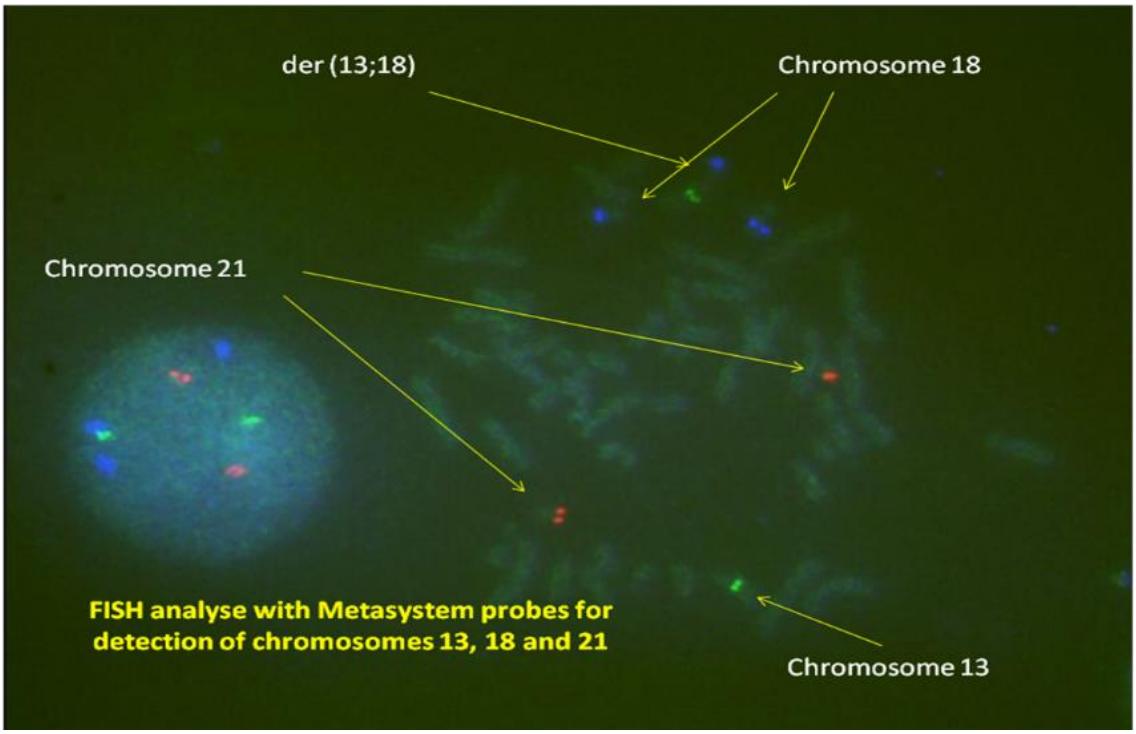


Figure II.8. FISH analysis with Metasystem® probes for chromosomes 13 (green) 18 (blue) and 21 (red)

To complete the investigation, the FISH test was also applied using subtelomeric probes from Cytocell® corresponding to the long arm of chromosome 18 and the short arm of chromosome 18, which confirmed the association of a partial trisomy 13 (containing almost all

of the long arm of this chromosome) with a small partial monosomy (determined by the absence of the terminal end of the short arm of chromosome 18) (figure II.9.).

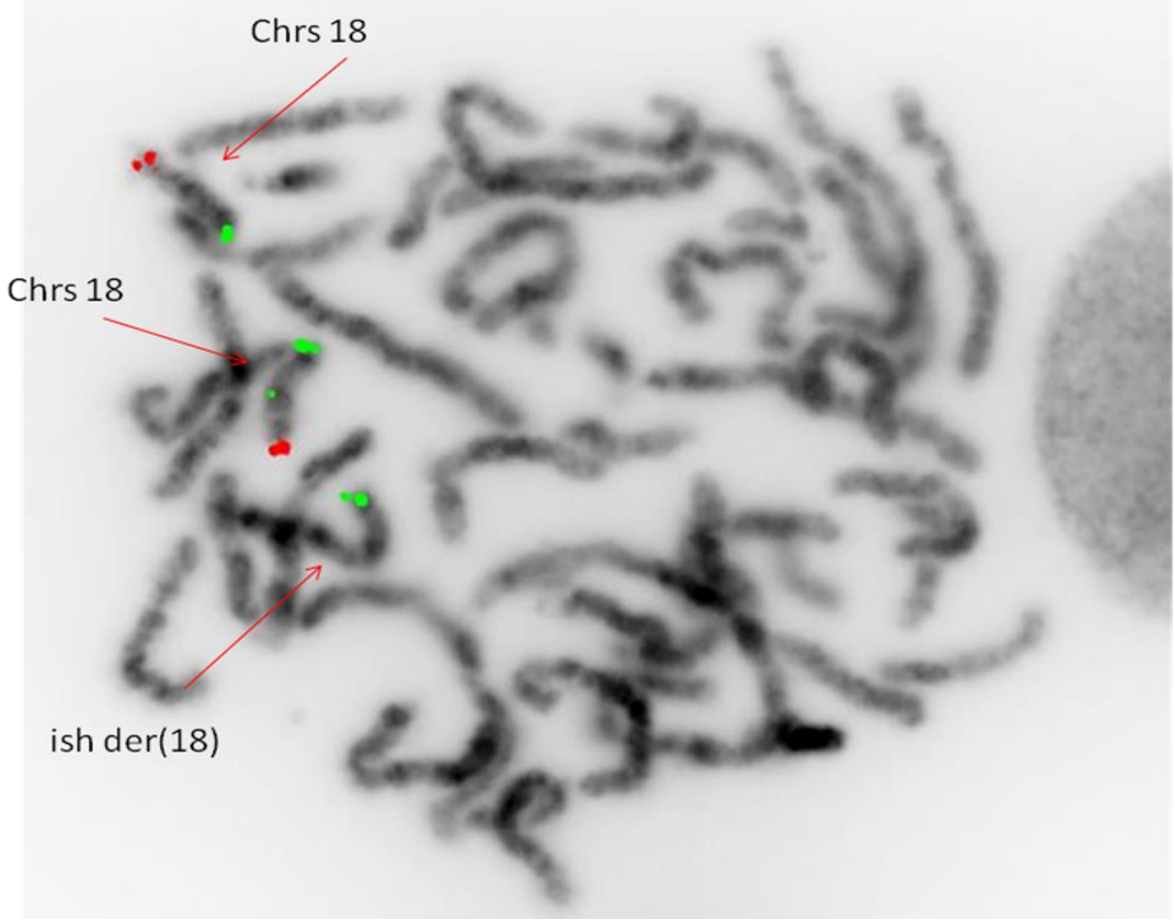


Figure II.9. FISH analysis with Cytocell® probes corresponding to the subtelomeric region of chromosome 18 - red signals 18p tel – 74G18; green signals – 18q tel – dJ964M9

II.1.7.4. DISCUSSION

In our investigations we used a sequential diagnostic strategy, increasing the resolution at each stage. We started with conventional karyotype (GTG banding), followed by the use of molecular cytogenetic methods like FISH or MLPA. In cases with particular chromosomal anomalies to increase the precision of diagnosis we applied DNA microarrays, but definitive results are not completed.

Conventional chromosomal analysis has some disadvantages: relatively long *duration* of analysis 4-10 days; relatively limited *resolution* to 5-10 Mb; requires specialized technicians to analyze GTG banding karyotype, all these meaning high costs and organizational difficulties in small laboratories. Karyotype is the only genomic analysis that identify a balanced structural chromosomal abnormality. Also, it identify the position of a genomic imbalance in the cases with partial trisomy or partial monosomy. Thus, GTG banding karyotype continues to be a part of protocol investigation of chromosomal diseases [117, 183, 502].

In cases with a suggestive phenotype for a microdeletion syndrome / microduplication syndrome, some protocols indicate the use first of FISH or MLPA technique with locus - specific probes bound to a particular region of a chromosome. Even in these cases, the karyotype must be made because in some cases a derivative chromosome could be present. If

the diagnosis is not confirmed by FISH or MLPA technique, the diagnostic strategy imposes a pan-genome analysis (array-CGH) able to identify subtle genomic imbalances [117, 200].

Microdeletion / microduplication syndromes are produced by the loss / gain of small chromosomal fragments (1-4 Mb) impossible to be detected by conventional cytogenetic methods. The detection of such genomic imbalances requires the use of a molecular technique: FISH, MLPA or array-CGH. In a minority of cases when the chromosomal fragment involved is large, it may also be highlighted by conventional G-banded chromosome analysis. The selection of cases for the FISH test is done using clinical diagnostic scores, because analyse is expensive, laborious and targeted [117, 542].

Genomic techniques based on DNA microarrays (array-CGH) increased the resolution to 50-100 kb. This technique can discovery a CNV (copy number variation). The CNVs represents 12% of the genome, and the classification in pathogen or not pathogen is difficult. If the CNV was already associated with a human disease, it is considered pathogen. If the CNV contains a high number of genes and its size is big, the CNV is considered potential pathogen. If the same CNV is present in one of the patient's parents, it is benign. In the other situation when the anomaly is *de novo* and it is not associated with a specific phenotype, the CNV is considered as VOUS (variants of uncertain significance) [132, 133, 582, 592].

Fourteen of the cases with clinical suspicion of a microdeletion syndrome were confirmed by molecular techniques (FISH and MLPA). We consider that the confirmation of approximately 60% of such cases is a proof of good clinical diagnosis. The absence of confirmation in other cases could have different explanations. In some diseases it is present a genetic heterogeneity. In other cases, the genomic imbalance is very small and its identification requires the use of array-CGH technique.

For example, in Wolf-Hirschhorn syndrome different types of abnormalities were described. About 55% of patients have a "pure deletion" identifiable by conventional G-banded chromosome analysis or FISH technique. In 40-45% of cases, the phenotype is generate by an unbalanced translocation resulting in chromosome 4p distal segment deletion. The rest of the cases were generated by complex rearrangements. [17 - 36]. The FISH test detects 95% of WHS cases using a WHSCR critical region specific-probes. A false negative result can be obtained when the probe hybridizes outside of the critical region. The array-CGH technique has a good detection rate (95%) and for this reason it is recommended for the diagnosis of complex rearrangements involving the 4p16.3 critical region. [37, 614].

The majority of cases (90-95%) with velocardiofacial syndrome present a small genomic imbalance of 22q11.2 region. For this reason, the karyotype is not useful for diagnosis. The commercial FISH probes used for the detection of abnormalities in the critical region for the velocardiofacial syndrome are TUPLE1 and N25. The FISH technique detection rate of deletions at this level is approximately 95%, the two probes being equivalent to the detection rate, but if the deletion is less than 40 kb then it cannot be highlighted. In the 22q11.2 region six duplicate segments (A-F) were identified. The FISH probes are located in regions A and B, and for this reason the cases with other type of deletion will be not detected with 22q11.2 commercial FISH probes. In such cases, the use of array-CGH technique represents the best solution [293, 361].

Prader-Willi syndrome is caused by genomic imbalances in 15q11-q13 region. Molecular classes of PWS are: I a (deletion of 5-6 Mb, 65-75%), I b (chromosomal rearrangements, <1%), II a (maternal 15q11-q13 UPD, 20-30%), II b (maternal UPD with predisposition to parental translocation/chromosome marker, <1%), III a (imprinting defect with imprinting center deletion), III b (epimutation: imprinting defect without imprinting center deletion). Approximately 70% of individuals with PWS have a deletion involving the 15q11-q13 region, so that the proportion of cases detected by FISH method is 65-75%. If the deletions are small or atypical the FISH test result may be false negative. A very good detection rate

(99%) has MLPA because it is able to detect both microdeletions and methylation status. In approximately 1% of cases, both FISH and MLPA methods do not confirm the clinical suspicion of Prader-Willi syndrome, and the identification of genomic abnormality requires the application of array-CGH technique [145, 512].

Trisomy 21 - the most common chromosomal disease (1/650 newborns), requires epidemiological surveillance mainly through three types of actions: monitoring the impact of intellectual disability, screening programs evaluation and adapting medical services to associated comorbidities [96, 108, 202, 337].

The results of chromosomal analysis in subgroup of Down syndrome are concordant with other studies. Thus, the majority of cases (92-95%) present a free homogenous trisomy 21. The rest of cases have a mosaic trisomy 21 or a trisomy 21 due to a Robertsonian translocation. In the last case the most frequent translocation is rob(14q;21q), that is concordant with our data. In less than 0,1% of cases, Down syndrome is generate by a partial trisomy 21q, chromosomal anomaly being an unbalanced reciprocal translocation, duplication, dicentric chromosome 21, marker chromosome 21 etc. [200, 202]

The clinical suspicion of Down syndrome was not confirmed in approximately 6.31% of investigated cases. This result is concordant with data previously presented by Gorduza et al. (2007) that indicated a 5.77% of cases with clinical suspicion of Down syndrome that are not confirmed by karyotype. A possible explanation is genetic heterogeneity, but also could be take in consideration the presence of somatic mosaicism or a mosaic trisomy 21 with a small number of abnormal cells [202, 437].

The cytogenetic results in patients with Turner syndrome (subgroup D) are relatively concordant with literature data. We found a high rate of X monosomy (complete or partial) mosaicism – 10/16 cases (62.5%) that are similar to data presented by Abulhasan et al. (1999) that discovered 22 cases with X monosomy mosaicism from 37 cases with Turner syndrome cytogenetically confirmed (59.45%) [30 – 36]. Other reports that used large cohorts of patients with Turner syndrome presented different results, with an approximately frequency of X homogenous complete monosomy of 45% [31, 32 – 36].

We consider that confirmation of clinical diagnosis in only 16 from 39 patients with Turner syndrome suspicion is correlated with the age of patients. The majority of our patients were children and during childhood the only major symptom of Turner syndrome is the growth delay.

II.1.7.5. CONCLUSIONS

Elucidation of the chromosomal etiology of congenital anomalies is an important objective in their management, due to different recurrence risks depending on the chromosomal abnormality, and for identifying other individuals in the family at risk of carrying a balanced chromosomal abnormality. Our study showed the importance of use of different chromosomal analysis for confirmation of ~~a~~etiology of congenital anomalies. In 148 from 331 cases (44.71%) we discovered a chromosomal abnormality using only the karyotype or using the association of karyotype and FISH or MLPA. The strategy of genetic testing is sequential, and usually must be started with the most simple and inexpensive technique that remains, the karyotype. In cases with clinical suspicion of a syndrome caused by a microdeletion, it is necessary to perform FISH or MLPA test for confirmation. If karyotype and/or FISH (MLPA) method does not identify a genomic imbalance it is important to apply array-CGH method that is able to confirm small genomic imbalances.

However, because some congenital anomalies are produced by other factors, the application of all cytogenetic techniques – conventional cytogenetic methods (karyotype) molecular techniques (FISH, MLPA, array-CGH) – could remain without results and supplementary investigations are necessary.

II.1.8. RING CHROMOSOMES

II.1.8.1. BACKGROUND

Ring chromosomes are rare chromosomal abnormalities with an overall frequency of 1/30000-1/60000 [303, 355]. They are described for all human chromosomes, vary in size, and show a variable number of centromeres and almost 50% of ring autosomes are originated from acrocentric chromosomes [485]. The ring chromosome can be present in homogenous or mosaic form. Usually it is the novo and is formed during meiosis [182, 302, 304, 615, 628]. The mosaic abnormality can be explained by a mitotic nondisjunction associated with ring instability. An argument for postzygotic origin of anomaly is the detection of a monosomic line in the case of small chromosomes (ring/monosomy mosaicism) and the presence of diploid normal line in the case of chromosomes rich in euchromatin (ring/normal line mosaicism) [281, 302, 304].

The most frequent mechanism implies a double chromosomal breakage on both arms, followed by the loss of terminal fragment and fusion of the ends of the centromeric fragment. The ring chromosome may manifest a structural instability resulting in its' loss with formation of an aneuploid cell and appearance of a mosaic. Another mechanism presumes a telomere-to-telomere fusion that generates a pseudo-complete ring chromosome, associated with a small loss of genetic material that generates cryptic deletions, but with a near normal phenotype [38, 194, 527]. Other mechanism implies duplication with inversion associated with a terminal deletion [290, 467] or a sister chromatide exchanges that produce interlocked, broken rings or double rings [457].

In absence of apparent loss of chromosomal material could be discovered the "ring syndrome" characterized only by short stature and mild cognitive impairment [182, 302]. The mechanism proposed for growth retardation was ring instability which produces death cells. This process occurs following sister chromatid exchange during the mitotic cell cycle, and is more frequently associated with larger ring chromosomes [292, 345, 428]. In the largest study on ring chromosomes, Kosztolanyi showed that ring syndrome has a frequency of 20% [304].

The severity of phenotype depends on length of chromosome, amount of euchromatin deleted, ring stability, presence of monosomic lines and rate of mosaicism [194, 232, 443, 518, 567, 628].

II.1.8.2. METHODS

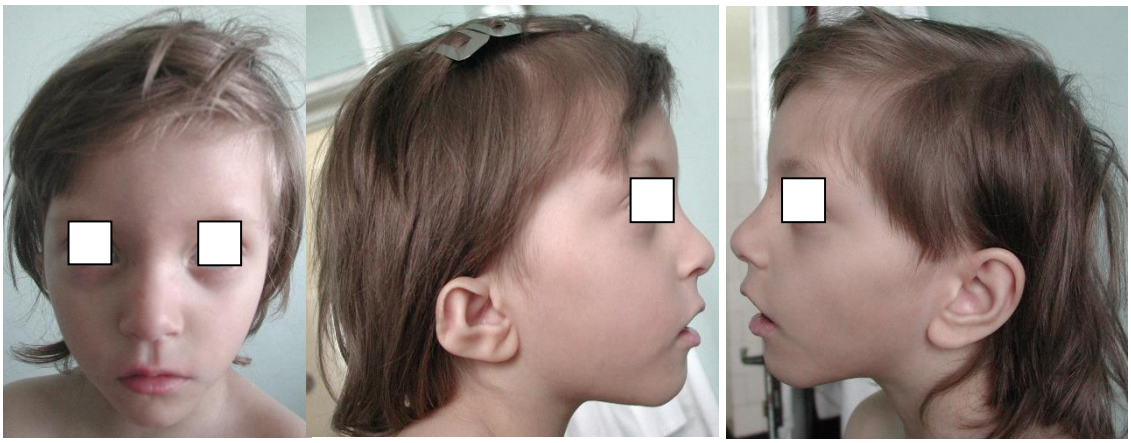
Our study is based on conventional cytogenetic analysis and FISH analyse. The chromosomal analysis was performed in all cases on a short time T lymphocyte culture stimulated by phytohaemagglutinin. The chromosomes are G-banded using trypsin and Giemsa according to standard techniques. Conventional cytogenetics analysis was performed both for propositus and his parents in all cases. The FISH technique protocol was applied only in cases with ring chromosome 18. In this case FISH was performed using chromosome-18-specific direct-labeled probes: telomeric probes for chromosome 18 (Aquarius) and Aquarius®Whole Chromosome Painting Probes.

II.1.8.3. RESULTS

Case 1:

A girl born at term with a weight of 1550 g, length of 38 cm and head circumference (HC) of 28 cm. Postnatal development: severe developmental delay, hyperactivity, aggression, mewing cry in neonate period. Clinical examination at 3 years old revealed: short stature, microcephaly, dysmorphic face (triangular face, mild facial asymmetry, hypertelorism, abnormal, low set, asymmetric ears) (figure II.10.).She presented moderate mental retardation (IQ 40) and behavioral difficulties. Echocardiography showed: subaortic VSD, tricuspid

insufficiency; foramen ovale apertum without pulmonary hypertension. The clinical diagnostic suposition was "*cri du chat"* syndrome. Blood karyotype: 46,XX,r(5)(p14q35)[80]/45,XX,-5[8]/47,XX,r(5)(p14q35),r(5)(p14q35)[3] (figure II.11-13.). The karyotype of both parents is normal.



a b c

Figure II.10. The phenotype of patient 1

a. facial asimetry; b. microtia ; c. low set, normal conformed ear

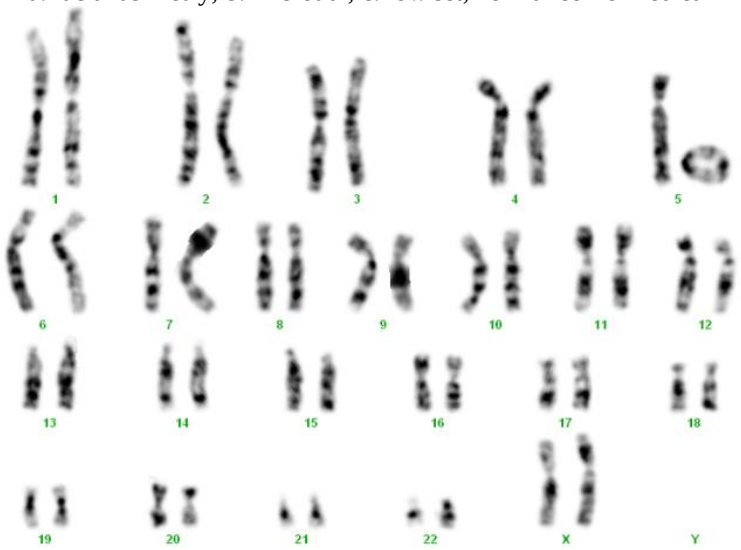


Figure I.11. Karyotype of patient 1 – Cellular line 46,XX,r(5)(p14q35)

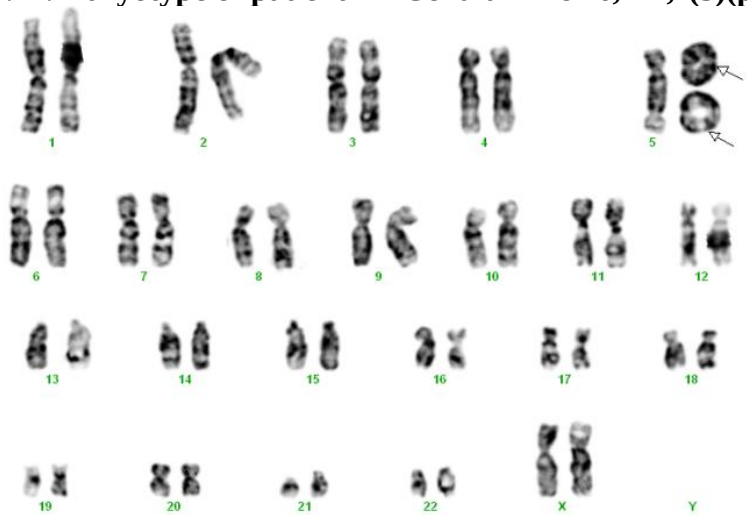


Figure I.12. Karyotype of patient 1 – Cellular line 47,XX,r(5)(p14q35), r(5)(p14q35)

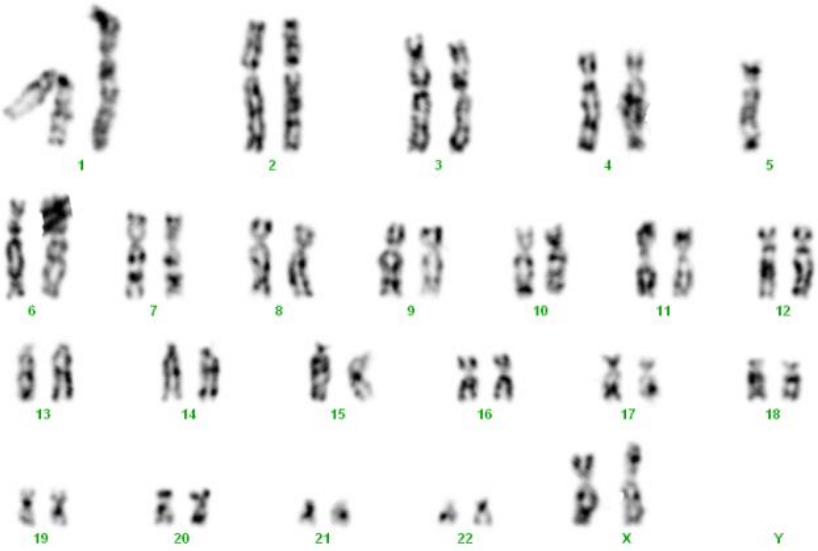
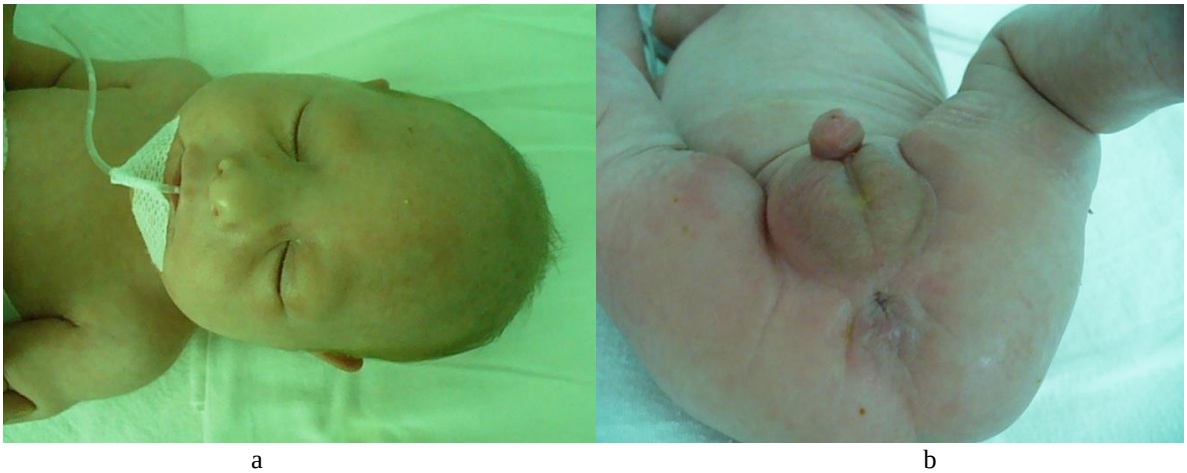


Figure I.13. Karyotype of patient 1 – Cellular line 45,XX,-5

Case 2:

Our second case is a boy examined first time at the age of 2 days for a plurimalformative syndrome. The patient was the first child of a young, apparently healthy and unrelated couple. The pregnancy was uneventful with delivery at age of 39 weeks of gestation by caesarian section. The boy had a severe intrauterine growth retardation (weight – 1600 g, length – 42 cm, HC - 28 cm) and presented microcephaly, dolicocephaly, hypertelorism, epicanthic folds, small nose with anteverted nostrils, broad and prominent nasal bridge, ogival palatine vault, long philtrum, micrognathia, big low set ears with posterior rotation, short neck. Phallus was small, embedded in the bifid scrotal folds and gonads were palpable in the scrotal folds bilaterally. The anus was anteriorly displaced with a deep sacral dimple (figure II.14.). Cardiac echography showed a patent ductus arteriosus, persistent foramen ovalae and diastolic dysfunction of left ventricle.

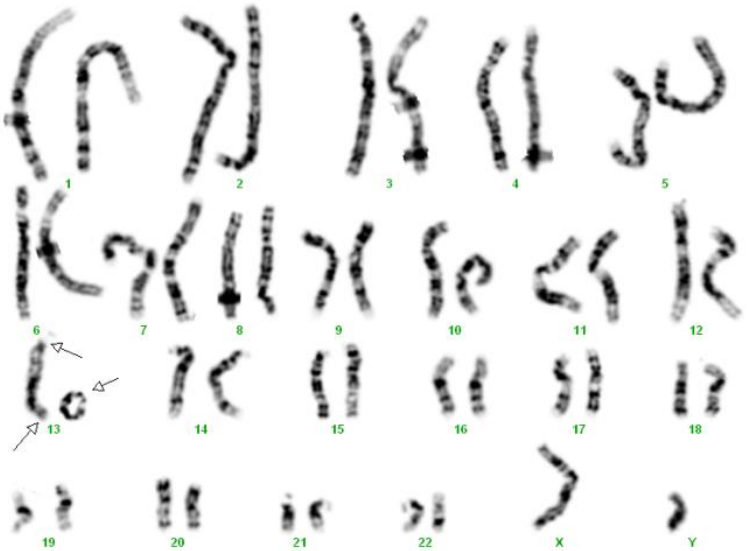


a b

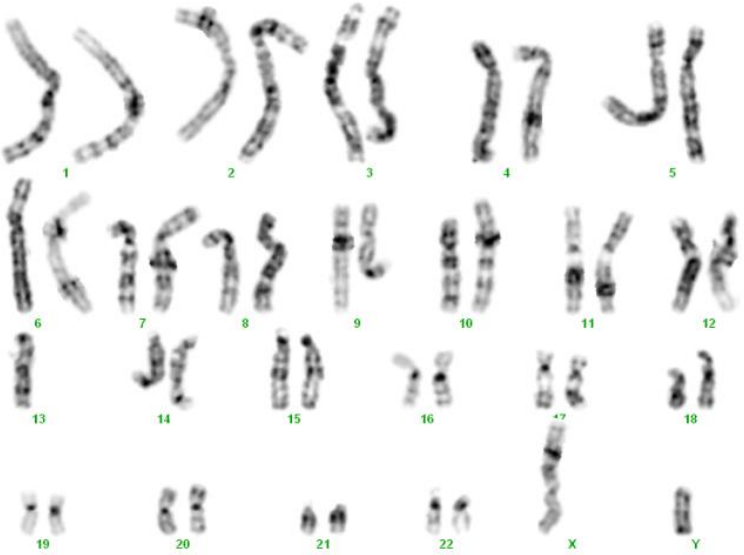
Figure II.14. The phenotype of patient 2

a. facial dysmorphism; b. aspect of urogenital region

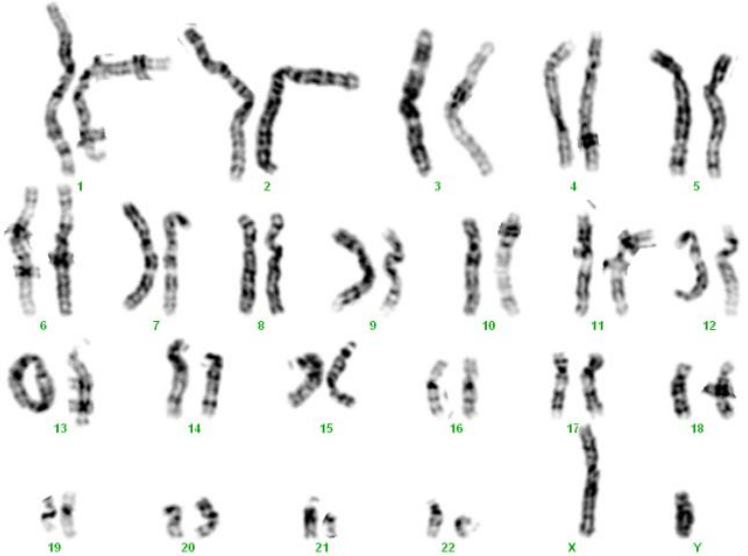
The karyotype indicated a mosaic with three cell lines - 46,XY,r(13)(p11.2q34)[51]/45,XY,-13[12]/46,XY,dic r(13)(p11.2q34)[1] (figure II.15.). The chromosomal analysis of both parents were normal.



46,XY,r(13)(p11.2q34)



45,XY,-13

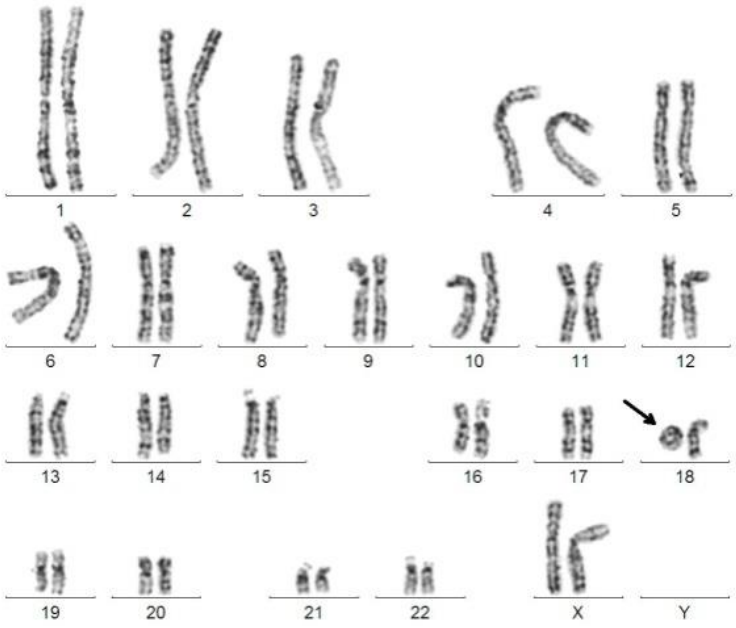


46,XY,dic r(13)(p11.2q34)

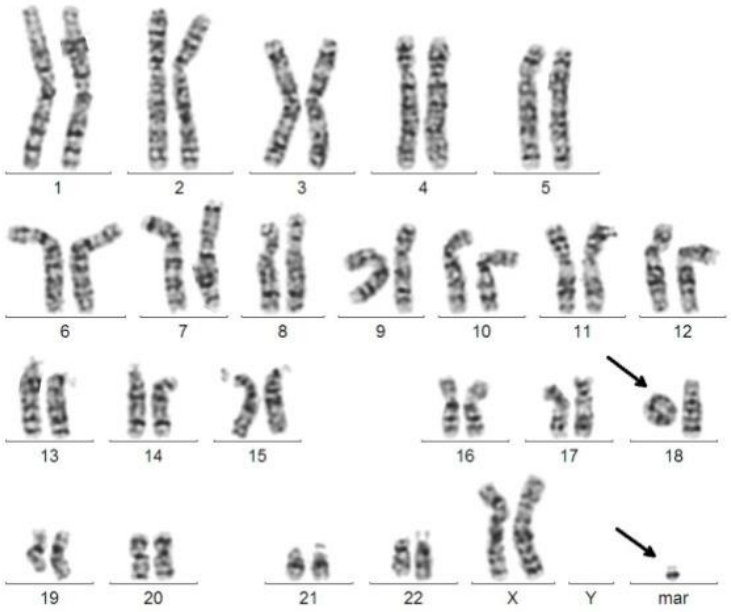
Figure II.15. Karyotype of patient 2

Case 3:

A girl born at term without intrauterine growth retardation was examined at age of 13 years 7 moand we found dysmorphic features (hypertelorism, facial scar after surgical correction of cleft lip) and mental retardation. Medical history presented: left cleft lip surgical corrected, neurosensory hypoacusis, bilateral stenosis of external auditory canals, clubfoot. Cranial MRI showed an acquired lesion of corpus callosum. Blood karyotype: mos 46,XX,r(18)(p11.32q23)[43]/47,XX,r(18)(p11.32q23),+mar[7] (figure II.16.). Blood karyotype of parents was normal. Using FISH probes for telomeres of chromosome 18 (Aquarius) we identified presence of telomeres on normal chromosome 18 (green signal) and absence of these on the ring chromosome 18 (figure II.17.).



46,XX,r(18)(p11.32q23)



47,XX,r(18)(p11.32q23),+mar

Figure II.16. Karyotype of patient 3

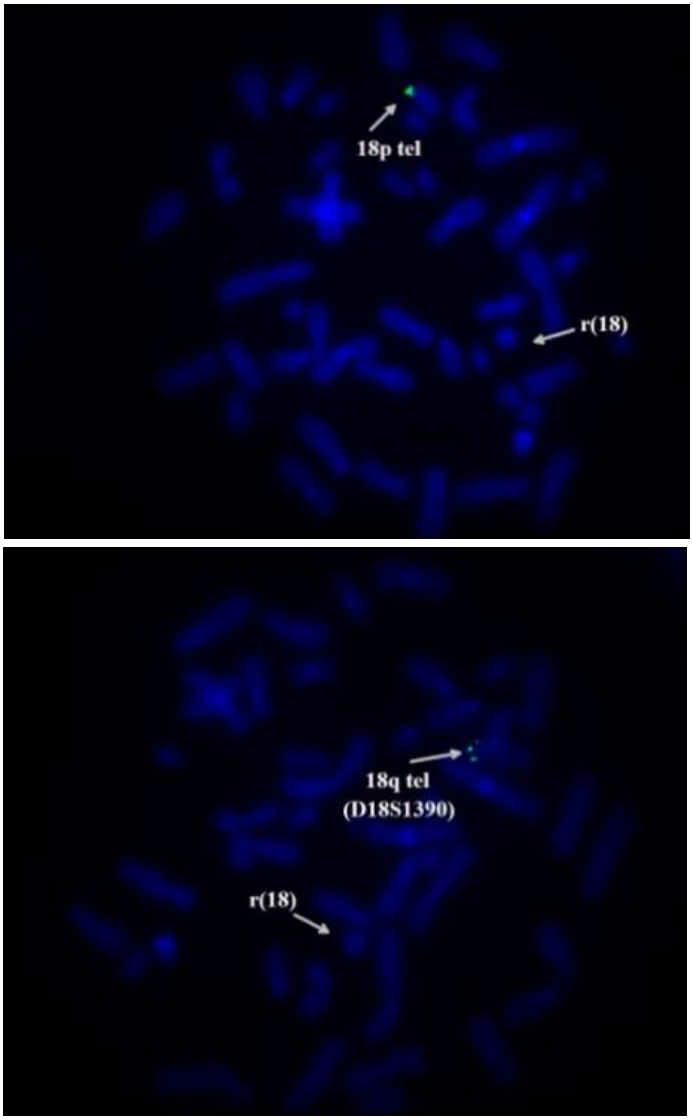


Figure II.17. The FISH analysis in patient 3.

a. Probe for telomere 18p b. Probe for telomere 18q; Absence of signals on the the ring chromosome 18

Case 4:

A girl born at term without intrauterine growth retardation was examined at age of 2 years and we identified dysmorphic features (hypertelorism, narrow ear canals, micrognathia), club foot varus, impaired hearing, developmental delay. Abdominal ultrasound revealed transversal kidney. Blood karyotype of proband was 46,XX,r(18)(p11.32q21.3). Using FISH probes for both subtelomeres 18p (D18S552 - red) and 18q (D18S1390 – green) (Aquarius®) we found the presence of subtelomeres FISH signals on normal chromosome 18 and absence on the ring chromosome 18 (figure II.18.). Using Aquarius®Whole Chromosome Painting Probes for chromosome 18 we identified only green signal that attested the origin of the ring chromosome (figure II.19.).

Case 5:

It is a boy examined at the age of 2 months because of dysmorphic features (short neck, narrow bitemporal diameter, preauricular pits, particular ears, bulbous nose, anteverted nostrils, long philtrum, macrostomia, thin upper lip, short lingual frenula), hypospadias. Echocardiography discovered VSD and PDA. Blood karyotype 46,XY,r(18)(p11.32q23). Blood karyotype of parents: normal.

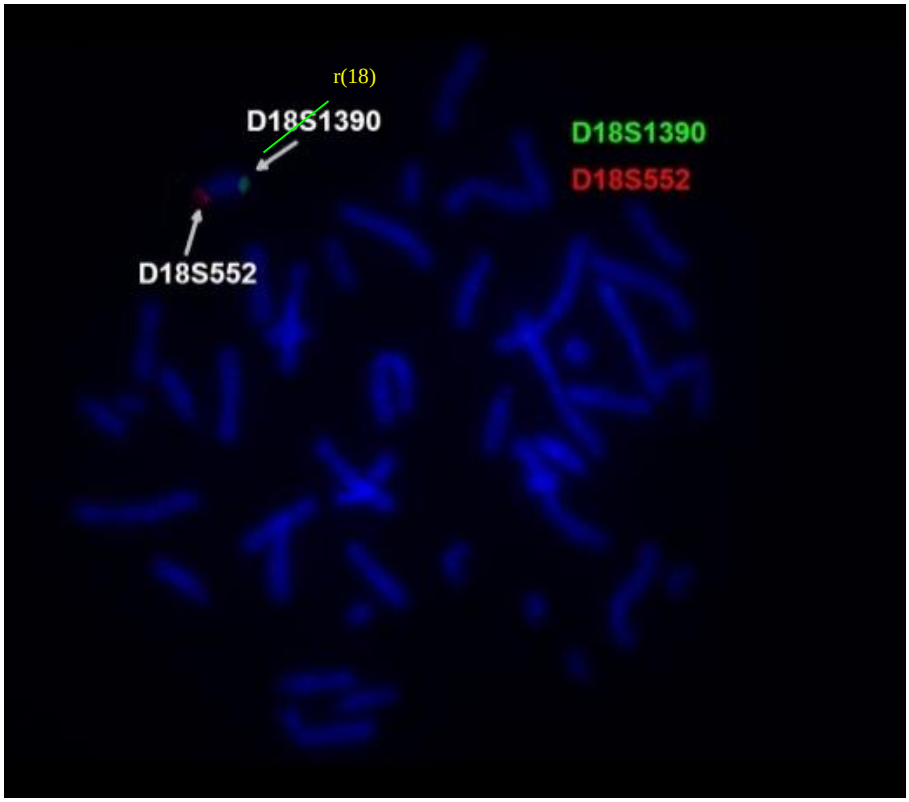


Figure II.18. The FISH analysis with probes for subtelomeres 18p (D18S552 - red) and 18q (D18S1390 - green) in patient 4.

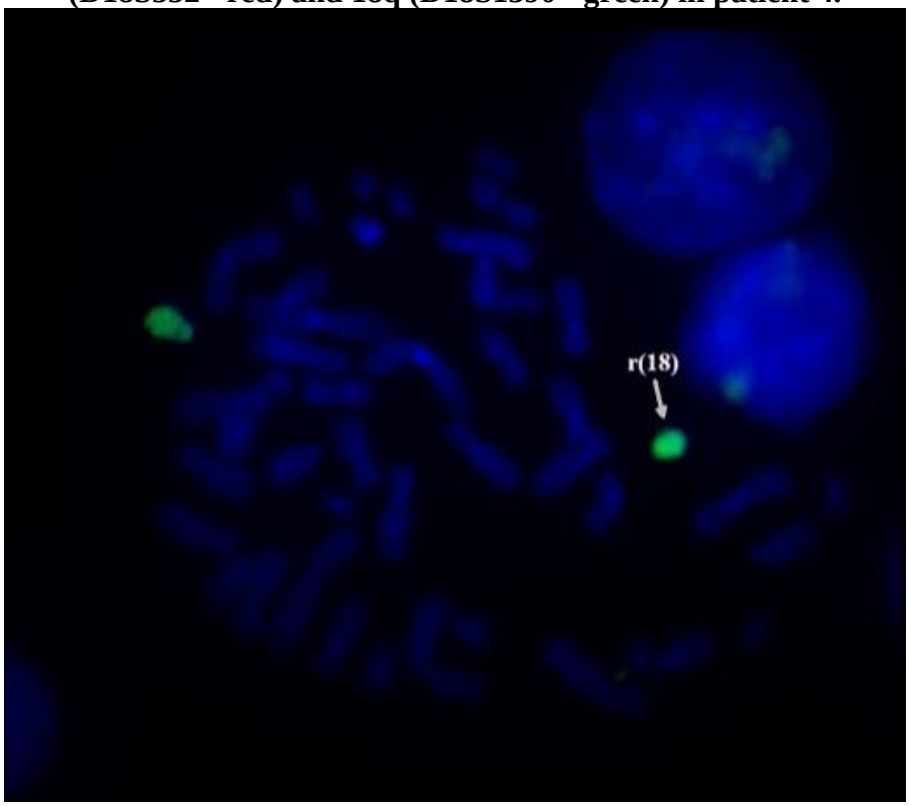


Figure II.19. The FISH analysis with Aquarius®Whole Chromosome Painting Probes using a green fluorophore for chromosome 18 in patient 4

Case 6:

It is a boy born premature after an obstetrical history of imminent abortion. Clinical examination after birth revealed: microcephaly, dysmorphic cranio-facial with tall forehead,

down-slanted palpebral fissure, low set ears, prominent antihelix, flattened helix, large concha, micrognathia, microstoma, palate cleft, muscular hypertonia, systolic heart murmur, umbilical and inguinal bilateral hernia, undescended right testis, right simian crease, left Sydney crease. The patient died at three months. The postmortem examination revealed a large septal atrial defect. Karyotype 46,XY,r(21) (figure II.20.). Blood karyotype of parents: normal.

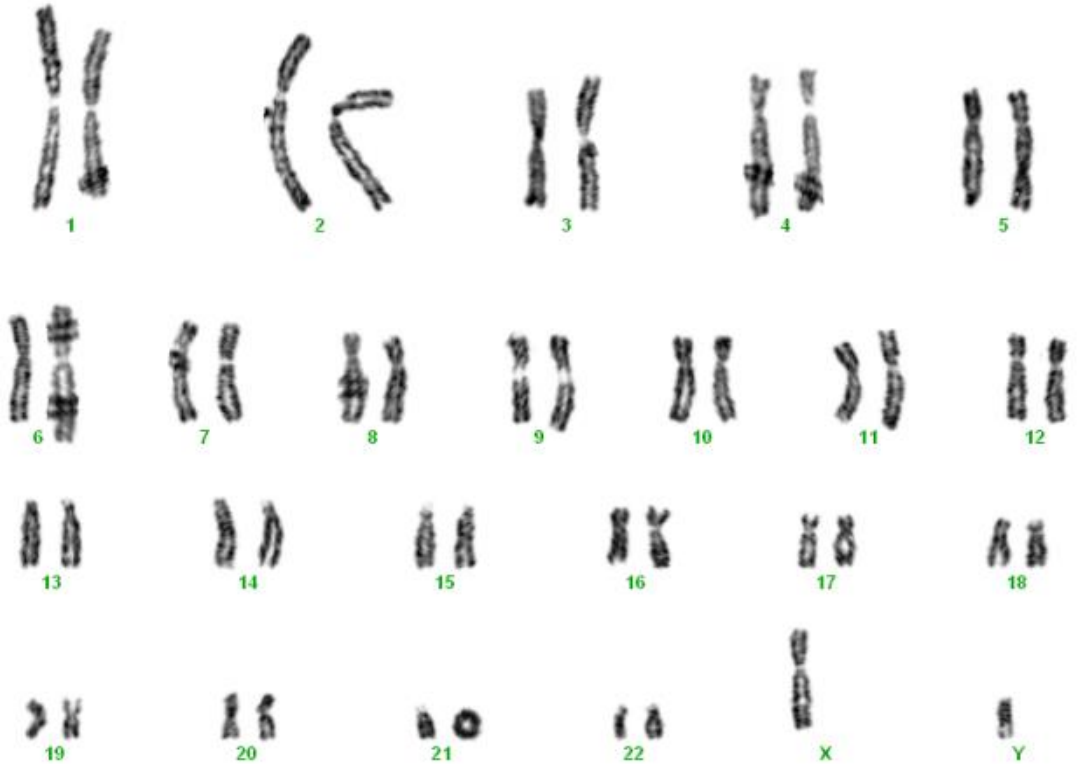


Figure II.20. Karyotype of the patient 6: 46,XY,r(21)

II.1.7.4. DISCUSSION

Ring chromosome is an unbalanced abnormality. In some cases one chromosome is replaced by the ring and this generates a deletion/monosomy of implicated genes. In other cases a supernumerary ring is present, generating a duplication/trisomy of that part of chromosome in association with partial monosomy [194].

Sometime one normal chromosome is replaced by two rings derived from the same chromosome [370]. We found a similar situation in patient no. 1 that had eighty cells (88%) with a ring chromosome with breakpoints in the short arm at band 5p14 and in the long arm at band 5q35, eight cells (8.8%) with monosomy 5 and three cells (3.29%) with two 5 ring chromosomes and a normal 5 chromosome in the same cell. In case 1 lack of normal diploid line it's an argument for meiotic origin. It's very likely that the line with one ring is the first one and the others are the expression of the mitotic instability ring. Monosomic line could be the result of an anaphase lag in mitosis of cell with ring chromosome 5. But the more likely mechanism is the chromatidian nondisjunction that explains the formation of both secondaries lines: one with 5 monosomy and one with two ring chromosome 5. The patient's phenotype is suggestive of "*cri-du-chat*" syndrome. As there are three different lines in karyotype we made a comparison between clinical signs specific for each of them and clinical features of our patient (table II.9.).

The association between 5p monosomy and OAVS was described and some authors suggested there were some genes on 5p implicated in OAVS pathogeny [103, 147, 311, 388]. Heart defects have been reported in OAVS in 5-58% in different studies [141]. These types of

heart defects also appear in the "*cri-du-chat*" syndrome and they have been frequently associated with deletions of the distal part of 5q and in rings with 5q35-5qter deletions (like in our case) [35].

Table II.9. Comparison of phenotypes in 5p monosomy, 5p trisomy , 5q trisomy and clinical features of our patient.

Clinical feature	5p monosomy	5p trisomy	5q trisomy	Our case
Low birth weight	+		+	+
Early death		+		
Cat-like cry	+			+
Microcephaly	+		+	+
Macrodolichocephaly		+		
Hydrocephalus		+		
Round face	+			
Abnormal skull shape			+	+
Micrognathia	+		+	
Downward slanting palpebral fissures	+		+	
Upward slanting palpebral fissures		+		
Hypertelorism	+	+		
Epicanthic fold	+	+	+	
Strabismus	+		+	
Bulbous nose		+	+	
Broad nose bridge	+		+	
Low set ears	+	+	+	+
Posteriorly rotated ears			+	+
Preauricular tags	+			
Down-turned corners of the mouse	+			
Narrow, high arched palate	+			+
Cleft palat			+	
Transverse flexion creases	+			
Clinodactyly	+	+	+	+
Brachydactyly	+		+	
Cerebral malformations		+		
Congenital heart defect	+	+	+	+
Lung malformation			+	
Gut malformations		+		
Kidney malformations		+	+	
Limb malformation			+	
Club foot	+	+		
Flat arches of feet	+			
Anteriorly placed anus		+		
Feeding difficulty	+			
Recurrent infections	+	+		
Hipotonia	+			+
Seizures	+	+		
Psychomotor retardation	+	+	+	+
Lack of speech			+	
Schizophrenia			+	
Myelodysplastic syndrome			+	
Acute lymphocyte leukemia			+	

The mild facial asymmetry and unilateral ear abnormality represent minimal diagnostic criteria for oculo-auriculo-vertebral spectrum (OAVS). Isolated microtia is considered the mildest form of OAVS [208]. Tasse et al. proposed the following diagnostic criteria: hemifacial microsomia with preauricular tags or microtia [545]. Marked facial asymmetry is present in just 20% of cases, but the overall frequency is about 65%. It becomes more apparent by the age of 4 years. Our patient was examined at 3 years old and we would expect the facial asymmetry to be emphasized in the future.

The clinical features of our case 2 – growth retardation, microcephaly with dolicocephaly, hypertelorism, epicanthic folds, small nose with anteverted nostrils, but with a broad and prominent nasal bridge, ogival palatine vault, long philtrum, micrognathia, big low set ears with posterior rotation, short neck, micropenis and malposition of the anus – are conform with characteristics of 13q- syndrome described first by Allerdice in 1969 [14].

The patients with 13q partial deletions may have widely varying phenotypes, but the common clinical features are moderate to severe mental and growth retardation, craniofacial dysmorphisms, hand and foot anomalies, and brain, heart and kidney defects [12, 15, 34, 77, 344, 393]. Brown et al. tried to make a delimitation of phenotype in 13q- syndrome and divided the patients in 3 groups: patients with proximal deletions not extending into the q32 band and variable dysmorphic features, mild/moderate mental and growth retardation (group 1); patients with deletions of band 13q32 and the most severe phenotype (mental and growth retardation, major malformations involving the brain, eyes, distal limbs, genitourinary and gastrointestinal traits) (group 2); and patients with distal deletions in bands q33–34, who usually show growth and severe mental retardation, but without gross malformations (group 3) [76].

The presence of a ring chromosome 13 produces a similar phenotype generated by deletion 13q syndrome, because the short arms of acrocentric chromosomes contain inactive genetic material [71, 355]. The clinical features of ring chromosome 13 include growth deficiency (with prenatal onset), mental deficiency, brain malformations, microcephaly, facial dysmorphism (prominent nasal bridge, hypertelorism, ptosis, epicanthal folds, microophthalmia, coloboma, retinoblastoma, prominent maxilla, micrognathia, prominent slanting and low set ears) and short webbed neck. In addition, cardiac defects, hypospadias, cryptorchidism, limb anomalies such as small to absent thumbs, clinodactyly of fifth finger, fused metacarpal bones 4 and 5, talipes equinovarus, short big toe and focal lumbar agenesis have been described [329].

Lorentz et al. tried to make a classification of cases with ring chromosomes 13. Thus, they identified 4 categories of chromosomal anomalies. The group A is represented by mosaic partial monosomy 13q [30, 342, 589]. The group B is represented by nonmosaic rearrangements (rings or deletions) that lead to a net deletion for distal 13q [76, 171, 225, 445, 535]. Group C contains the cases with mosaicism for a distal 13q deletion and complete monosomy 13 [61, 71, 198]. Finally, group D is represented by complex chromosome rearrangements that result in mosaicism for partial duplications and partial deletions of chromosome 13 [186, 263]. We consider that our case is part of group C: mosaicism for a distal 13q deletion and complete monosomy 13. The presence of dicentric ring chromosome 13 in the karyotype of our patient can be explained by a sister chromatides exchange of monocentric ring after replication. In our case we consider that dicentric ring chromosome 13 has a „*Tai Chi*" configuration in correlation with mechanism proposed by Hoo et al. [237].

The frequency of familial cases with ring chromosome 13 is very low and might be due to the fact that the phenotypes are generally too severe for reproductive fitness. Analysis of the breakage/ fusion junctions using other molecular techniques may provide further insight into the mode of ring formation. The case of Bedoyan et al. have similar breakage points with our case, but we consider that the severity of phenotype in our patient is due to the presence of monosomy 13 [42].

For our case 3, GTG-banded karyotype showed two cell lines, both with 18 ring chromosome. The less frequent line (7/50 metaphases) has a marker chromosome unidentified by conventional cytogenetics or FISH. FISH analysis with telomeric probes for both arms of chromosome 18 demonstrates the presence of telomeric region just in the normal chromosome and its absence in ring chromosome. For cases 4 and 5, GTG-banded karyotype revealed a homogeneous abnormality: 18 ring chromosome. In case 4, by using FISH probes for chromosome 18 we certified the origin of the ring chromosome and demonstrated a lack of 220 kb on terminal 18p and absence of 290 kb on terminal 18q.

Phenotype in ring 18 syndrome has some main elements: developmental delay/mental retardation, typical facial feature, major abnormalities and immunological problems [338]. The phenotype in all three cases has specific features for 18p- syndrome or 18q- or a combination between them (table II.10.).

Table II.10. Comparison of phenotypes in 18p-, 18q- and clinical features of our patients.

18p- syndrome	***Case 4***	***Case 5***	***Case 6***	***18q- syndrome***
Short stature	-	-	-	Short stature
Brachycephaly, microcephaly	-	-	-	Brachycephaly microcephaly,
Developmental delay, mental retardation, dystonia	Mental retardation	Mental retardation	-	Mental retardation seizures, abnormal myelination
Broad face, micrognathia Midface hypoplasia, mandibular overbite small mandible	-	Micrognathia	Narrow bitemporal diameter	Midface hypoplasia, mandibular overbite
Hypertelorism	Hypertelorism	Hypertelorism	-	Deep-set eyes
Inner epicanthic folds, ptosis	-	-	-	Epicanthic fold
Strabismus	-	-	-	Strabismus, nystagmus
Broad flat nose	-	-	Bulbous nose	Short nose, depressed and wide bridge
	-	-	Narrow upper lip, long philtrum	Narrow upper lip, absent philtrum
Macrostomia, downturned corners of the mouth	Cleft lip	-	Macrostomia	Downturned corners of the mouth, Cleft palate with/without cleft lip
Large protruding, posteriorly rotated, poorly formed ears	-	-	Dysmorphic ears, preauricular pits	Peculiar ears: prominent anthelix and antitragus
	Hearing loss,	Hearing loss	-	Hearing loss
Broad hands, short fingers, clinodactyly of fifth fingers	-	-	-	Proximally placed thumbs, tapering fingers, clinodactyly of finger V
	-	-	-	Transverse palmar crease
Short and broad neck	-	-	Short neck	Short neck
	-	-	-	Dimples over various joints
	-	-	-	Vertebral anomalies
	-	-		Kyphosis, scoliosis or both
Coxa valga	-	-	-	Coxa valga
	Clubfeet	Clubfeet		Toe position anomalies, clubfeet
Micropenis	-	-	Hypospadias	Hypospadias
	-	-	VSD, PDA	Cardiac abnormalities

VSD - Ventricular septal defect, PDA - patent ductus arteriosus

Stankiewicz et al (2001) suggested there were two types of 18 ring chromosomes according to the breakpoints. The first category has the breakpoints at the centromere or nearby and loss of p arm and the second one has the breakpoints on the long arm [527]. Our cases with 18 ring chromosome belong to second category. Feenstra et al. in 2007 realizes an update of the phenotypic map for chromosome 18q deletions. They identify some critical regions for cardinal signs in 18q deletions [163]. Some of the clinical features in our cases are in accordance with them. Lack of normal diploid line suggests that the 18 ring chromosome is formed in meiosis and the appearance of marker chromosome is the result of mitotic instability [280].

The ring chromosome 21 was reported as de novo or inherited. The phenotypes were different: almost normal in familial cases and characterized by mental retardation or developmental delay, short stature, microcephaly, epicantus, short neck and small ears. Three types of 21 ring chromosome were described. Clinically type I associates normal development, no malformations, short stature, infertility, and slightly delayed puberty in boys. The breakpoint is at the very end of q arm with minimal loss of genetic material [363]. The second type is characterized by: hypertonia, proeminent occiput, protruding forehead, down-slanting palpebral fissures, large ears, ocular anomalies, micrognathia, cleft lip or palate, heart defects, inguinal hernias, hypospadias, undescending testes, learning disabilities or mental retardation, defects in the immune system. This is generated by loss of region 21q22.3 during the ring formation [130]. The third type shared features with Down syndrome because these patients have three copies of 21 chromosome. It has been suggested that the mechanism was the break of a 21q isochromosome in two points followed by fusion in a circular configuration [363]. According to these features we concluded that our case is part of the second category.

II.1.7.5. CONCLUSIONS

Ring chromosomes are rare abnormalities, most of the time of *de novo* origin, presenting a different phenotype according to the loss of genetic material and genetic instability. The karyotype represents the main analysis for detection of ring chromosomes, but other molecular techniques are necessary for complete characterization. Also, parental investigation is required for proper genetic counselling.

II.1.8. INVERTED DUPLICATION DELETION

II.1.8.1. BACKGROUND

Inverted duplication deletion of 8p – inv dup del(8p) - is a recurrent chromosomal rearrangement first described by Weleber et al. [598]. It has an estimated prevalence of about 1:20,000 newborns [174], around 50 cases being reported worldwide. Clinical manifestations include: intellectual disability (ID), agenesis of the corpus callosum, minor facial abnormalities, congenital heart defects, hypotonia with a tendency to develop progressive hypertonia and severe orthopedic problems [127, 180]. Inverted duplications with terminal deletions on 8p is favorised by a paracentric inversion polymorphism in 8p23.1, found in ~26% of the European population, that may trigger meiotic misalignment and non-allelic homologous recombination between the inverted OR gene repeats (OR-REPD and OR-REPP) [191].

II.1.8.2. CASE PRESENTATION

The patient provided from an uneventful pregnancy. She presented neonatal hypotonia, seizures and cyanotic crises. Echocardiography showed a patent foramen ovale. Brain ultrasound and MRI revealed agenesis of the corpus callosum without other abnormalities. She achieved head control at 9 months and spoke her first word at the age of 12 months. Clinical examination at the age of 4 years and 8 months showed height of 106 cm (+0.43 SD), weight of 13.5 kg (-2.64 SD), and HC of 47 cm (-2.58 SD). She had facial dysmorphic features

including square face, high forehead, hypoplastic zygomatic bones, large mouth, thin upper lip and everted lower lip, high palate and abnormal dental development, large ears, and dry, curly hair that receded from the temples. Other features were short neck, pectus excavatum, slender arms and legs with long, tapering fingers, joint contractures in the legs and hips. She showed severe developmental and speech delay.

Standard G banding chromosomal analysis identified an additional material on chromosome 8 at band p21: 46,XX,add(8)(p23.1). Parental karyotypes were normal. A SNP array was performed using HumanCytoSNP-12 v2.1 BeadChip platform (Illumina Inc., San Diego, CA) containing approximately 300.000 SNPs per sample. The data was processed using Genome Studio V2010.1 software (Illumina). Genomic positions were defined using GRCh37/hg19. SNP array analysis detected a terminal deletion of approximately 6.8 Mb, from 8p23.3 to 8p23.1 (52,041-6,928,066), and a duplication of approximately 31.3 Mb, from 8p23.1 to 8p11.1 (12,599,114-43,951,038) (figure II.21.). The deleted and duplicated regions contained 15 and 144 OMIM genes, respectively.

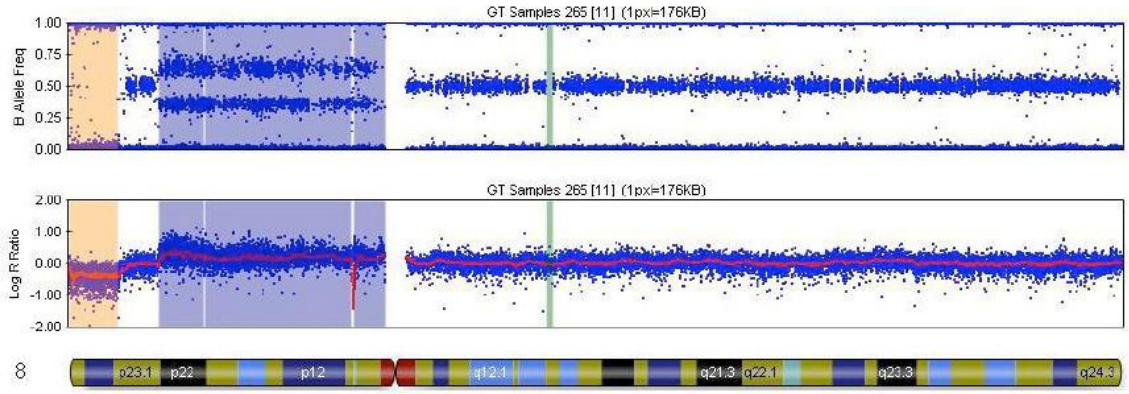


Figure II.21. SNP array results of chromosome 8 in the patient, showing a 6.8 Mb deletion in 8p23.3-p23.1 and a 31.3 Mb duplication in 8p23.1-p11.1

II.1.8.3. DISCUSSION

Our patient is severely affected compared with patients reported in the literature. The concomitant presence of deletion and duplication makes genotype/phenotype correlations difficult. Considering the size of the duplicated region and the large number of OMIM genes involved, the phenotypic findings in our patient are mainly due to duplication. Three mechanisms may explain the formation of this abnormality: recombination within a paracentric inversion, recombination between inverted low copy repeats, or U-type exchange following a double strand break. All three mechanisms involve the formation of a dicentric chromosome 8 which subsequently breaks either during a meiotic division or during early stages of embryonic development to produce an inv dup del(8p). Observation of a region of disomy between duplication and deletion can distinguish the U-type exchange from the other two mechanisms. The inv dup del(8p) in our patient can be explained by the second mentioned mechanism because the duplication was separated from the 8pter deletion by a ~5.6 Mb single copy region flanked by OR-REPD and OR-REPP regions.

Stabilization of the broken chromosome ends can be achieved by: direct addition of telomeric repeats (‘‘telomere healing’’) [605]; ‘‘telomere capture’’, in which broken chromosomes obtain the telomeric end of another chromosome [27]; or by formation of a ring chromosome [290]. Using high-resolution microarray no additional duplications could be identified, therefore in our patient the broken chromosome end of 8p has been stabilized by telomere healing.

The rearrangement is *de novo* as both parents had normal karyotypes. Dual colored FISH can be performed in the parents to investigate whether the meiotic recombination event

among the OR gene clusters could have been stimulated by the presence of an inversion heterozygosity at 8p23.1. Even if inv dup del(8p) is a recombinant product of a parental 8p23.1 heterozygous inversion, there is no recurrence risk for this rearrangement and no invasive prenatal investigations are indicated for the next pregnancy.

II.1.8.4. CONCLUSIONS

Microarray analysis enabled *precise molecular* characterization of the rearrangement and indicated that non-allelic homologous recombination between segmental duplications is the most likely mechanism for this abnormality. Also, it indicated that the terminal deletion was stabilized by telomere healing.

II.1.9. CONGENITAL ANOMALIES IN PATIENTS WITH UNBALANCED TRANSLOCATION t(14;18)

II.1.9.1. BACKGROUND

Some partial trisomies and monosomies are impossible to detect by karyotype, requiring a molecular cytogenetic techniques. In most of these cases, patients present a typical craniofacial dysmorphia and visceral anomalies [182, 513].

Partial monosomy 18p, first reported by de Grouchy et al. [129] has an incidence of 1:50,000 live-born infants [568] and about two-thirds of cases are de novo deletions. The rest come from familial translocations, inversions, complex translocations or direct transmission [485]. The monosomy 18p is characterized by mild to moderate ID, postnatal growth retardation, and dysmorphic features including ptosis, hypertelorism, strabismus, broad flat nose, micrognathia, and low-set large ears [602].

We report one girl with ID, mild dysmorphic face and masculine body habitus who had a de novo 14;18 translocation. Copy number analysis with single nucleotide polymorphism (SNP) array detected a terminal 18p deletion flanked by a duplication on 18p and a duplication of 16p11.2.

II.1.9.2. CASE REPORT

The patient is a 20-year-old female, the first child of non consanguineous, healthy and young parents. The familial history is negative. The pregnancy was uneventful and ended with the normal delivery of baby girl weighing 2,200 g. All developmental milestones were delayed: she achieved head control at 6 months, walked without support at 2 years, spoke first clear words at 1.5 years. The girl was referred for genetic evaluation at the age of 5.5 year old, due to ID and single central maxillary incisor. At the time, she presented a growth retardation and dysmorphic features: triangular face, horizontal palpebral fissures, blue sclera, short, slightly protruding philtrum and upper lip, blunted Cupid's bow, slightly everted lower lip, mild microretrognathia, bilateral preauricular sinus (figure II.22.). We found an absent maxillary and mandibular frenulum and single central maxillary incisor. Her language was limited to single words. She had nocturnal and diurnal enuresis. Echocardiography showed an ASD. Abdominal ultrasound, biochemical, hematological and endocrine investigations, CT and MRI of spine were normal.

At the age of 20, we noticed walking difficulties (leaning slightly forward, with widened base of support) and slowness in motion and action. The face became elongated, mature for age, with slightly coarse features (figure II.23.). She had mild webbed neck, broad chest and narrow hips, normal posterior hairline, kyphoscoliosis, pectus excavatum, short and wide hands and feet, with mild brachydactyly. Puberty was normal, but she developed asymmetric mammary glands and excessive hair growth in presternal, circumareolar and subumibilical regions. Psychological testing established a moderate ID (IQ 45), with impaired

speech and language skills, difficulties with interpersonal relationships and oppositional behavior. She presented giggle incontinence. We found normal levels of FSH, LH, estradiol and prolactin, but elevated plasmatic levels of testosterone, dehydroepiandrosterone (DHEA)-sulfate, and 17-hyroxyprogesterone (OH).

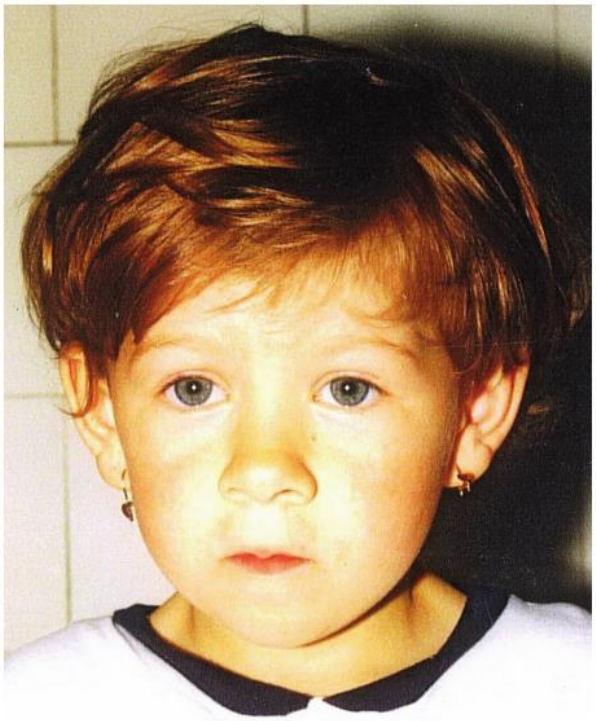


Figure II.22. Patient at age of 5.5 years.

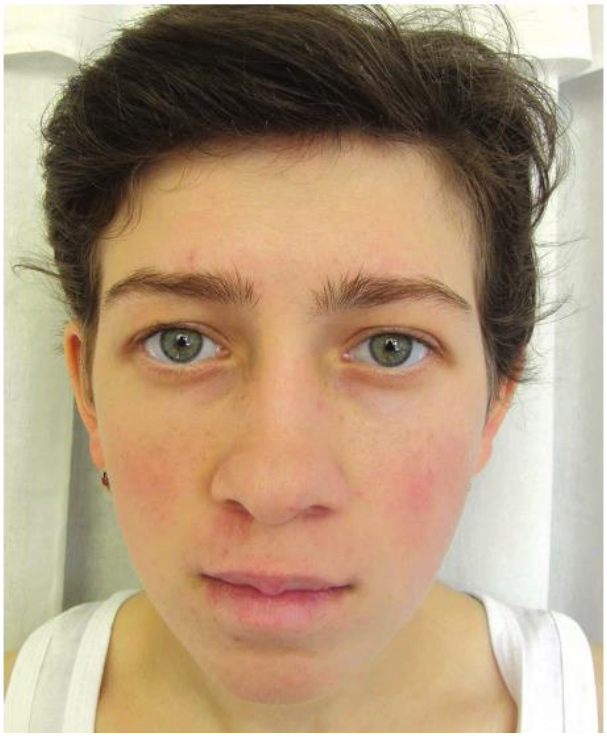


Figure II.23. Patient at age of 20 years.

The chromosome analysis was performed for the patient and her parents using the G-banding technique on metaphase chromosomes from peripheral blood lymphocytes, according

to standard protocol. Chromosome C-banding was performed by the standard BSG (barium hydroxide/saline solution/Giemsa) method with slight modifications.

Genomic DNA was purified from peripheral blood using Wizard Genomic DNA Purification Kit (Promega, USA). The SNP array was performed using Human CytoSNP-12 v2.1 BeadChip platform (Illumina Inc., USA), containing approximately 300,000 SNPs per sample, according to the manufacturer's instructions. The data was processed using Genome Studio V2010.1 software (Illumina). Genomic positions were defined according to the GRCh37/hg19 Assembly of the Human Genome (February 2009).

II.1.9.3. RESULTS

The cytogenetic G-banding revealed an unbalanced translocation between chromosomes 14 and 18 (figure II.24.). The C-banding showed two centromeres on the derivative chromosome (figure II.25.). The karyotype was 45,XX,psudic(14;18)(p11.1;p13.1). The parental karyotypes were normal.

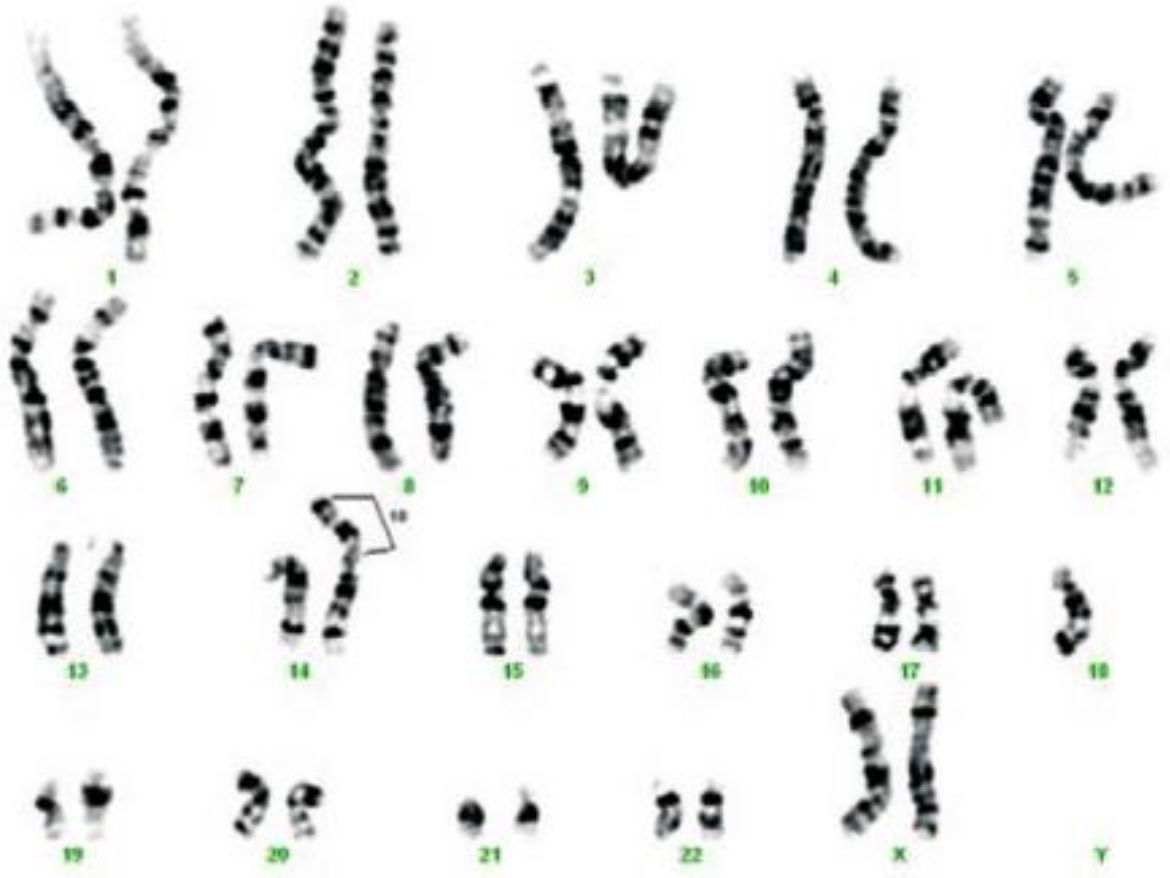


Figure II.24. Karyotype of the proband

Figure II.25. C-banded derivative chromosome from two different metaphases showing the presence of two centromeres.

The SNP array analysis detected a terminal deletion of approximately 10.24 Mb, from 18p11.32 to 18p11.22 (274-10,242,742), flanked by a duplication of 1.15 Mb, from 18p11.22 to 18p11.21 (10,249,343-11,401,062) (figure II.26.). Also, SNP array revealed a duplication of 516,590 bp in 16p11.2 (29,568,718-30,085,308). The relatively small size of the duplications did not allow for FISH to determine the orientation. Blood samples from the parents were not available for SNP array analysis.

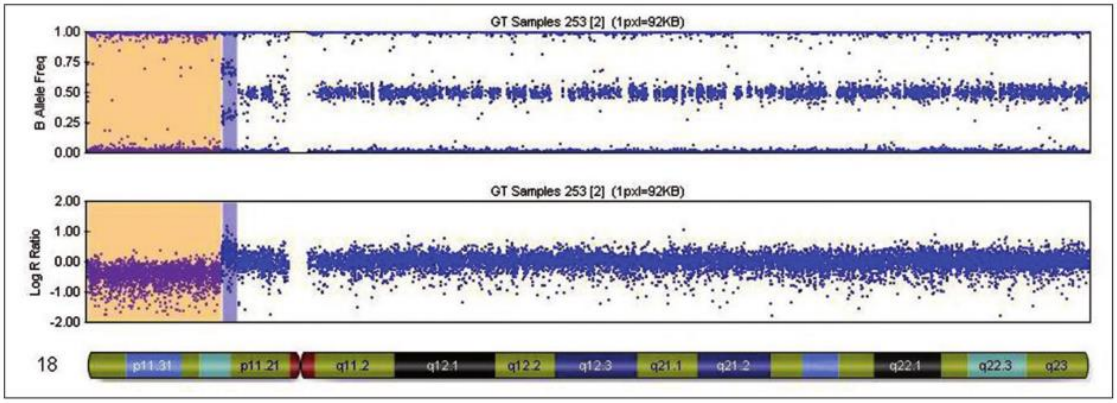


Figure II.26. The SNP array results of chromosome 18 in the patient, showing a 10.24 Mb deletion in 18p11.32-p11.22 and a duplication of 1.15 Mb in 18p11.22-p11.21.

II.1.9.4. DISCUSSION

We described a female patient with some signs of Turner syndrome, mild dysmorphic face, minor features of holoprosencephaly (HPE), small hands and feet, excessive hair growth on anterior trunk and ID. The karyotype showed an unbalanced translocation between chromosomes 14 and 18 resulting in the formation of a dicentric derivative chromosome. Single nucleotide polymorphism array analysis revealed three abnormalities: an 18p deletion flanked by an 18p duplication, and 16p11.2 duplication. The translocation is de novo.

Non Robertsonian dicentric autosomes are rare findings, reported in only 26 cases in a review by Lemyre et al. [345]. Except the acrocentric chromosomes, the most frequent such anomaly involve chromosome 18. Most of the heterodicentric autosomes have only one primary constriction and the constriction is noticed mostly at the site of the non-acrocentric centromere, as in our case.

The 18p duplication (1.15 Mb) is not likely to contribute to the phenotype, since most patients with trisomy 18p have normal or mild phenotypes, usually without ID [352]. Our patient displays some features of 18p- syndrome (ID, mild microcephaly, single central maxillary incisor) and some features of Turner syndrome (short stature, mild webbed neck, pectus excavatum, broad trunk and narrow hips). Facial dysmorphism (triangular face, blue sclera, bilateral preauricular sinus) is different from that described for 18p deletion, except for the oromandibular region. Facial appearance has changed over time, becoming elongated (figure II.23.), as described also by Tsukahara et al. [566]. Congenital cardiac defects have been observed in 10% of cases of 18p- [142]. However, the standing position with widespread legs and leaning slightly forward as well as marked slowness in motion and action are very suggestive for monosomy 18p.

Recurrent 16p11.2 microduplications were initially characterized by normal to ID, autistic spectrum disorders and psychiatric issues [168, 310, 358, 597]. After, to this duplication were associated microcephaly, torticollis, cleft lip and palate, pectus excavatum/carinatum, mild scoliosis, hypospadias, phimosis, tethered cord, pes planus and seizures [508]. Jacquemont et al. [261] showed that 16p11.2 duplication is associated with a growth retardation. Among the features mentioned above, our patient exhibited mild

microcephaly, pectus excavatum, mild scoliosis and ID, but these features are also described in 18p deletion. She was underweight during childhood, but recovered later, her BMI being within normal range as an adult. However, because penetrance of proximal 16p11.2 duplication is 27.2%, and the likelihood of a normal phenotype is ~73.0% [465], we cannot clearly conclude how this copy number variation (CNV) influences the phenotype. More recently, a patient with thoracolumbar syringomyelia and 16p11.2 duplication has been described [483]. Although our patient presented kyphoscoliosis and nocturnal enuresis, MRI of the spine showed no changes.

In a study of three patients with 18p deletion, Portnoi et al. [435] suggested that there might be a critical region for GH deficiency between 18p11.23 and 18pter. Our patient has a deletion which includes that region, but the level of GH is normal and the craniocerebral CT did not show any pituitary gland anomalies. The critical region for ID has been mapped between 18p11.1 and 18p11.21 [602]. Our patient has a deletion distal to this point and moderate ID, but this feature may be due to the 16p11.2 microduplication.

Brenk et al. [74] proposed round face to map to the distal 1.6 Mb of 18p, and postnatal growth retardation and seizures to the distal 8 Mb. Our patient has a terminal deletion larger than 10 Mb, but she had no history of seizures, and face was triangular in childhood and elongated in adulthood. Because pointed chin was observed in five out of 13 patients with 16p11.2 duplication with facial anomalies [168, 508], we appreciate that the triangular aspect of the face may be due to this rearrangement. Ptosis and short neck, frequently associated with 18p- [568], were attributed by Brenk et al. [74] to the proximal half of 18p. These features were absent in our patient, in whom the proximal 5.1 Mb of 18p was not deleted. Thus, haploinsufficiency of genes located in this region may be responsible for these features.

Our patient has a microform of HPE, although only 10% of patients carrying an 18p deletion present HPE [485]. A study of a large group of HPE patients demonstrated a high frequency of submicroscopic anomalies involving some novel HPE loci, including 16p11.2 [44]. Therefore, the 16p11.2 microduplication present in our patient can be a second genetic event contributing to HPE manifestation.

II.1.9.5. CONCLUSIONS

We described a female patient with a pseudodicentric 14;18 chromosome that carries two additional CNVs. These CNVs confer phenotypic variability to 18p- syndrome, leading to difficulties in establishing the contribution of each abnormality to the phenotype. Although the phenotype of 18p- syndrome is not as typical as for other syndromes, HPE microform and Turner stigmata associated with characteristic posture and marked slowness in motion and action is very suggestive for this syndrome. Microarray analysis of our patient allowed us to define precise molecular characterization of the translocation breakpoints and to uncover two unsuspected cryptic abnormalities, improving genotype-phenotype correlations and management.

II.2. MULTIFACTORIAL DISORDERS

The researches concerning multifactorila disorders were published in the following papers
Pânzaru M., Rusu C., Volosciuc M., Braha E., Butnariu L., Gramescu M., Popescu R., Caba L., Bujoran C., Ivanov I., Macovei M., Sireteanu A., Covic M., **Gorduza E.V.**, *Benefits of cytogenetic testing in diagnosis of plurimalformative syndromes with congenital heart defects*, Rev. Rom. Med. Labor., 2012, 20 (3/4) 265-272
Caba L., Rusu C., Voloşciuc M., Butnariu L., Braha E., Grămescu M., Bujoran C., **Gorduza E.V.**, Covic M., *Retardul mental idiopatic – importanţa scorurilor de diagnostic clinic pentru selecţia cazurilor*, Rev. Med. Chir. Soc. Med. Nat. Iaşi, 2009, 113 (2), 523-526
Sireteanu A, Popescu R, Braha EE, Bujoran C, Butnariu L, Caba L, Graur E., **Gorduza E.V.**, Grămescu M., Ivanov I.C., Pânzaru M., Rusu C., *Detection of chromosomal imbalances using combined MLPA kits in patients with syndromic intellectual disability*, Rev Rom Med Lab. 2014;22(2):157-64
Resmerita I, Cozma RS, Popescu R, Radulescu LM, Panzaru MC, Butnariu LI, Caba L, Ilie O-D, Gavril E-C, **Gorduza EV**, Rusu C. *Genetics of Hearing Impairment in North-Eastern Romania—A Cost-Effective Improved Diagnosis and Literature Review*. Genes. 2020; 11(12):1506
Resmeriță I, CobzeanuBM, Popa S, Gavril EC, Martiniuc V, Grămescu M, **Gorduza EV**, Rusu C, *Oculo-auriculo-vertebral spectrum: Clinical features in a cohort of 37 patients*, Rev Med Chir, 2021, 125 (4): 578 -584, https://www.revmedchir.ro/index.php/revmedchir/article/view/2499/1793, https://doi:10.22551/MSJ.2021.04.13

II.2.1. INTRODUCTION

Human disorders are generated by different etyological factors. Some are genetic and other are environmental. In concordance with implication of genetic and are environmental factors, human diseases are divided in three categories: genetic, multifactorial and environmental. Genetic diseases are produced 100% by different types of mutations and are classified in chromosomal, monogenic and mithocondrial. Multifactorial disorders are generated by implication of a great number of factors that are both genetic and environmental. Multifactorial diseases are represented by isolated congenital anomalies, isolated mental retardation, majority of neoplasia and common disorders of adulthood.

II.2.2. STUDY OF ETIOLOGY OF CONGENITAL HEART DISEASE

II.2.2.1. BACKGROUND

Congenital heart diseases (CHD) represent major birth defects with a prevalence of 14/1,000 live births. CHD may be isolated or associated with multiple congenital anomalies (MCA), mental retardation (MR), craniofacial dysmorphism or growth abnormalities [43, 82].The overall frequency of associated extracardiac anomalies in newborns with CHD is 20% with the chromosomal abnormalities being the common cause [167]. Karyotype and molecular techniques, like FISH, revealed chromosomal abnormalities in 12.3% of cases with CHD and MCA; the most common observed were: trisomy 21, trisomy 18, 22q11.2 deletion and trisomy 13 [124, 221, 269].Certain types of CHD are more frequently associated with a chromosomal abnormality: interrupted aortic arch, atrioventricular septal defect, and double-outlet right ventricle [221, 314, 521, 549]. The identification of genetic etiology of CHD is important for a proper genetic counselling in families with affected members.

II.2.2.2. MATERIALS AND METHODS

We analyzed retrospectively the patients from Iasi Genetics Medical Centre (GMC), in order to evaluate the benefits of genetic tests in cases with CHD and MCA. Between 2000 and

2010 we evaluated 1123 patients with MCA±MR at clinical, paraclinical and cytogenetic level. In all patients we performed the karyotype. The clinical examination included anthropometric measurements, evaluation of cardiac anomalies, craniofacial dysmorphism and psychomotor development. We found 321 patients with CHD through 3D echocardiography. The selection for cytogenetic evaluation was based on clinical guidelines from literature and GMC Iași [43, 140, 343, 362, 418, 429, 537, 557].

We identified 749 cases with unbalanced chromosomal abnormalities (group A) and 374 cases with normal karyotype (group B). Group A was subdivided into subgroup A1 (699 cases), including patients with suggestive phenotype for known chromosomal syndromes, which were cytogenetically confirmed and subgroup A2 (50 cases), patients with MCA ± MR, in which cytogenetic analysis revealed unbalanced chromosomal abnormalities. Group B, with normal karyotype, was divided, based on phenotype, into subgroup B1 (34 cases) with suggestive phenotype for microdeletion syndromes and subgroup B2 (340 cases), with MCA±MR, without presumptive clinical diagnosis. For subgroup B1 patients, we applied FISH technique with specific probes to confirm clinical diagnosis. For patients with confirmed clinical diagnosis (subgroups A1 and B1) we analyzed the frequency of different types of CHD corroborated with chromosomal abnormality type.

Chromosomal analysis was based on a short-term culture of activated T- lymphocytes stimulated with phytohemagglutinin [43, 85, 200]. The slides were stained and examined on an optical microscope, directly and after application of G- banding. For each case a minimum of 32 cells were analyzed. When we detected more than 2 cell lines, the number of analyzed cells was increased to 64 or 96. FISH was performed on metaphase chromosome spreads using the Kreatech Diagnostics® cell samples and the Vysis FISH microdeletion probes kit® [43, 200]. DNA probes used were specific for microdeletions: velo-cardio-facial syndrome (22q11/22q13), Williams-Beurren syndrome (7q11/7q22), Wolf-Hirschhorn (4p16/SE 4) and Cri du chat (5p15/5q31).

II.2.2.3. RESULTS

Of the 1123 patients with MCA + MR cytogenetically tested, 749 (66.7%) cases had chromosomal abnormality (group A - table II.11.). Of these, 699 (93.33%) had a suggestive phenotype for a chromosomal abnormality and karyotype confirmed clinical diagnosis (subgroup A1). In 50 cases (6.67%) with abnormal phenotype (MCA ± MR) without specific diagnosis (subgroup A2) karyotype revealed: addition material of unknown origin, deletions, insertions, translocations or ring chromosomes (figure II.27., II.28.).

In group A, a CHD was identified in 223 patients (29.77%). In subgroup A1, CHD was more frequent in: trisomy 18 (60%), trisomy 21 (31.72%) and monosomy X (14.86%). In subgroup A2, we found a CHD in 17 cases (34%) with MCA ± MR and chromosomal abnormalities. The CHD percentage is significantly higher in group A2 than in group B2 (340 cases) – patients with MCA ± MR and normal karyotype, in which CHD were identified in 74/340 cases or 21.76% ($\chi 2 = 6.371$; $p < 0.01$; $p < 0.05$) (table II.11.).

In group B, from 374 patients with MCA ± MR and normal karyotype, 34 cases (subgroup B1) have a suggestive phenotype for a microdeletion syndrome. In 12/34 cases the clinical diagnosis was confirmed by FISH and the majority had a CHD. In subgroup B2, 340 cases with MCA ± MR and normal karyotype, 74/340 cases (21.76%) had CHD (table II.12.). Frequency of chromosomal abnormalities varied by type of CHD and was higher in cases with atrioventricular canal, persistent ductus arteriosus, ventricular septal defects and atrial septal defects, than in pulmonary stenosis, aortic stenosis and Fallot tetralogy (table II.13.).

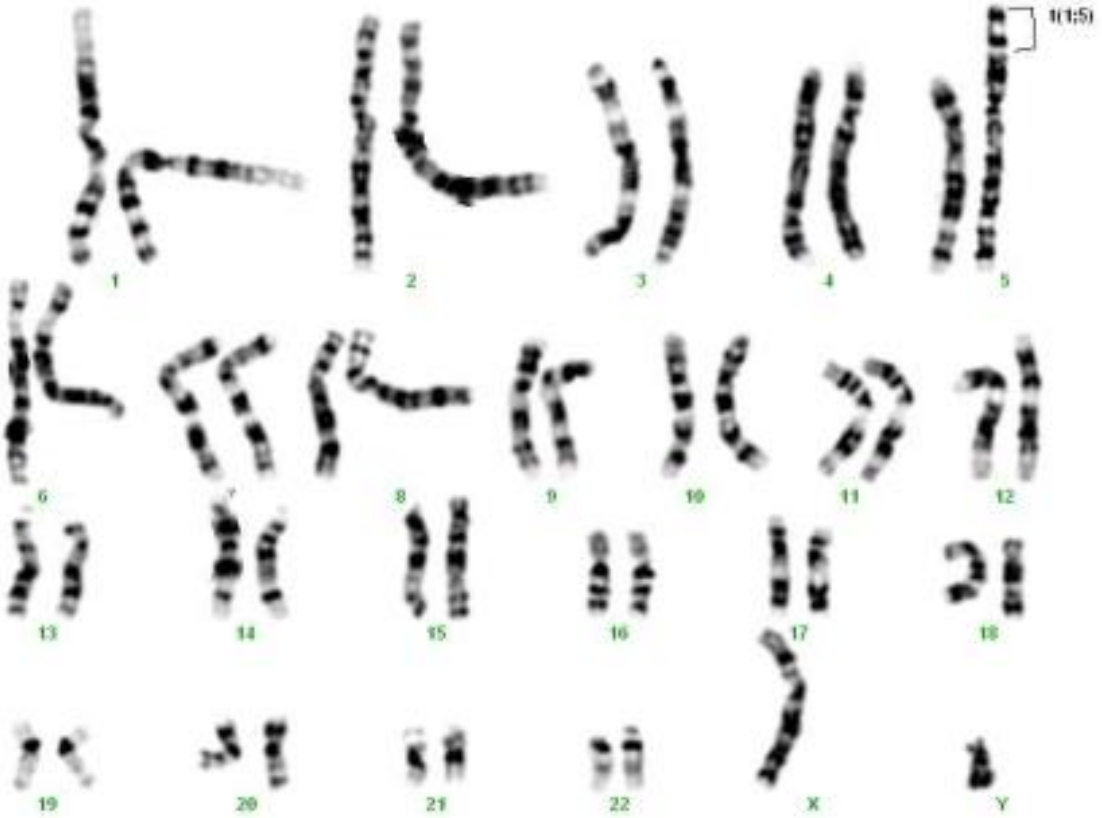


Figure II.27. Karyotype 46,XY,der(5)t(1;5)(q32;p15.3)

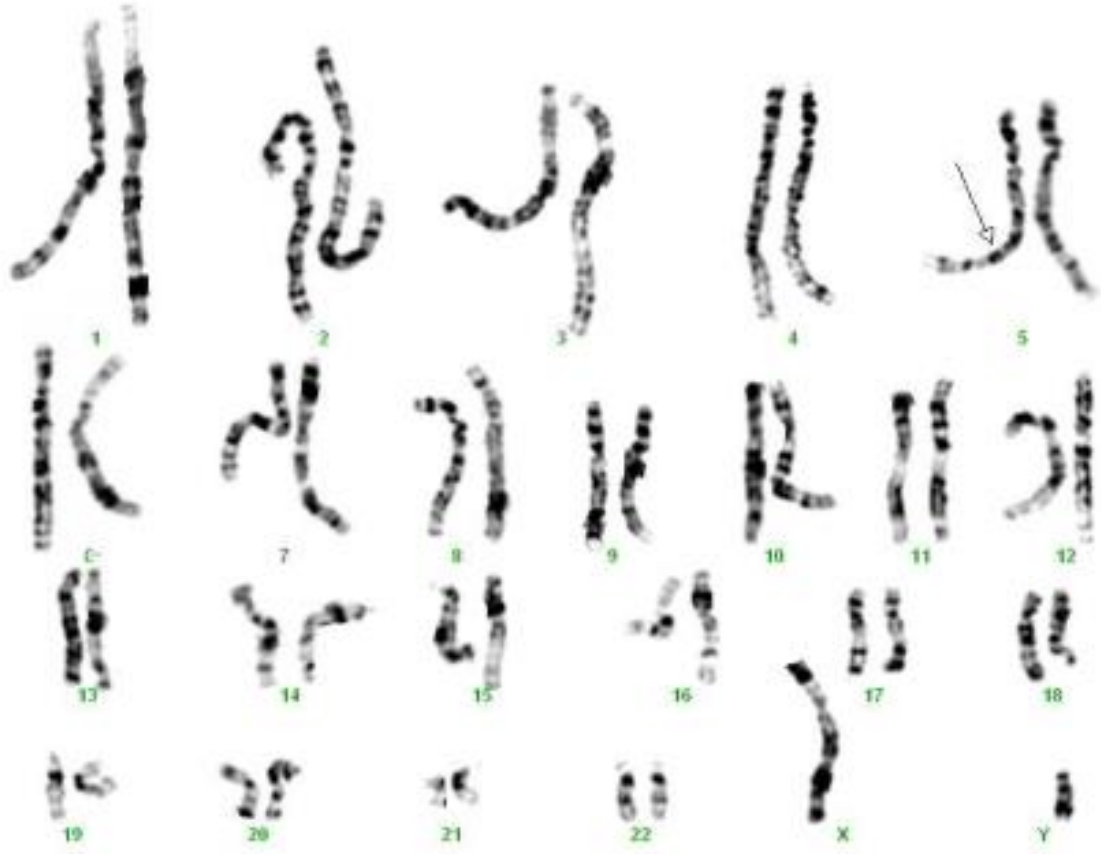


Figure II.28. Karyotype 46,XY,del(5)(q22.2;q31.22)

Table II.11. Results of chromosomal analyses and frequency of CHD

Phenotype	Karyotype			CHD	
	Anomaly	**No. cases**	**Total**	**No. cases**	**Total**
MCA + MR Chromosomal disease phenotype (subgroup A1)	21 trisomy	558	699 (93.33%)	177 (31.72%)	206 (29.47%)
	18 trisomy	15		9 (60%)	
	13 trisomy	12		4 (25%)	
	X monosomy	74		11 (14.86%)	
	XXY trisomy	34		1 (2.94%)	
	Deletion 4p	2		1 (50%)	
	Deletion 5p	3		2 (66.66%)	
	Deletion 7q11.2	1		1 (100%)	
MCA + MR Without chromosomal disease phenotype (subgroup A2)	add	22	50 (6.67%)	7	17 (34%)
	del	16		7	
	ins/t	11		2	
	r	1		1	
			749		**223 (29.77%)**
MCA + MR Without chromosomal anomalies (group B)	Normal	374	374		99 (26.47%)
Total			**1123**		**322 (28.67%)**

Table II.12. Results of FISH analyses and frequency of CHD

Phenotype	No. cases	FISH	No. cases	CHD
Microdeletion 22q11.2	22	positive	**7**	**6**
		negative	15	10
Microdeletion 7q11.23	8	positive	**3**	**2**
		negative	5	4
Microdeletion 4p16	4	positive	**2**	**1**
		negative	2	2

Table II.13. Frequency of chromosomal anomalies in different types of CHD

Type of CHD	AVC	PDA	VSD	ASD	PS	AS	FT
No. cases with chromosomal anomalies/ total cases	34/38	44/50	43/55	82/112	15/23	9/15	5/18
%	88.23	88	78.18	73.21	65.21	60	27.77

AVC – atrioventricular canal; PDA – persistent ductus arterious; VSD – ventricular sept defect; ASD – atrial sept defect; PS – pulmonary stenosis; AS – aortic stenosis; FT – Fallot tetralogy

II.2.2.4. DISCUSSION

Between 2000 and 2010, in Iaşi GMC cytogenetic tests were requested for 1123 patients with MCA and MR. CHD was present in 322 (28.67%) cases. G banding karyotype revealed chromosomal abnormalities in 749 cases, representing 66.7% of all patients tested. This high percentage proves a correct clinical selection of cases for chromosomal analysis, based mainly on association of MCA±MR. For 34 of remaining 374 patients, with suggestive phenotypes for a certain microdeletion, we applied FISH test that confirmed diagnosis in 12 cases.

From patients with CHD 29.77% had chromosomal abnormalities. The percentage is not statistically significant compared with patients with MCA±MR and normal karyotype in which only 26.47% had CHD. CHD was present in 232 (30.49%) of 761 patients with chromosomal abnormalities (749 group A and 12 subgroup B1), a similar result of those found in other studies [221]. CHD was more frequent in 22q11.2 microdeletion (6/7 cases), trisomy 18 (9/15 cases), trisomy 21 (177/558 cases or) and X monosomy (11/74 cases). In our study, CHD frequencies in different types of chromosomal abnormalities are lower than in other studies [221]. For example CHD frequency in trisomy 21 in our study was 31.72%, lower than reported by other studies (33 – 48%) [177, 202, 284, 537, 599]. This is probably due to higher mortality in severe CHD and lack of complete investigations in many of our cases.

We detected chromosomal abnormalities in 232 cases (72.04%) from 322 cases with CHD. Septal defects and patent ductus arteriosus were more frequently associated with a chromosomal abnormality, probably due to participation in complex CHD. Association of AVC with chromosomal abnormalities, especially trisomy 21, was reported in other studies The lack of association of FT with microdeletion 22q11.2 is surprising, and this can be the result of a population characteristic [82, 177, 284, 537, 599].

II.2.2.5. CONCLUSIONS

Our study showed a high frequency of chromosomal abnormalities among cases with MCA and MR and proved the benefits of cytogenetic testing in plurimalformative syndromes with CHD. When "standard" chromosome analysis shows a normal karyotype, molecular cytogenetic techniques are useful to detect submicroscopic chromosomal abnormalities.

II.2.3. STUDY OF ETIOLOGY OF INTELECTUAL DISABILITY

II.2.3.1. BACKGROUND

Intellectual disability (ID) is a major public health issue, with prevalence of 1-3% in general population [459]. ID etiology is complex, including exogenous, genetic or unknown factors. The introduction of FISH and MLPA techniques [489] enabled the detection of microdeletions/ microduplications in syndromic ID patients. In the past decade, the introduction of the microarray technology has allowed the detection of submicroscopic CNVs in 15-20% of ID cases [112, 369, 511]. However, this technology requires expensive equipment and consumables that are hardly accessible to all diagnostic centers. Some of the chromosomal abnormalities identified by microarray tests can also be detected using different MLPA kits. Screening of ID patients using the MLPA technique represents a reasonable option in the diagnostic evaluation of ID, especially in the developing countries.

II.2.3.2. MATERIAL AND METHOD

We made a retrospective study concerning the etiology of intellectual disability. In a first step we made a clinical analysis of a group of 36 children with variable ID associated with other congenital abnormalities to verify the validity of de Vries score (table II.14.) [5, 134].

Table II.14. Criteria for ID by subtelomeric rearrangements [134]

Criterion	Score
Familial history of MR	
• Monogenic inheritance	1
• No monogenic inheritance	2
Growth retardation with prenatal onset	2
Postnatal growth disorder (each feature 1 point, maxim 2 points)	
• Microcephaly	1
• Shortstature	1
• Macrocephaly	1
• High height	1
2/> facial dysmorphia (especially hypertelorisme, anomalies of nose and ears)	2
Extrafacial anomalies (each feature 1 point, maxim 2 points):	
• Anomalies of hands	1
• Cardiac malformations	1
• Hypospadias+/- cryptorhidisme	1

In the second part of study we applied the MLPA method to identify the presence of some chromosomal imbalances. The second study group consisted of 369 patients with syndromic ID of unknown etiology, with normal (358 patients) or uncertain karyotype at 400-550 band resolution (11 patients). Patients that were clinically suggestive for aneuploidies (21, 13 and 18 trisomies) and Fragile X syndrome were not included in the study. All patients have been clinically evaluated by a geneticist that searched family and medical history, anthropometric measurements, detailed physical examination and psychological examination. A written informed consent was obtained prior to evaluation from either patients' parents or legal representatives.

All patients were assessed for chromosomal imbalances using commercially available SALSA MLPA kits® (MRC-Holland). Patients with a clinical suspicion of a microdeletion syndrome (186 patients, subgroup A) were tested with P064 kit (156 patients) or P096 kit (30 patients), which contain probes for common microdeletion/microduplication syndromes. For all other patients - 183 patients, subgroup B – we used the P036 and P070 kits developed to screen for subtelomeric CNVs and contain one MLPA probe for each subtelomeric region, except for the short arms of acrocentric chromosomes, for which a probe on the q arm, close to the centromere is included instead.

Abnormal results detected by these MLPA kits were further characterized using appropriate follow-up MLPA kits (SALSA® MLPA®Telomere Follow-up set, P029-A1 Williams-Beuren Syndrome probe-mix, P250-B2 DiGeorge probe-mix and ME028-B1 Prader Willi/Angelman probe-mix). The gene content of aberrations was analyzed using the UCSC genome browser (NCBI36/ hg18, http://genome.ucsc.edu/).

The DNA extraction from peripheral blood was performed using Wizard Genomic DNA Purification Kit® (Promega, USA). The standard MLPA analysis was performed according to the manufacturer's instructions. Briefly, 200 nanograms of genomic DNA was denatured and hybridized with SALSA probes at 60°C for 16-18 hours. After 15 minute ligation at 54°C, PCR was performed in a Gradient Palm-Cycler (Corbett Research, Australia) using Cy5 universally labeled primers. Fluorescent amplification products were subsequently separated through capillary electrophoresis, in a CEQ 8000 GeXP Genetic Analysis System (Beckman Coulter), and were analyzed using the default software. The number of DNA copies was estimated using the Coffalyser.Net software, which calculates the ratio of peak areas in test samples over those of normal controls for each target sequence.

II.2.3.3. RESULTS

The clinical evaluation of the first 36 patients with ID included in study was made using de Vries score (table II.14.). According to de Vries a value higher than 3 is suggestive to a chromosomal imbalance. To compare the utility of algorithm we chose an equal number of children with chromosomal abnormalities (group I) and without chromosomal abnormalities (group II) detected by karyotype. Group I met 18 patients with chromosomal abnormalities: 4 numerical anomalies and 14 structural abnormalities that involved chromosomes 1, 4, 5, 7, 8, 9, 17 or X and were represented by deletions (9 cases), inversions (1 case), insertions (1 case), ring chromosomes (1 case) and additional material (2 cases). By applying clinical score Vries in these cases we found the following values: 4 (6 cases) 5 (2 cases) 6 (4 cases) or 7 (6 cases). The main anomalies found in group I were: shortstature (56.7%), microcephaly (72.3%), nasal abnormalities (72.3%) ear abnormalities (66.7%) or hand abnormalities (83.4%). Applying de Vries score in group II (18 patients with normal karyotype) we found lower values, 14 patients having a score inferior to 3. The main clinical signs identified were: microcephaly (38.9%), shortstature (38.9%), abnormal ears (33.4%), abnormal hands (61%) and heart defects (44.5%).

In a second part of study different MLPA kits were used to detect chromosomal aberrations in a group of 369 patients with unexplained syndromic ID. In subgroup A, using P096 kit, we identified reduced ratios for all of 16 probes targeting 4p telomeric region and confirmed the Wolf-Hirschhorn syndrome in 1 of the 30 patients suspected. Using P064 kit and follow-up kits we detected abnormalities in 24/156 (15.3%) patients: fourteen with 22q11.21 deletions (Velocardiofacial/DiGeorge syndrome), seven with 7q11.23 deletions (Williams syndrome) and three with 15q11.2 deletions (Prader - Willi syndrome). In subgroup B, using P036 and P070 kits, we found subtelomeric rearrangements in 30 patients, 24 confirmed by both kits and 6 identified by only one kit.

II.2.3.4. DISCUSSION

The clinical analyse of patients with ID with chromosomal imbalances (group I) and without chromosomal imbalances (group II) confirmed the utility of de Vries score [134] in the selection of patients with ID for supplementary investigations by molecular analysis. In cases with chromosomal imbalances the de Vries score are higher than 4 in all cases and shortstature, microcephaly, nose, and ear or hand anomalies were identified in more than 50% of cases. In patients without chromosomal imbalances de Vries score was <3 in 14 from 18 patients and the most common signs were hand abnormalities and heart defects.

In subgroup A of second study, the incidence of microdeletion/microduplication syndromes was 13.4%, most of them (24 out of 25) being identified using the P064 kit. The low detection rate of P096 kit might be explained by lower frequency of the syndromes covered by P064 kit. Other studies that have used MLPA P064 kit had detection rates of 5.8-9.2% when patients were selected based on the presence of ID and/or multiple congenital anomalies [266, 289], and of 14,1%, when patients were selected based on phenotype suggestive of a microdeletion syndrome [289]. Submicroscopic anomalies are involved in many cases with ID/multiple congenital anomalies, and clinical selection of patients may increase the detection rate.

The 22q11.21 deletion was the most frequent abnormality in our study, being detected in 7.5% of patients. The outcome is concordant with other studies, in which the 22q11.21 deletion was detected in 4.6-7% of patients [266, 289]. Using the P250 kit we found the common ~3.0 Mb deletion with breakpoints between low copy repeats A and D in all 14 patients.

In patients with Williams syndrome, using P029-A1 follow-up kit we found a complete deletion in five patients and a smaller deletion that does not extend telomerically further than LIMK1 gene in two sisters, which do not display a classical phenotype. Only few patients with

Williams's syndrome and atypical deletions have been reported [181, 538], and they are important for genotype–phenotype studies.

Using methylation-specific MLPA kit for Prader Willi/Angelman syndrome we identified full deletion in all patients with Prader-Willi syndrome that are concordant with the literature data [84].

In cases with suspected subtelomeric rearrangements the use of two different probes is recommended for identification of abnormal regions [288, 398, 460]. However, the clinical significance of subtelomeric rearrangements is quite complicated, especially in cases where the DNA samples of the parents are not available. In 24 patients (from 183 tested) we found subtelomeric rearrangements identified by both kits and most of them were concordant with the phenotype. Regarding only the patients with normal karyotype (172 cases), cryptic subtelomeric rearrangements was detected in 13 cases (7.5%). Previously reported studies that performed subtelomere analysis showed an overall rate of 6% (2 - 29%) [10, 11, 54, 288, 298, 366, 452, 460, 530, 611]. The differences are correlated with inclusion criteria, assay used in the study, size of the cohort and exclusion (or not) of the polymorphisms. A general overview upon the main previous studies using MLPA to identify subtelomere imbalance is presented in table II.15.

Table II.15. A general overview upon the main studies using MLPA to identify subtelomere imbalance

Reference	Number of cases	Selection criteria	Number of patients with clinically significant abnormalities (%)
Koolen et al. [298]	210	ID	9 (4.3%)
Kirchhoff et al. [288]	258	ID + D	13 (5%)
Rooms et al. [460]	275	ID	8 (2.9%)
Ahn et al. 2007 [11]	455	DD ± D	27 (5.9%)[a]
Ahn et al. 2008 [10]	403	DD ± D	22 (5.5%)[a,b]
Stegmann et al. [530]	466	ID ± CA	18 (3.9%)
Wu et al. [611]	451	ID	23 (5.1%)
Medina et al. [366]	112	ID	5 (4.2%)

ID – intellectual disability, D – dysmorphy; DD – development delay; CA – congenital anomalies; [a] Polymorphisms are included; [b] Abnormalities detected by karyotype are included.

Out of 30 patients displaying abnormal MLPA results, 6 were detected by only one kit. Such rearrangements were previously reported in the literature and could be explained by the fact that the probes of P036 and P070 kits hybridize to sequences in different positions of the same subtelomeric region. The imbalances detected by only one probe can be either a false-positive result (due to a mutation or a polymorphism in the sequence or to a bad application of method) or a true positive result (the genomic imbalance is small or the breakpoint is situated between the regions covered by the two probes). Half of the rearrangements identified by a single kit in our study were 4q deletions detected by older versions of the P036 kit, and they

were due to the presence of a SNP in the probe site, an aspect that the manufacturer confirmed as well. Most abnormal results detected by a single kit are clinically irrelevant inherited polymorphisms that can also be detected in a healthy parent [288, 298, 530]. In our study the parental DNA samples were not available, but considering the size and gene content of the 21q duplication and the fact that 5q duplication and 16p deletion could not be confirmed by follow-up kits, we considered them as non-causative variants.

The follow-up kits allowed identification of unknown chromosomal material in 10 out of 11 patients with abnormal karyotype and established the size of 14 of the 31 (45%) singular anomalies. In the case of two patients for which the screening kits indicated the presence of deletions (5pter, and 2qter respectively), the use of follow-up kits (P358, and P264 respectively) has also indicated the presence of duplications of the same chromosome arms. We hypothesize the presence of inverted duplications contiguous to terminal deletions, but further studies are needed for confirmation. In other four patients with normal karyotype we detected both a deletion and a duplication in different chromosomes, which suggests the presence of a cryptic unbalanced translocation. Our data is concordant with that reported in previous studies [266, 381, 611].

By comparation with FISH method, MLPA follow-up kits offer better information regarding the position and breakpoints, but FISH allows detection of smaller abnormalities (like microduplications). Moreover, the price of the follow-up MLPA kit is lower compared to the corresponding FISH test.

The combined use of the MLPA kits led to the diagnosis in 38 out of 358 cases with normal karyotype results (10.6%) and helped to establish the origin of the additional material and the type of rearrangement in 10 out of 11 cases with extra segments of unknown origin. Other studies that used a combination of MLPA kits had detection rates of 14% [432], respectively 20.7% [266], but the inclusion criteria in the study were different: patients with ID with or without dysmorphic features or additional congenital abnormalities in the first study and patients with multiple congenital malformations with or without ID in the second study. A study performed on patients with ID and/or dysmorphic features where the same three kits were used separately, on groups of patients, had a detection rate of 7.2% [10]. All these studies show that the combined use of MLPA kits has a relatively high detection rate in ID patients, close to the ~19% reported by a review of 29 microarray-based studies of unselected multiple congenital anomalies/ID patients [335]. A study that compared different investigation approaches regarding ID [307] has suggested that the replacement of chromosomal analysis with MLPA, followed by microarray can prove to be efficient as far as the detection rate and the cost-efficiency balance are concerned.

II.2.3.5. CONCLUSIONS

Clinical de Vries score is useful for identifying major chromosomal abnormalities. We believe that it could be discussed and other signs such as obesity, labio-maxillo-palatine cleft or renal abnormalities present in some patients included in the study and could provide other evaluation criteria.

Using MLPA test, the detection rate of subtelomeric abnormalities was 7.5%, higher than the average value reported in the literature, which illustrates the efficiency of using two subtelomeric screening kits for all patients and of assessing the abnormal results by means of follow-up kits. The follow-up kit allows confirmation of the abnormality and estimation of its size, which established the clinical significance of subtelomeric anomalies. Further characterization of additional material of unknown origin noticed in the standard karyotype is also possible by use of follow-up subtelomeric kits.

The combined use of karyotype and MLPA kits for the screening of the most frequent submicroscopic anomalies represents an efficient strategy for establishing the etiologic diagnosis in ID patients, particularly when microarrays are unavailable as a first line approach.

II.2.4. STUDY OF ETIOLOGY OF HEARING LOSS

II.2.4.1. BACKGROUND

Hearing impairment (HI) is the most common and heterogeneous sensory deficiency. It is defined by a unilateral or bilateral decrease in hearing acuity, more precisely a decrease in the hearing threshold in decibels (dB), at different frequencies. World Health Organization estimates that HI affects 466 million people around the world (6.1% of the world's population), of which 34 million children.

It is considered that 1/1000 newborns have a form of congenital hearing impairment [48, 610]. More than 50% of cases of deafness are due to genetic causes [121] out of which 67% are classified non-syndromic hearing impairment (NSHI), whereas a specific syndrome can be identified in 33% of cases [209].

In the last 5 years, progress has been made in identifying new hearing impairment genetic causes, due to research and new technology. Approximately 121 loci for NSHI have been currently mapped: 49 autosomal dominant, 76 autosomal recessive and 5 X-linked [573].

GJB2 (NM_004004.5) or Gap Junction Protein β 2, situated on chromosome 13q12 (DFNB1 locus), is the most common cause of congenital hearing loss in many populations [444] including European and Mediterranean countries [89, 296, 368, 7–9 - 23]. More than 150 different pathogenic variants in *GJB2* have been reported. The most frequent variant in the Caucasian populations is c.35delG, representing about 60 % of all cases of NSHI [67, 89, 169, 296, 297]. *GJB3* (Gap Junction Protein β 3) and *GJB6* (Gap Junction Protein β 6) are the next frequent genes that can cause hearing impairment but they are less common, with less than 10 mutations cited [308, 383, 539].

The aim of this study was to identify and investigate the main genetic causes for NSHI in the hearing impairment subjects from the North-Eastern Romania and to convince other specialties to advice for genetic testing and counseling.

Subsequently we verified a possibility to use Multiplex Ligation-dependent Probe Amplification (MLPA) as a cost-effective diagnosis protocol for developing countries and as a first intention genetic method. Genetic screening is feasible, *GJB2* being accountable for a large proportion of NSHI.

In patients with hearing impairment, the diagnostic approach starts with personal medical history, physical examination and family history for at least three generations and should continue with genetic tests and appropriate management.

II.2.4.2. MATERIALS AND METHODS

The patients included in the study were registered under a numerical code in order to maintain anonymity. Study was approved by the Ethics Commission of "Grigore T. Popa" University of Medicine and Pharmacy Iasi and the Ethics Commission of "Saint Mary" Emergency Children's Hospital Iasi.

Informed consent was signed by from patients, parents or legal guardians before beginning the research. All subjects included in this study were offered voluntary entrance.

In the study (2015–2019), were enrolled 395 subjects with mild to profound and bilateral hearing impairment from the Iasi Regional Center for Medical Genetics and Audiology Department of Iasi Rehabilitation Clinical Hospital. All the subjects were clinically characterized by physical and auditory examinations.

A number of 104 individuals were excluded from this study based on: syndromic or environmental/infectious etiology for hearing loss.

Audiologic Assessment

Auditory functional assessment was performed only in the absence of the pathology of the middle ear, confirmed by otomicroscopy and wideband tympanometry. In cases identified with otitis media, the appropriate treatment was recommended and the child was rescheduled for repeated controls until the condition of the middle ear allowed audiological testing (normal otomicroscopy with wideband tympanogram of type A).

The audiological evaluation was adapted to the age and to the psycho-intellectual development of children. Thus, in children over 6 years of age, the auditory thresholds were measured by standard liminal tonal audiometry. In those under 6 years of age, as well as in some children over 6 years of age but who could not collaborate in subjective audiometric testing, the identification of hearing thresholds was done by objective audiological assessment of cross check type. The auditory steady state response and brainstem evoked response audiometry using insert headphones and distortion product otoacoustic emissions were measured in natural sleep. We performed visual reinforced audiometry or/and free field audiometric examination for subjective threshold confirmation in children who collaborated. In these cases, the conduction hearing loss was excluded based on normal otomicroscopy accompanied by type A wideband tympanometry. The audiologic evaluations were performed in soundproof rooms, using Interacoustics equipment. The audiologic follow-up was made periodically with the same methods adapted to each child's particularity (age and medical condition), mainly at 4-, 6- or 12-months intervals, in order to identify the dynamic evolution of hearing impairment and for the fitting of the conventional hearing aids. Children with progressive hearing loss received the indication for cochlear implantation when they were included in the category of severe or profound hearing loss. All subjects had cranial computed tomography (CT) scan and none showed ear malformation.

DNA genomic extraction

DNA was extracted from 3 mL of peripheral blood samples stored with EDTA agent, using Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA).

MLPA

The probe mix P163 *GJB-WFS1-POU3F4* was used for the detection of deletions or duplications in the *GJB2*, *GJB3*, *GJB6*, *POU3F4* genes, genomic microdeletions upstream of *POU3F4* and the presence of six specific variants in the *GJB2* gene: c.313_326del14, c.235delC, c.167delT, c.101T>C and c.35delG. The MLPA analysis was performed according to the manufacturer's protocol. Briefly, 100 nanograms of genomic DNA was denatured and hybridized with SALSA probes at 60°C for approximately 17 h. PCR was performed after 15' ligation at 54°C, using Cy5 labeled primers. Fluorescent amplification products were separated based on their length by capillary electrophoresis in a CEQ 8000 GeXP Genetic Analysis System (Beckman Coulter, Brea, CA, USA) and the results were analyzed using Coffalyser.NET V9 program (MRC-Holland, Amsterdam, The Netherlands). The probe ratio of deletion and duplication were fixed at 0.7 and 1.3 respectively. Genomic regions of the *GJB2* gene were sequenced bidirectionally in heterozygous or normal individuals.

Sanger Sequencing

The amplification using 125ng genomic DNA (25 µL reaction volume) was performed in a Sensoquest Thermocycler (Sensoquest, Göttingen, Germany), using Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA). PCR conditions included: initial denaturation (10' at 95°C), followed by 35 cycles of denaturation (30'' at 94°C), annealing (30''at 57°C) and elongation (60'' 72°C), with a final elongation at 72°C for 5 min [449]. The sequencing was performed using GenomeLab™ Dye Terminator Cycle Sequencing (DTCS) and a Quick Start kit (Beckman Coulter, Brea, CA, USA). A modified protocol was used with 10 µL reaction volume [22]. The Agencourt system (Beckman-Coulter) was used to purify PCR amplicons (Agencourt AMPure XP, Brea, CA, USA) and sequencing products

(Agencourt Cleanseq® system, Brea, CA USA). The final products were subsequently separated and detected on a CEQ 8000 GeXP Genetic Analysis System (Beckman-Coulter). Sequences were analyzed in both directions (forward and reverse) and compared with the NCBI reference sequence NM_004004, using Mega6 software. The variants were verified for pathogenicity in Mutation taster, ClinVar and PolyPhen for the evaluation of disease-causing potential of sequence alterations [384, 492].

Statistical Analysis

Experiment results were analyzed in Excel and presented in descriptive statistics.

II.2.4.3. RESULTS

A total sample of 291 patients from North-Eastern Romania were collected in period 2015–2019. HI was reported to be congenital and without other accompanying clinical features. All patients included in this study showed different pathologic levels of auditory thresholds, from mild to profound bilateral hearing impairment. The patients' age ranged from 1 month to 52 years (median age 12.31). Among the 291 probands, 74.6% (217/291) were sporadic cases of HI (simplex probands) (of which 15 with parental consanguinity) and 25.4% (74/291) had at least one first degree affected relative with bilateral HI (multiplex probands), of which 4 with parental consanguinity.

Mutations in *GJB2*, *GJB3*, *GJB6*, *POU3F4* and *WFS1* genes were analyzed by MLPA that revealed abnormal results in 141 cases (48.45%). Out of the total of 141 abnormal cases, 4 (2.84%) had variants in *WFS1* gene and 137 (97.16%) in *GJB2* gene: 57 (40.43%) were c.35delG homozygous, 26 (18.44%) were c.35delG heterozygous, 30 (21.28%) were compound heterozygous and 28 (19.86%) had other types of variants. No mutations were identified by MLPA in *GJB3*, *GJB6* and *POU3F4* genes. Referring to the *WFS1* gene, all of the 4 patients with variants in this gene had exon 8 deletion (table II.16.). They had non-progressive mild to moderate hearing impairment and the age ranges from 15 to 20 years. We included these patients in a different study.

Regarding the *GJB2* gene, the most common pathogenic variant in the Romanian population is c.35delG, found in 97 patients in our study (33.3%). Of these, 57 patients (58.76%) had the c.35delG variant in homozygous state, 26 (26.84%) in heterozygous state and 14 (14.4%) were compound heterozygous for 3 different 35delG/non-35delG variants.

The entire coding region of *GJB2* was sequenced in all individuals included in this study. Out of 26 patients with c.35delG variant in heterozygous state, 10 patients (38.46%) were in fact compound heterozygous. Among 150 patients with normal results at MLPA, 44 patients (29.33%) had abnormal results: 25 patients with variants in heterozygous state: 7 with c.71G>A (28%), 5 with c.457G>A (20%), 3 with c.269T>C (12%), 3 with c.109G>A (12%), 3 with c.100A>T (12%), 2 with c.551G>C (8%). All the patients with c.35delG in homozygous state were confirmed with Sanger Sequencing.

Genotype-phenotype correlation was performed based on the distribution of the severity of HI in c.35delG and non-35delG genotype categories as shown in table II.17. Most cases had hearing loss before age 18. A small proportion of patients with mild hearing impairment showed a sequence variation in *GJB2*. Out of 18 patients with mild hearing loss, only 2 of them had c.35delG in homozygous state (diagnosed before age 4) and 4 patients had c.35delG in heterozygous state (diagnosed after the age 4). 81 patients had *GJB2* biallelic mutations and severe or profound hearing impairment: 20 (24.6%) of them had severe HI and c.35delG in homozygous state, 28 (34.5%) had profound HI and c.35delG in homozygous state, while 8 patients (9.87%) with severe HI had c.35delG in compound heterozygous state and 14 patients with profound HI had c.35delG in compound heterozygous state.

Table II.16. Variants spectrum found in this study.

	Variants	Protein Change	Clinical Significance	Patients (no)
MLPA	c.35delG, rs80338939	p.Gly12Valfs	Pathogenic	97
	c.101T>C, rs35887622	p.Met34Thr	Pathogenic	19
	c.313_326del14, rs111033253	p.Lys105Glyfs p.Trp3Ter	Pathogenic Pathogenic	12 6
	c.-23+1G>A, rs80338940			
	Del *WFS* 1-8		Pathogenic	4
	Del ex1 *GJB2*		Pathogenic	3
SANGER SEQUENCING	c.71G>A, rs104894396	p.Trp24Ter	Pathogenic	15
	c.551G>C, rs80338950	p.Arg184Pro	Pathogenic	4
	c.109G>A, rs72474224	p.Val37Ile	Pathogenic	3
	c.269T>C, rs8033894	p.Leu90Pro	Pathogenic	3
	c.100A>T, rs564084861	p.Met34Leu	Uncertain significance	3
	c.457G>A, rs111033186	p.Val153Ile	Likely benign	5
	c.380G>A, rs111033196	p.Arg127His	Benign	10
	c.39G>A	p.(=)	Benign	4
	c.341C>G	p.Glu114Gly	Benign	4
	c.79G>A, rs2274084	p.Val27Ile	Benign	6

Table II.17. Correlations of *GJB2* genotypes and severity of hearing loss.

	Genotypes	No of Subjects	Mild (21–40 dB)	Moderate (41–70 dB)	Severe (71–90 dB)	Profound (>90 dB)
c.35delG Homozygous	c.35delG/c.35delG	57	2	7	20	28
c.35delG Heterozygous	c.35delG/wt	26	4	11	7	4
c.35delG Compound Heterozygous	c.35delG/c.101T>C	10	-	6	2	2
	c.35delG/c.313_326del14	6	-	1	2	3
	c.35delG/c.-23+1G>A	4	-	-	1	3
	c.35delG/c.71G>A	8		1	3	4
	c.35delG/c.551G>C	2	-	-	-	2
Non-35delG Compound Heterozygous	c.79G>A/c.380G>A	1	-	1	-	-
	c.79G>A/c.341C>G/C.380G>A	4	1	3	-	
	c.79G>A/c.39G>A	4	2	2		
Non-35delG Heterozygous	c.101T>C/wt	9	5	2	1	1 1
	c.71G>A/wt	7	-	5	1	-
	c.457G>A/wt	5	1	2	2	-
	c.313_326del14/wt	2	-	1	1	1
	c.269T>C	3		-	2	
	c.109G>A/wt	3	-	-	2	1
	c.551G>C/wt	2	-	1	1	-
	c.380G>A/wt	2	2	-	-	-
	c.100A>T	3	2	1	-	-
	Total	158	19	44	45	50

II.2.4.4. DISCUSSION

Hearing impairment a heterogeneous conditions and each population has a different etiologic profile based on ethnic, geographic, social and medical background. It is diagnosed in 1–2 of 1,000 newborns [487], genetic factors are responsible to up to 2/3 of HI cases (70% non-syndromic and 30% syndromic deafness)[503]. The remaining one-third of cases can be caused by environmental and unidentified genetic factors. The prevalence of *GJB2* gene mutations can vary according to ethnicity: more than 50% in the European population [283], 16% in China [332] and Iran [187], and 9.6% in Mexican population [339]. Among the European population, the c.35delG variant represents 2/3 of the total mutations in the *GJB2* gene [184, 576]. In other populations variants such as: c.235delC variant in Japanese and other Asian populations [403, 506], c.167delT in the Ashkenazi Jews [143], c.71G>A in Indians and Roma [449, 475] are prevalent.

We performed a genetic screening of *GJB2* gene (responsible for the major etiologies of hereditary hearing impairment among Romanians), *GJB3*, *GJB6*, *POU3F4* and *WFS1* genes. Genetic diagnosis was confirmed in 174 (59.7%) of the 291 patients with different degrees of hearing impairment, most of them being accounted for *GJB2* gene. *GJB2* mutations are frequent in all studied populations [31, 58, 63, 279, 546, 612]. In some populations *GJB2* mutations are prevalent due to consanguineous marriages. In Turkey autosomal recessive inheritance is responsible for 76.9 % of the studied cases [547].

In this study we did not found any significant difference in the severity and evolution of hearing impairment when comparing the 74 multiplex probands with 217 simplex probands.

The c.35delG variant (rs80338939) is responsible for approximately 70% of autosomal recessive NSHI and is the most common cause of hearing loss in Caucasian populations [165, 296]. The carrier rate is estimated to be the highest in Europe with a mean rate of 1.89% and a variation across countries with a higher rate of 2.48% in Southern Europe compared with 1.53% in Northern Europe [347]. This frequency was found also in hearing-impaired population from Hungary, Czech Republic, Poland and Austria, where c.35delG was prevalent [404, 448, 564, 10,42–44 - 23]. In Romania there are relatively few data about the frequency and audiological features of *GJB2* gene sequence variants [319].

Because c.35delG is the most frequent variant in the coding region of the *GJB2* gene, it has become the first intention genetic investigation for patients with non-syndromic hearing loss. In our study, the 35delG variant was present in 97/291 (33.3%) patients with different degrees of hearing impairment. The results are in accordance with previous reports in Romania and Central Europe [319, 481]. All patients with c.35delG variant were diagnosed by MLPA and confirmed with Sanger Sequencing of *GJB2* gene. The study revealed that subjects with 35delG in homozygous state present more severe hearing impairment, compared with the 35delG/non-35delG compound heterozygotes. The subjects with two non-35delG variants have an even less degree of hearing impairment. This observation is in accordance with other studies which conclude that c.35delG in homozygous state is associated with a higher risk for severe hearing impairment [144, 319, 320, 555, 10,45,47–49 - 23].

The next frequent variant was c.101T>C (rs35887622), accounting 19/291 (6.5%) patients. In Caucasian population the frequency of the c.101T>C variant is up to 6.5% [627] and was initially reported as a polymorphism. Different studies of *GJB2* have determined that the c.101T>C variant is more frequent in individuals with mid-west American, UK and German [627] origins, in comparison with those with French, Spanish, Italian and Japanese origin. A possible explanation may be that these variants are found in an ancestral mutation event that occurred in UK or Ireland. More than 50% of our patients with c.101T>C variant had moderate to profound hearing impairment. Also, at the time of the diagnosis, the age of the patients with c.101T>C was greater than the age of the patients with c.35delG. The results of a large study on the UK population affirmed that this variant is associated with mild/moderate HI [217]. The

lower pathogenicity of the mutation that leads to later and milder manifestation of hearing impairment may sustain this finding. The majority of diagnosed cases with c.101T>C in our study were older than 18 years. One possible explanation can be that adults with mild forms of HI may not pursue audiology or genetic investigations. The progression of HI was found in few cases, because it was slow and long-term follow-up information was not possible. The incomplete penetrance of c.101T>C variant was not confirmed because the study included only subjects with HI. The phenotype of the patients with hearing impairment was variable: the individuals with biallelic c.101T>C and c.35delG had moderate to profound hearing loss and the heterozygous c.101T>C had mild to moderate forms of hearing loss. No individuals with c.101T>C in homozygous state were found.

The c.313_326del14 variant (rs111033253), called in the past c.310del14, c.312del14 or c.314del14, truncates the *GJB2* gene and disrupts the integrity of connexons. In many European populations, this variant has been identified previously with a variable frequency 0.47-28.3% [368]. The c.313_326del14 variant in our group was 8/291 patients (2.75 % of pathogenic alleles). 6 patients were compound heterozygous with moderate to severe hearing impairment and the age of diagnosis being under 18 years and 2 patients had c.313-326del14 in heterozygous state with mild to moderate hearing impairment.

The c.71G>A variant is the fourth most common in our study and it had over five times lower frequency than the c.35delG variant. This mutation, previously called W24X, was first described in a Pakistani family [322] and later on was also discovered in several Asian families [41, 125, 212, 309, 455, 501]. This mutation is prevalent also in Roma population with autosomal recessive NSHI [371]. In this study, it was found only in Roma patients: 15 individuals of 291 (15.5%) had this variant: 8 were compound heterozygous with c.35delG and 7 were in heterozygous state. The c.71G>A frequency in different Roma subgroups is variable: it ranges from 0.0% to 26.1% in Slovak subgroups [60 - 23] and up to 4.0% in Spanish subgroups [66]. This finding is a result of the social structure of the Roma people, they being a conglomerate of genetically isolated founder populations, with a high degree of consanguinity [61 - 23]. Our data is not concordant with other Central European series or the study from North-Western Romania, where 35delG and c.71G>A were the most common mutations [46 - 23]. We can explain this fact by problems to access health care, from financial constraints, mobility issues or simply because they do not speak Romanian language. The findings in Roma population confirm the ethnic origin of this mutation.

Another result determined in the present study was the presence of c.-23+1G>A, rs80338940 formerly called IVS1+1G>A, which is a splice site mutation found in exon 1 and intron 1 of *GJB2* gene in patients with hearing impairment. The mutation is present in compound heterozygous and allele frequency was determined as 1% [62, 135, 137]. In our study, this variant was found in 4/291 patients (1.37%) in association with c.35delG variant and the subjects had severe forms of HI. Previous studies showed that such association determines moderate HI [119] and profound HI [123]. To date, to our knowledge, homozygotes for the c.−23+1G>A variant have not been reported.

In 6 patients of 291 included in the study were identified two variants: c.109G>A and c.100A>T that have not been reported in any study from Romania. Out of these, 3 had c.109G>A variant and presented the same pattern of HI (progressive, bilateral and profound to severe) while the other 3 had c.100A>T (non-progressive, moderate, bilateral hearing impairment).

Our results contribute to define the mutation spectrum in the Romanian individuals with hearing impairment. Despite the genetic heterogeneity of NSHI, 217 patients were diagnosed out of a cohort of 291 patients. MLPA confirmed the genetic diagnosis in 141 cases (48.45%). We selected for further study the patients to which the *GJB2* mutations did not explain their hearing impairment and the patients with variants in *WFS1* gene.

Regarding our second aim of the study, we concluded that MLPA can be used as first intention genetic test for patients with HI due to some advantages over the Sanger Sequencing method: it is time saving, has a low price for consumables, the initial investment is lower for the platform than for Sanger Sequencing, the interpretation is much faster and it could easily detect the number copies variation and most frequent pathogenic variants [236, 580].

This is the first report of the utility of MLPA and Sanger sequencing of HI in Romania; the results show notable findings in comparison to other European populations. However, some limitations should be noted: the samples included in this study are not truly representative for the entire Romania as all samples were collected from individuals with different degrees of hearing impairment, born in North-Eastern Romania, we did not have access to all of the parental samples to confirm compound heterozygosity. Our results need further studies on larger patient groups, especially Roma-population, in order to estimate the real incidence of the disease and to make more accurate predictions about the genotype phenotype correlation in our population.

The genetic diagnosis in hearing impairment is important for many reasons: allows us to determine the etiology of deafness, offers the possibility to provide genetic counseling and prenatal diagnosis and not at least, based on the genotype-phenotype correlation provides prognostic information and facilitates an adequate management.

II.2.4.5. CONCLUSIONS

In this study, 217 patients had pathogenic/likely pathogenic variants, 141 being confirmed by MLPA. We identified two variants: c.109G>A and c.100A>T that have not being reported in any study from Romania. The most common variant in our study is c.35delG followed by c.101T>C, c.313_326del14 and c.71G>A.

All of the patients had been confirmed with Sanger Sequencing, proving that MLPA can be a cost-effective diagnosis method, useful for every patient with hearing impairment. MLPA is an inexpensive, rapid and reliable technique that could help as first intention genetic test for every individual with NSHI. Moreover, it can be adaptable for the mutation spectrum in every population and can be followed by Sanger sequencing for *GJB2* gene in cases of normal results.

II.3. MEDICINE OF REPRODUCTION

The researches concerning medicine of reproduction were published in the following papers
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II.3.1. INTRODUCTION

Reproductive disorders (RD) are an important component of human diseases, affecting 10-15% of couples of reproductive age. Primary sterility (SP) is generated by the inability of an individual to form gametes or the inability of a couple to conceive. Frequently, SP is the result of a gonadal dysgenesis (GD), a complex disease with a variety of clinical, cytogenetic and histological features. GD is caused by a primary defect in the gonad formation, which is manifested by primary gonadal failure or hypergonadotropic hypogonadism, characterized by a decreased function which may impair production of gametes and sex hormones. The most frequent causes of GD are the gonosomal aneuploidy: X monosomy and XXY trisomy [33]. Infertility occurs in conditions where the pregnancies end with recurrent miscarriages (ASR) or the birth of a child with multiple congenital anomalies. According to the American Society for Reproductive Medicine [2], the RD affects both sexes equally and is produced by different causes: masculine (25%) feminine (49%) or no known cause (26%). The RD may be caused by endocrine, anatomic, infectious, immunological or genetic factors [207]. The genetic factors produce 50-60% of the early ASR in the first quarter of pregnancy, 1/3 of ASR in the second quarter of pregnancy and 5% of intrauterine deaths [3, 120, 204, 301, 595].

In the last decades, were developed differents techniques that allow the obtaining of pregnancies in couple with RD. All these foetuses are very precious and to analyse the healthy status require investigations in prenatal period. Such investigations are represented by screening methods or prenatal techniques.

Prenatal screening represents an ensemble of methods that allows the separation of embryos/foetuses in two categories: with high risk and with low risk. All these methods are not invasive and are focused to analyse some biochemical or genetic parameters in maternal blood or to evaluate the anatomical features of embryo/foetus by ultrasonographical examination.

Prenatal diagnosis is a complex medical act, allowing the detection of birth defects or genetic diseases during pregnancies of couples with genetic or malformative risk. The indications for performing prenatal chromosomal analysis are: advanced maternal age, positive biochemical screening, ultrasonographic signs of alarm, the existence of a previous child with a chromosomal disease, the presence of a parent with a balanced structural chromosomal anomaly. Techniques of prenatal diagnosis are invasive and require the collection of fetal cells. Tests should be performed early, and the results are required to be obtained quickly thus that pregnant women can opt for selective abortion in a period established by law. Classical methods of prenatal chromosomal analysis involve a cell culture that need 2-3 weeks. An alternative method for studying fetal and embionic cells is the FISH technique, which allows a quickly result [205].

II.3.2. STUDY OF REPRODUCTIVE DISORDERS

II.3.2.1. INTRODUCTION

Human reproductive disorder (RD) is characterized by the inability of an individual / couple to conceive naturally (primary sterility) or the inability to maintain a pregnancy or give birth to a healthy baby (infertility) [348, 488]. It is represented by primary sterility (ST) or infertility - spontaneous abortions (AS), recurrent miscarriages (ASR) (≥ 2 AS) or birth of dead babies (NM) [272, 488, 623]. RD has multiple causes: genetic, malformative, endocrine,

immunological and environmental [348, 448]. RD can affect both partners of the couple with 25% male causes, 27% ovulatory causes, 31% uterine/fallopian causes, and 17% idiopathic causes [448]. Genetic causes are the chromosomal and, more rarely, monogenic or multifactorial [24]. About 50-60% of early spontaneous abortions (RSA) are determined by a aneuplody of fetus. Chromosomal etiology is implied in 1/3 of ASR in the second quarter of pregnancy and only 5% of newborn deaths in the third quarter of pregnancy [110, 120, 301]. In 5% of recurrent abortions, were identified segmental aneuploidies, which mostly were the result of a malsegregation of derivatives chromosomes, when a balanced structural chromosomal anomaly (ACSE) was present in one of the parents [179, 620].

II.3.2.2. MATERIAL AND METHOD

In the period 1998 - 2009 in Genetic Center Iaşi, we evaluated 266 couples with reproductive disorders: 80 cases (30.07%) with primary infertility (ST), 149 cases (56.01%) with recurrent spontaneous abortions (RSA) and 37 cases (13.90%) with neonatal deaths (NM). In each couple with RD was performed chromosomal analysis using G banding technique, after a short culture of stimulated T lymphocytes performed according to the Moorhead technique (1960) [374], adapted in our laboratory. Metaphases were examined with a Nikon microscope, and images were captured with automatic Cytovision Karyotyping software (Applied Imaging). For each case were analyzed at least 16 metaphases. In cases where we detected more than one anomaly, the number of analyzed cells was increased to 32, 64 or even 96.

II.3.2.3. RESULTS

The karyotype revealed the presence of chromosomal abnormalities in 20 cases (7.51%) in couples with ST, 13 cases (4.88%) in couples with ASR and 10 cases (3.75%) in couples with NM. In 23 cases (8.64%) the anomaly was detected in women, and in 20 cases (7.51% in men (table II.18.).

The most common abnormalities were balanced structural chromosomal abnormalities, presented in 23 couples (8.64%), followed by numerical or structural anomalies of chromosome X, identified in 18 couples (6.76%), most of them being represented by X monosomy mosaic in female and XXY trisomy in men. In other 2 cases we detected a karyotype 46,XX in male and in 3 couples presented a polymorphic chromosomal variant: 9qh + (2 cases) and 1qh + (1 case).

Table II.18. Types of chromosomal anomalies

		Woman	**Man**
Chromosomal anomaly	**Number (%)**	**Number (%)**	**Number (%)**
Gonosomal aneuploidies	**14 (5.26)**	7 (2.63))	7 (2.63)
Balanced structural anomalies	**23 (8.64)**	10 (3.75)	13 (4.88)
- Robertsonian translocations	**3 (1.12)**	3 (1.12)	**-**
- reciprocal translocations	**2 (0.75)**	1 (0.37)	1 (0.37)
- insertions	**3 (1.12)**	1 (0.37)	2 (0.75)
- inversions	**15 (5.63)**	5 (1.87)	10 (3.75)
Gonosomal structural anomalies	**4 (1.50)**	4 (1.50)	-
Other anomalies	**2 (0.75)**	-	2 (0.75)
Total anomalies	**43 (16.16)**	**21 (7.89)**	**22 (8.27)**
Normal polymorphic variants	**3 (1.12)**	**1 (0.37)**	**2 (0.75)**

In 20 couples with infertility (25% from couples with ST) we detected a chromosomal anomaly, their frequency being almost twice as high in men (13 cases) than women (7 cases). The most common anomalies detected in this case were numerical structural anomalies of

chromosome X (12 cases), followed by inversions (6 cases). In 13 (8.72%) of the 149 couples with recurrent spontaneous abortions we detected chromosomal anomalies, 8 of which being present in women. The most common anomalies were inversions (6 cases) and monosomy X in mosaic (4 cases). In couples with stillborn we found 10 cases with chromosomal anomalies (27.02%), the most common being ACSE (8 cases), followed by gonosomal aneuploidies (2 cases).

II.3.2.4. DISCUSSION

In our group of couples with RD we found more chromosomal anomalies (16.16%) compared with other similar studies: 8.9% - Pal [415] 9.04% - Nazmy [385] 5.53% - Espinoza et al [158] and 1.97% Clementine et al [107] probably due to a rigorous selection made before cytogenetic investigation. Overall, we found more ACSE (8.64%) compared with the numerical and structural abnormalities of the X chromosome (6.76%), but aneuploidy and structural abnormalities of the X chromosome have been the main cause of sterility, while ACSE affected frequently couples with ASR and NM. Our data is consistent with the data from literature [488]. The chromosomal anomalies were present in similar proportions in women and men (48.83% vs. 51.16%) and these findings are consistent with literature data [107, 158].

Inversions (15 cases) were the most common balanced structural chromosomal anomalies, followed by translocation (5 cases). The most common inversion implied chromosome 9 (7 cases), with a frequency similar to that found in the general population [488]. Inversion of chromosome 9 was seen in all three categories of couples with RD, both in women (4 cases) and men (5 cases). Involvement of pericentric inversion of chromosome 9 in RD is controversial and usually they are considered normal variants. Heterochromatin from pericentromeric region of short arm (9p12) and long arm (9q13-21.1) of chromosome 9 presents homology, thus in this region chromosomal rearrangements [529] are produced frequently. In these regions were identified segmental duplications and the two chromosomal regions contained 97% identical sequences, which favoured aberrant chromosomal recombinations [252].

The most common translocations were Robertsonian translocations (3 cases), all identified in women. These results were concordant with those of Espinoza, et al. [158] or Orozco Quinono et al [408].

II.3.2.5. CONCLUSIONS

The results of our retrospective study are consistent with the literature, and highlight the role of chromosomal anomalies in human RD, emphasizing the importance of chromosomal analysis for etiologic diagnosis, management and genetic counseling for such couples.

II.3.3. STUDY OF AMENORRHEA

II.3.3.1. BACKGROUND

Amenorrhea (A) is the absence or abnormal cessation of the menses. It can be classified as primary amenorrhea (PA) – absences of menses after age of 16 years - or secondary amenorrhea (SA) – in which the menses appeared at puberty but subsequently ceased [488]. The incidence of PA in the United States is less than 1% and the prevalence of SA is 5-7% [8]. The main causes of PA are: pituitary / hypothalamic disorders (27.8 %); gonadal /ovarian disorders (50.4%); outflow tract abnormalities (21.8 %) [488]. In gonadal /ovarian disorders anomalies of sex chromosomes are the main cause. Such disorders are Turner Syndrome, X trisomy, pure 46,XX gonadal dysgenesis, Swyer syndrome, complete androgen insensitivity syndrome (CAIS) or mixed gonadal dysgenesis (MGD). In majority of cases, SA is not produced by chromosomal abnormalities [8].

II.3.3.2. MATERIAL AND METHOD

In period 1985-2009 we performed a clinical, paraclinic and cytogenetic evaluation (by X chromatin test and karyotype) of 531 patients with PA and early SA (5 to 10 years after menarche). The age of petients ranged between 13 and 45 years; 493 patients (92,84%) had PA and 38 patients (7,15%) had SA**.**

The clinical examination included anthropometric measurements, evaluation of pubertal development and evaluation of presence of typical features of Turner syndrome.

The cytogenetic investigations were the *X -chromatin test* and the *chromosomal analysis*. The *X - chromatin* was analyzed on buccal mucosa smear, fixed in a mixture of absolute ethanol and glacial acetic acid (3:1), and stained with a carbol-fuchsin solution. In every case we analyzed 300 cells. The test was considered abnormal: in patients with absent Barr body (negative test); in cases with low percentage (<10%) of Barr bodies and in patients with abnormal number or size of Barr bodies [206]. *Chromosomal analysis* was based on a short-term culture of activated T-lymphocytes stimulated with phytohemagglutinin. The slides were stained and examinated on an optical microscope, direct or after aplication of G- banding. The metaphases were analyzed with a Nikon microscope and imagines were captured with an automated image analysis system (Cytovision, Applied Imaging). For each case a minimum of 32 metaphases were analyzed. If we detected more than 2 cell lines, the number of analyzed cell was increased to 64 or 96.

II.3.3.3. RESULTS

In the sample group of 493 (92.84%) patients with PA the results of X chromatin test was abnormal in 201 cases (40.8%) [Barr test negative - 154; positive <10% - 21; 2 Barr bodies - 16; Barr body > 1 μm - 10] and normal (positive >20%) in 292 cases (59.2%). The karyotype in patients with PA was abnormal in 269 cases (54.56%) and normal in 224 cases (45.43%).

The most frequent anomalies discovered in patients with PA (table II.19.) were X chromosome homogeneous monosomy - 45,X (137 cases – 27.78%) and different forms of X chromosome monosomy mosaicism (105 cases -21.29%).

The frequency of X chromosome homogeneous monosomy in cases of patients with PA was statistically significant than frequency of X chromosome monosomy mosaicism and X chromosome structural abnormalities (χ^2 (2,269) = 71.40: p < 0, 01; p< 0, 05). In 80 cases (16.22%) we discovered a mosaic with 45,X cell line. Other 19 cases (3.85%) with TS presented a mosaicism with 1 cell line with X monosomy and the second line with an unbalanced structural abnormality of X chromosome: the most frequent abnormality detected was isochromosomes Xq (10 cases), followed by ring X chromosomes (7 cases) and Xq deletions (2 cases) (figures II.29-II.32.).

In 3 cases (2 with i(Xq) and 1 with del(Xp)) we identified a homogeneous unbalanced structural abnormality of X chromosome.

In 23 patients with PA we found a cell line with normal / abnormal Y chromosome. The karyotype result was 46,XY in 14 cases, wich had the following clinical diagnosis: pure gonadal dysgenesis (8 cases) and CAIS (6 cases). In other 9 cases with with Y chromosome material the diagnosis was: MGD (4 cases) and hermaphroditism (5 cases).

In 4 cases we detected a X trisomy (47,XXX or 46,XX/47,XXX) and in 2 cases we identified a balanced structural chromosomal abnormality: 46,XX,ins(1;X)(q21.1;q11.3-21.2) and 46,XX, ins(4;6)(q31;q33). One patient with PA had an unbalanced structural chromosomal abnormality: 46,XX,add(1)(q21), the origin of supplementary chromosome 1 band could not been identified.

In patients with SA (38 cases – 7.15%) the X chromatin test was normal in 31 of cases and abnormal (positive, but with low percentage of Barr body, or two Barr bodies) in 7 cases.

Table II.19. Chromosomal abnormalities in patients with primary amenorrhea

Type of chromosomal abnormalities	X Chromatin*	Diagnosis	No. of cases	Frequency % *****
X chromosome homogenous monosomy			137	27,78
45,X	negative	TS	136	27,58
45,X, inv(5)(p14;q11)**	negative	TS	1	0,20
X chromosome monosomy mosaicism			80	16,22
45,X/46,XX	positive<10% (21 cases) positive (45 cases)	TS	66	13,38
45,X/46,XX/47,XXX	positive-2B	TS	7	1,61
45,X/47,XXX	positive-2B	TS	6	1,21
45,X,inv(9)(p13;q21)/47,XXX,inv(9)(p13;q21)***	positive-2B	TS	1	0,20
X chromosome unbalanced structural abnormalities			3	0,60
46,X,i(Xq)	positive B>	TS	2	0,40
46,X,del(Xp)(p11.2-p11.4)	positive	TS	1	0,20
Aneuploid mosaics with X chromosome unbalanced structural abnormalities			19	3,85
45,X/46,X,i(Xq)	positive B>	TS	8	1,62
45,X/ 46,X,i (Xq)/46,XX	positive	TS	1	0,20
46,X,i(Xq)/46,XX	positive	TS	1	0,20
45,X/46,X,r(X)	positive	TS	3	0,60
45,X/46,XX/46,X,r(X)/ 47,XXX	positive	TS	2	0,40
46,X,r(X)/46,XX	positive	TS	1	0,20
45,X/45,X,+min/46,XX,+min/47,XX,r(X) ****	positive	TS	1	0,20
45,X/46,X,del(X)(q22→qter)	positive	TS	2	0,40
46,XY sex-reversal syndrome	negative	PGD/CAIS	14	2,83
Aneuploid mosaics with one cell line with Y chromosome			9	1,82
45,X/47,XXY	positive	MGD	1	0,20
45,X/46,XY	negative	MGD	2	0,40
45,X/46,X,dic(Y)	negative	MGD	1	0,20
45,X/46,XX/47,XXY	positive	TH	1	0,20
45,X/46,XX/46,XY	positive	TH	1	0,20
46,XX/46,XY	positive	TH	1	0,20
46,XX/47,XXY	positive	TH	2	0,40
Trisomy X			4	0,80
47,XXX	positive-2B	TXS	2	0,40
46,XX/47,XXX	positive	TXS	2	0,40
Balanced X-autosom translocations			1	
46,X,ins (1;X)(q21.1;q11.3→21.2)	positive	PA	1	0,20
Balanced autosomal structural abnormalities			1	0,20
46,XX,ins(4;6)(q31;q32)	positive	PA	1	0,20
Others			1	0,40
46,XX,add(1)(q21)	positive	PA	1	0,20
Total			269	54,56

TS - Turner Syndrome; PA - Primary Amenorrhea; MGD - Mixed Gonadal Dysgenesis; TH - True Hermaphroditism; PGD - Pure Gonadal Dysgenesis; TXS - Triple X Syndrome; CAIS – Complete Androgen Insensitivity Syndrome; B- Barr body; * X chromatin: abnormal = negative; positive <10%; 2B - two Barr bodies; B> - Barr body >1μm; ** It is possible that inv(5)(p14;q11 was inherited from one of the parents; *** inv(9)(p13;q21) is a polymorphic chromosome variant. **** The origin of minute chromosome could not be identified. ***** percentage was in relation to the total number of patients with PA (493 cases)

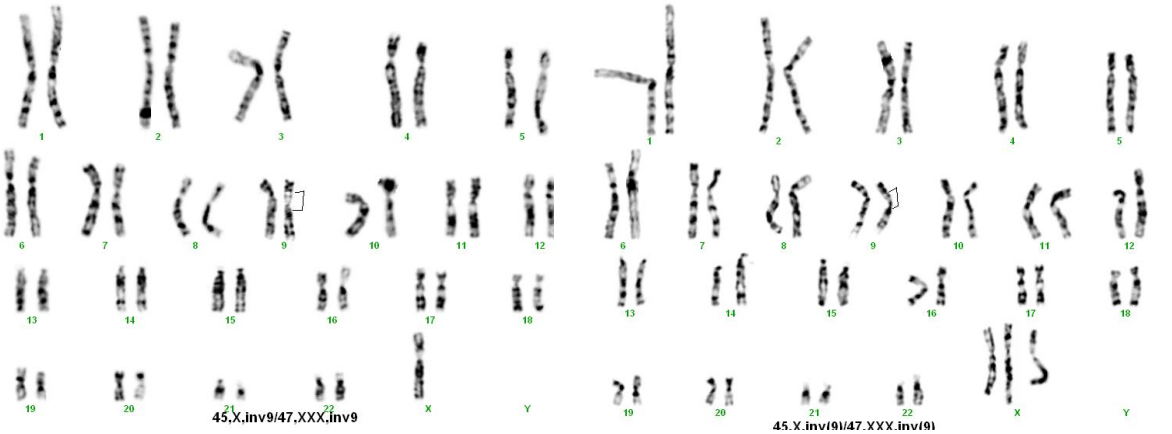


Figure. II.29. 45,X,inv(9)(p13;q12)/47,XXX, inv(9)(p13;q12)

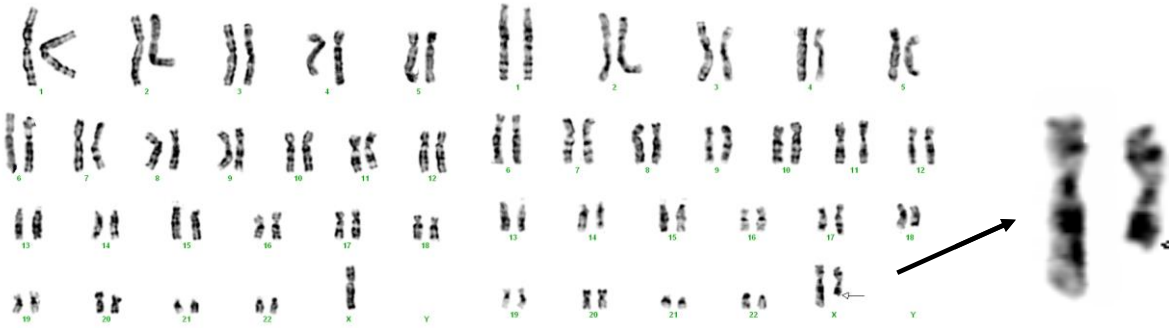


Figure II.30. 45,X/46,X,del (X)(q22→qter)

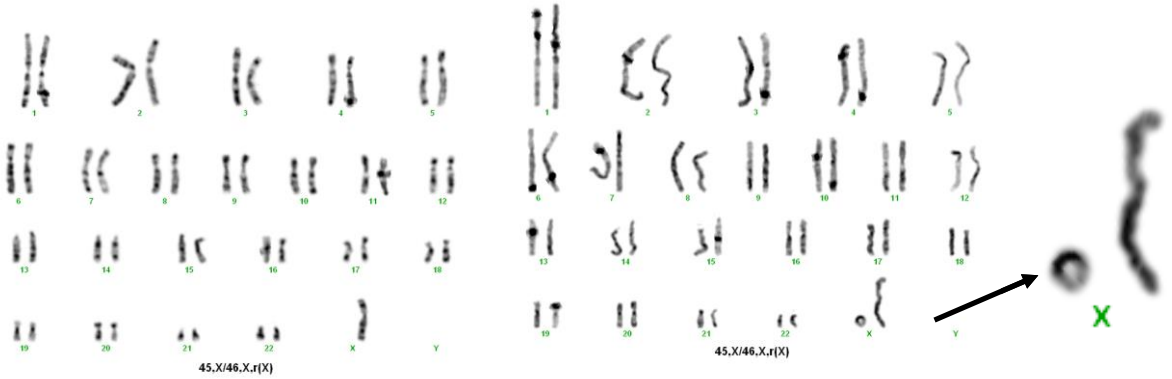


Figure II.31. 45,X/46,X,r(X)

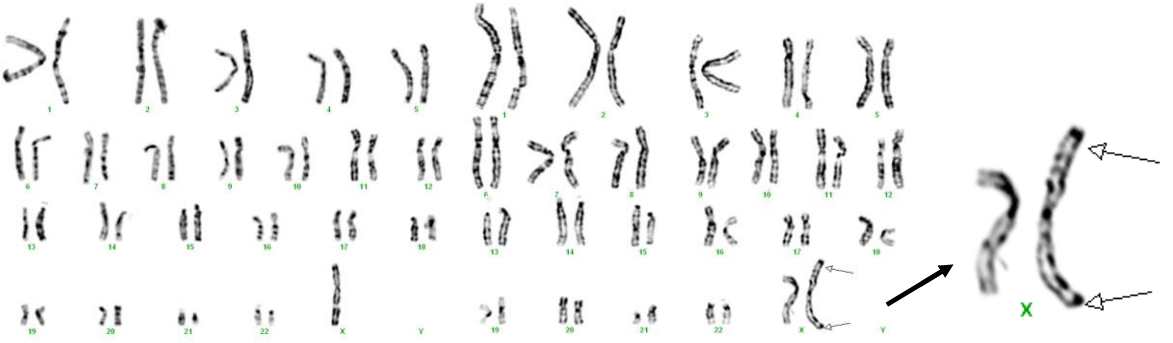


Figure II.32. 45,X/46,X,i(Xq)

Chromosomal analysis was normal (46,XX) in the majority of cases (31 patients), but in 7 cases with abnormal X chromatin test, we detected X monosomy mosaics: 45,X/46,XX (4 cases), 45,X/47,XXX (2 cases) and 45,X/46,XX/47,XXX (1 case). Thus, 6 patients with SA were diagnosed with Turner Syndrome and 1 patients (with 45,X/47,XXX karyotype) presented SA determined by premature ovarian failure (POF) (table II.20.).

Table II.20. The karyotype results in patients with Secondary Amenorrhea

Karyotype	X chromatin*	Diagnosis	No. of cases	Frequency %
46,XX	positive	SA	31	81,57 %
45,X/46,XX	positive <10%	TS	4	10,52%
45,X/47,XXX	positive – 2B	TS	1	2,63%
45,X/47,XXX	positive – 2B	SA/POF	1	2,63%
45,X/46,XX/47,XXX	positive - 2B	TS	1	0,35%

SA: Secondary Amenorrhea; TS: Turner Syndrome;POF: Premature Ovarian Failure; B: Barr body, * X chromatin: abnormal= negative or positive <10%; 2B - two Barr bodies.

Most patients with X chromosome homogenous monosomy, 45,X (137 cases -27.78%) presented clinical features of Turner Syndrome (short stature, primary amenorrhea, poor development of secondary sex characteristics, pterygium colli, cubitus valgus, craniofacial dysmorphism) and high plasma levels of FSH and LH (hypergonadotropic hypogonadism). In 25 cases (18.24%) we detected cardiac defects and 20 patients (14.59%) presented renal malformations. In cases with primary amenorrhea (PA) and mosaic or structural X chromosome abnormalities (108 cases – 21.90%), the clinical features of TS were present, but inconstant. The frequency of X chromosome homogeneous monosomy (27.78% -137 cases) in cases of patients with Turner syndrome was statistically more significant than the frequency of X chromosome monosomy mosaicism and X chromosome structural abnormalities ($\chi^2(2,245 = 8,31: p < 0,01; p< 0,05$).

The second frequent group of chromosomal abnormalities detected in patients with PA was the presence of Y chromosome (23 cases), but the clinical features were variable. We detected 14 patients with PA and 46,XY karyotype. Chromosomal analysis confirmed the clinical diagnosis of CAIS in 6 cases who presented female phenotype, the absence of the fallopian tubes, uterus and cervix, and the intraabdominal testis. In 8 cases with PA the patients had müllerian duct derivatives, sexual infantilism and low plasma testosterone levels. The diagnosis was Swyer syndrome.

Five patients with PA, presented a chromosomal mosaics with one of the cell lines containing a Y chromosome: 46,XX/47,XXY (2 cases), 46,XX/46,XY (1 case), 45,X/46,XX/47,XXY (1 case), 45,X/46,XX/46,XY (1 case). All these patients presented PA, clitoris hypertrophy, normal breast development, uterus, an ovotestis and a contralateral dysgenetic gonad (detected by laparoscopic gonad biopsy). The clinical diagnosis was: true hermaphroditism (TH). Four patients with PA and karyotypes 45,X/47,XXY (1 case), 45,X/46,XY (2 cases) and 45,X/46,X,dic(Y) (1 case) were diagnosed with mixed gonadal dysgenesis. They presented poor development of sex characteristics and variable TS stigmata: short stature, craniofacial dysmorphism, pterigyum colli, cubitus valgus and pigmented nevi. Ultrasound examination and exploratory laparoscopy showed a dysgenetic testis on one side and an ovarian tissue by the other side.

The last group of 7 patients with PA and chromosomal abnormalities presented different karyotypes, the association with PA being somewhat surprising. Four patients were diagnosed with triple X syndrome: 47,XXX (2 cases) and 46,XX/47,XXX (2 cases). They presented a nonspecific craniofacial dysmorphism, poor development of sex characteristics, short stature, and normal intellectual development. Other three patients with PA without any

special phenotypic features, presented structural chromosomal abnormalities*:* 46,XX,add(1)(q21), 46,XX,ins(4;6)(q31;q33) and 46,XX,ins(1;X)(q21.1;q11.3-21.2).

For investigation of cases with primary amenorrhea we propose an algorithm, presented in figure II.33.

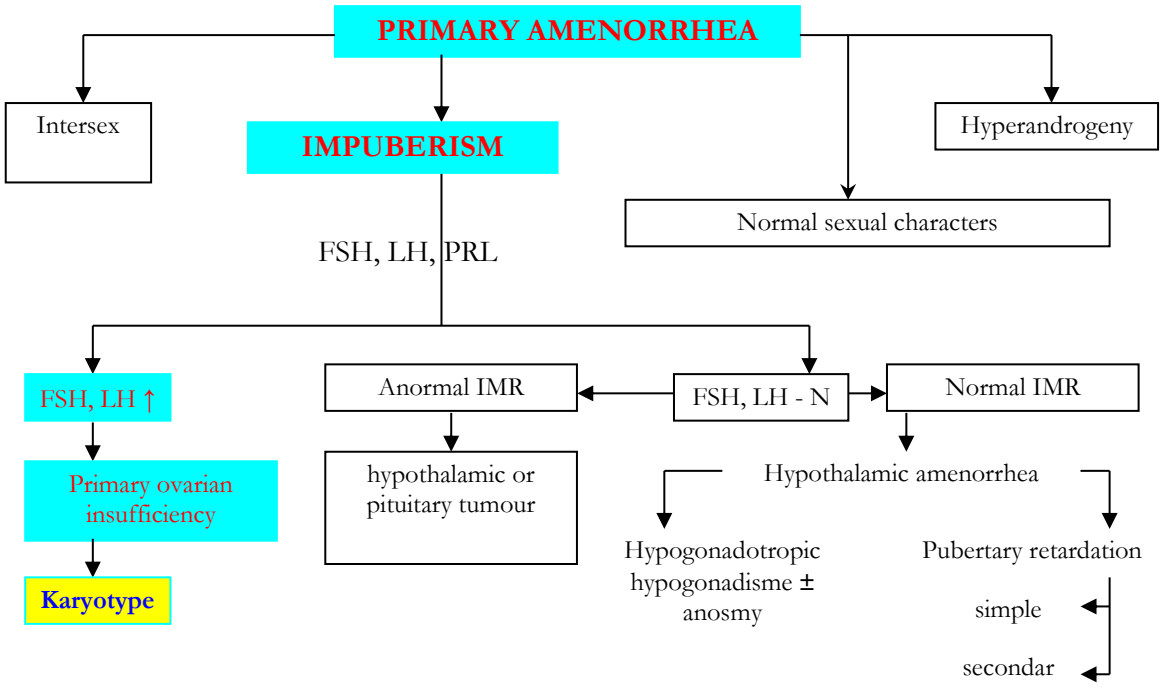


Figure II.33. Diagnostic algorithm in case of primary amenorrhea

In patients with *secondary amenorrhea* (38 cases), chromosomal analysis identified various chromosomal mosaics in 7 cases. Six cases presented clinical features of TS. In one case (karyotype 45,X/47,XXX) the patient presented a SA determined by POF. She presented: menarche at 16 years, irregular menstrual cycles, SA at 26 years, 161 cm, normal sex characteristics, uterus and ovaries with low dimensions, high plasma levels of FSH and LH. The presence of X monosomy generated secondary amenorrhea by premature ovarian follicular depletion.

II.3.3.4. DISCUSSION

In 201 cases with PA (40.8%) we identified a strong correlation between the results of Barr test and karyotype. The high percentage of chromosomal abnormalities (54.56%) detected in our patients with PA suggested the major role of chromosomal abnormalities in the abnormal gonadal development and function, as well as the importance of genetic investigation of patients with PA and SA. The frequency of abnormal karyotypes has been reported to vary between 15.9% and 63.3% among woman with primary amenorrhea [114, 608]. Our result (54.56%) was in accordance to the result obtained by Kong et al - 58.8% [295], and higher than other studies [275, 447, 474, 585, 608] (table II.21.).

We noticed the high frequency of chromosomal mosaics, with two or more cell lines, in cases with PA: 110/269 (40.9%) and SA: 7/31 (18.42%). The percentage of mosaicism ranged from 10% to 70% in diferrent studies [474]. Our result was similar to those of: Vijayalaksmi et al. – 51.2% [585] and Baros et al. – 33.8% [33]. The karyotype was normal in 224 cases with PA (45.43%), the absence of menarche having other etiology: hypothalamic/pituitary disorders or utero-vaginal malformations. In patients with SA the high frequency of normal karyotypes (81.57%) was expected, since SA was a frequent consequence

of the hypothalamic or pituitary disorders [488]. The percentage of chromosomal abnormalities reported for SA varies greatly, from 3.8% to 44.4% (table II.22.).

Table II.21. Chromosomal abnormalities in cases with primary amenorrhea in different studies

Karyotype results	**Present study**	**Wong et al [608]**	**Kong et al [295]**	**Vijayalaksmi et al [585]**	**Kalavathi et al. [275]**	**Ramirez et al [447]**	**Safaei et al [474]**
Frequency of abnormal karyotype (number of cases)	**54,56% (269)**	**24,5% (58)**	**58,8% (10)**	**27,8% (39)**	**25,82% (220)**	**36,7% (96)**	**20% (44)**
X chromosome (homogeneous/ mosaics) aneuploidies *	**82,15% (221)**	50% (29)	20% (2)	74% (29)	45,45% (100)	89,58% (92)	52,27% (23)
X chromosome unbalanced structural abnormalities	**8,17% (22)**	12,06% (7)	50% (5)	8,69% (3)	27,27% (60)	4,16% (4)	15,90% (7)
Marker chromosome	-	1,72% (1)	-	-	-	-	-
Mosaics X/XY and variants	**3,34% (9)**	1,72% (1)	-	-	3,63% (8)	-	4,54% (2)
46,XY	**5,20% (14)**	8,4% (20)	30% (3)	17,9% (7)	23,63% (52)	7,85% (3)	27,27% (12)
Other anomalies	**1,11% (3)**			-	7,23%	-	-

*were included 45, X and 47, XXX- -homogeneous and mosaics

Table II.22. Chromosomal abnormalities in patients with secondary amenorrhea in different studies

Karyotype results	**Present study**	**Wong et al [608]**	**Kalavathi et al [275]**	**Rajangam et al [446]**	**Ramirez et al [447]**	**Lin et al, [330]**	**Opitz et al, [407]**
No. of cases	**38**	312	127	245	30	18	15
46,XX karyotype	**31 (81,57%)**	281 (90,1%)	118 (92,91%)	215 (87,75%)	26 (86,7%)	10 (55,6%)	10 (66,7%)
Abnormal karyotype	**7 (18,42%)**	31 (9,9%)	9 (7,08%)	40 (16,32%)	4 (13,3%)	8 (44,4%)	5 (33,3%)
45,X	-	5 (1,6%)	1 (0,79%)	-	-	3 (16,6%)	-
X monosomy mosaic	**7 (18,42%)**	11 (3,5%)	8 (6,77%)	20 (8,32)	4 (13,3%)	2 (11,1%)	-
46, del (Xq)	-	6 (1,9%)	5 (4,23%)	-	1 (3,3%)	-	-
46,X,i(Xq)	-	1 (0,3%)	3	7 (2,85%)	1	-	-
t (X;A)	-	2 (0,6%)	-	-	2 (6,6%	1 (5,5%)	
47,XXX	-	3 (1,%)	1 (0,79%)	3 (7,5%)	-	2 (11,1%)	
46,XX/47,XXX	-	3 (1%)	-	10 (4,65%)	-		

We noticed that 239 cases with PA had a total or partial X chromosome monosomy: X homogeneous monosomy (57.32%); X monosomy mosaicism (33.47%); mosaic X structural unbalanced chromosomal abnormalities (7.94%) and homogeneous X chromosome structural abnormalities (1.25%).

In previous studies the most commonly-occurring karyotypes reported in Turner syndrome were: 45,X (45%), 45,X/46,XX (13%), 45,X/46,X,i(Xq) (8%), 46,X,i(Xq) (7%) and 45,X/46,XY (7%) [278]. Ramirez et al. reported similar results: X chromosome homogeneous monosomy (52.1%), X chromosome numerical mosaicism (45.74%) and X chromosome unbalanced structural abnormalities (4.25%) [447].

In cases of the X structural unbalanced chromosomal abnormalities there are discordant results: Kalavathi et al. – 27.27% [275] Wong and Lam – 12.06% [608], and Safaei et al. – 15.9% [474].

The PA was produced by presence of Y chromosome in 23 cases (8.55%). These patients presented: 46,XY pure gonadal dysgenesis (8 cases), CAIS (6 cases), true hermaphroditism (5 cases) and mixed gonadal dysgenesis (4 cases). Male karyotype and mosaics karyotypes having a cell line with Y chromosome were presented in a significant percentage of patients with PA in previous studies ranged from 3.3% to 13.7% [474]. Our results were similar to Vijayalaksmi et al, 2010 who detected 9 patients with Y chromosome material: 46,XY (7 cases), 45,X/46,XY (1 case) and 46,XX/46,XY (1 case) [585].

The clinical and cytogenetic diagnosis was Turner syndrome in the group of 245 patients (46.14% of all study patients) with PA (239 cases) and SA (6 cases). The frequency of cardiac abnormalities in Turner syndrome varies between 17 to 60% [541]. Similar data were found by: Proprawsky et al. – 21% [434], Korpal-Szczyrska et al. – 32.7% [300], Völkl et al. – 29.9% [588], Saenger - 45-65% [473]. In our patients we found cardiac abnormalities in 18.24% of cases that are inferior to those found by others. The frequency of renal abnormalities varies between 27-65% [56, 541]. Our study confirmed renal abnormalities in 14.59% of patients with Turner Syndrome, value lower than those reported in similar studies. A possible explanation of these features could be an incomplete evaluation of our patients or a population characteristic.

The cytogenetic evaluation revealed an X chromosome monosomy mosaicism or X chromosome structural abnormalities - homogeneous or in mosaics, in 108 patients (44.08%) with Turner Syndrome. These patients presented inconstant clinical features of TS, the chromosomal abnormalities were produced by postzygotic mitotic errors and the variable clinical features could be explained by absences of some genes located on Xp or Xq (Xq 13-q26) [603]. Based on these hypotheses the phenotype of patients with isochromosome (Xq) (Xp monosomy) can be correlated with short stature, sexual infantilism, gonadal dysgenesis and congenital malformations. The patients with chromosome X deletions involving Xq13-Xq26 region present primary amenorrhea determined by an ovarian dysgenesis [401, 603].

In patients with PA and Y chromosome (23 cases) is recommended prophylactic gonadectomy, because of the high risk (7% to 30%) of gonadoblastoma in the dysgenetic gonads [214, 541]. Our results underline the necessity of a good collaboration between different specialists and respecting of some standards and rigorous criteria of diagnosis.

In patients with SA we detected chromosomal abnormalities in 7 cases (18.42%) and our results were similar to other studies [300, 473, 541]. These findings are not surprising, because many SA cases are generated by the non chromosomal causes.

II.3.3.5. CONCLUSIONS

Our retrospective study confirms that sex chromosome abnormalities, numerical or structural, homogeneous or in mosaic, represents a major etiologic factor in primary or secondary amenorrhea (54.56% in cases of PA and 18.42% in cases of SA). The majority of patients presented ovarian dysgenesis and hypergonadotropic hypogonadism. Thus, we emphasize the importance of cytogenetic investigations in all patients with amenorrhea.

II.3.4. PRENATAL EVALUATION OF TRISOMY 21 BY QUANTIFICATION OF METHYLATED FOETAL DNA IN MATERNAL BLOOD

II.3.4.1. BACKGROUND

Down syndrome (DS) is a public health problem both by high frequency and by comorbidities and intellectual disability [202, 392]. Prevention of DS requires prenatal detection of the disease, by screening and prenatal diagnostic (PD). The screening identifies high-risk pregnancies by noninvasive methods, using biochemical and ultrasound parameters [392]. Prenatal diagnosis is based on genetic analysis of foetal cells obtained by invasive methods that generate risks for pregnant woman and foetus, like bleeding, miscarriage and foetal malformations [185, 543]. Prenatal chromosome analysis has 100% accuracy, allowing the detection of all chromosomal abnormalities in the resolution of optical microscopy. The major disadvantage is the long period required to complete cell culture [160]. The molecular cytogenetic tests reduced the time to achieve results within two days, but have the disadvantage of targeted detection of certain chromosomal regions. The FISH technique is applicable for the routine prenatal detection of aneuploidies of chromosome 13, 18, 21, X and Y. The method has a high specificity and sensitivity, but is expensive and time-consuming. QF-PCR and MLPA will produce rapid results at low cost, but are characterized by low sensitivity and specificity [160, 203].

Starting from these inconvenients in recent period were implemented noninvasive prenatal diagnostic methods (NPD) based on isolation of foetal cells or detection of free foetal DNA in maternal circulation [215].

Passage of foetal cells in maternal circulation is low, but the concentration methods, like magnetic cell sorting (MACS) or fluorescent activated cell sorting (FACS), could grow up the number of foetal cells, but also produce the lost of a large number of cells [51, 52, 215]. The amount of free plasma DNA in adult women is about 10-100 ng [57]. Free foetal DNA in maternal blood can be detected from 5 WA, after there is a steady increase during pregnancy to peak at the end of pregnancy, but not more than 3.4 to 6.2% of the total free circulating DNA [335]. Increased concentration of foetal DNA was identified in different obstetrical problems (preeclampsia, foetal-maternal hemorrhagia, polyhydramnios etc.) but also in trisomy 21 [318, 323, 336, 624, 625]. Cleavage of maternal DNA generated fragments longer than 201 bp. The foetal DNA is cleaved into short fragments, usually less than 193 bp [98].

Quantitative changes in DNA associated with foetal aneuploidies can be identified by indirect methods aimed at determining the allelic fraction and direct methods for molecular quantification [577]. Analysis of free foetal messenger RNA is targeted to genes expressed exclusively foetal as PLAC4 gene (located on chromosome 21) or MASPIN gene (located on chromosome 18) [102, 335].

Direct detection methods require expensive equipment and reagents, are laborious, require advanced statistical processing and have not been validated by extensive studies [335, 577]. Digital PCR accurately quantify very small amounts of DNA and allows detection of trisomy 21 by counting the number of target sequences on chromosome 21 compared to similar loci on other chromosomes, but is usefull in presence of more than 10,000 sequences DNA [215]. Massive parallel sequencing solves the problem of poverty of foetal DNA in maternal circulation, but has a low sensitivity and requires statistical methods for data processing [162].

Papageorgiou et al. (2009) focused on identifying localized regions on chromosome 21 that are hypermethylated in the placenta and hypomethylated in peripheral blood [419]. First they separated methylated and nonmethylated DNA, obtaining a methylated sample. For this sample was quantified the level of hypermethylation for several fetal/maternal differentially

methylated regions and the result relates to a reference value representing hypermethylation in pregnancies with normal fetuses [420].

II.3.4.2. MATERIAL AND METHOD

The study group consisted of 12 pregnant women investigated between January-November 2012 in Central Laboratory of Immunology and Genetics of University of Medicine and Pharmacy "Grigore T. Popa" Iaşi. The main reasons for prenatal diagnosis were: advanced maternal age and positive biochemical or ultrasound screening. All pregnant women agreed to participate in the study and signed an informed consent after providing genetic counseling, knowing the significance of prenatal diagnosis. In 7 cases we discovered a trisomy 21 confirmed by FISH or conventional chromosomal analysis (table II.23.).

Table II.23. The characteristics of patients included in study group

Sample	Gestational age - weeks	Motivation of performing prenatal diagnosis	Fetal status
PN 1	16	DTP	T21*
PN 2	18	DTP	T21*
PN 3	16	DTP	T21*
PN 4	11	nuchal fold > 3 mm	T21*
PN 5	16	AMA, pathological fetal ultrasound	T21*
PN 6	16	AMA, DTP, pathological fetal ultrasound	T21**
PN 7	17	pathological fetal ultrasound	N*
PN 8	19	AMA, TTP	N*
PN 9	16	AMA, DTP	N*
PN 10	16	DTP, pathological fetal ultrasound	N*
PN 11	16	AMA, pathological fetal ultrasound	T21*
PN 12	19	severe oligohydramnios	N*

DTP – patological double test, AMA – advanced maternal age, TTP –patological triple test; T21 –trisomy 21, N –euploid fetus; * confirmed by FISH; ** confirmed by fetal chromosome analysis

Working protocol, established by Papageorgiou et al. (2011) [420], has 6 stages: extraction of total DNA, DNA fragmentation, immunoprecipitation of methylated DNA, washing, isolation of DNA and qPCR for DNA immunoprecipitated. For each pregnant were collected on EDTA 2 ml of peripheral blood and DNA extraction from whole blood has been done up to 6 hours after blood sampling, using a kit QIamp DNA Blood Mini Kit ® (Qiagen, UK). The DNA was quantified, divided into samples, labeled and stored at -20 ° C in the collection of DNA in compliance with the quality and quantity of DNA evidence, traceability, anonymity, and the ergonomics.

The genomic DNA was fragmented by sonication at 40 Hz, using a sequence of 15 sonication and 15 s rest alternative for 10 minutes, using a MRC Scientific Instrument Ultrasonic Cleaner. The DNA sample was diluted in GenDNA TE to reach 0.1 µg/µl, to get a final volume of 300 µl of DNA (30 µg). Verification was done by migration in Agarose gel using a 50 bp ladder. Because there was not complete fragmentation of all samples, the procedure was repeated, DNA fragments containing 150-500 bp. Methylated fetal DNA extraction was done by using magnetic beads and 5-methylcytosine antibody extraction according to the protocol - MagMeDIP (*Magnetic Methylated DNA Immunoprecipitation kit* - Diagenode®). The DNA from each fetus was divided into three samples: two used for immunoprecipitation (IP) and one considered as evidence input (input - IMP 10% of the sample). Each IP contained 1 µg of DNA. For immunoprecipitation was carried out a mixture of 75 µl DNA, 5µl mixture of 20 µl of diluted antibody of magnets (100 µl per reaction) which was stirred magnetically at 4°C for 12 hours. Washing was carried out at cold, in magnetic stirrer in four successive stages. At the end of washing, centrifugation was performed by keeping the sediment.

After that we isolated immunoprecipitated DNA and sample input. To IP samples added 100 µl of buffer DIB with proteinase K 1%. To input sample added 92.5 µl buffer DIB to 7.5 µl of DNA. The samples IP and input were incubated 15 minutes at -55°C, then another 15 minutes at 100°C, followed by rapid stirring 5 minutes, centrifugation at 4°C with 14,000 rpm and the supernatant was finally transferred to labeled tubes, stored at -20°C, representing prepared DNA for qPCR.

To achieve qPCR analysis of immunoprecipitated DNA we used a kit containing four pairs of specific primers validated for four types of DNA: methylated control DNA (primer pair 1) unmethylated control DNA (primer pair 2), human DNA methylated (*TSH2B*) and human DNA unmethylated (*GAPDH* promoter). DNA samples was diluted in 25 µl solution containing: 1µl of primer pair (Forward and Reverse) 12.5 µl SYBR Green PCR master mix, 5 µl DNA sample and 6.5 µl water. PCR was carried out in the following sequence of steps: amplification at 95^0C for 7 minutes (one cycle), amplification at 95^0C for 15 s (40 cycles), amplification for 60 seconds at 60^0C (40 cycles), amplification at 95^0C for 1 min (one cycle). After amplification was done 60 successive cycles of melting of 1 minute each, with gradual increase of temperature by 0.5^0C, the first cycle at 65^0C. The analysis of efficiency for immunoprecipitation was done by calculating the ΔCT between IP and input for all four types of DNA: methylated DNA, unmethylated DNA, *TSH2B* and *GAPDH* promoter (figure II.34., table II.24.).

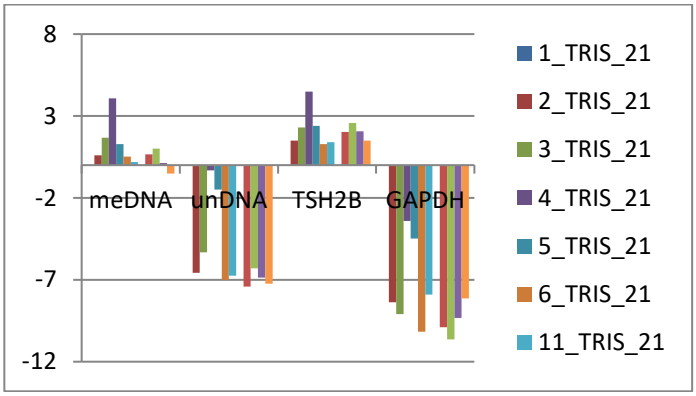


Figure II.34. The results of analysis of imunoprecipitation efficiency by calculation of ΔCT between IP and input for methylated DNA, unmethylated DNA, *TSH2B* (methylated human DNA), and *GAPDH* promoter (human unmethylated DNA)

Table II.24. The results of imunoprecipitation efficiency for different types of DNA

Probe	Type of pregnancy	ΔCT for methylated DNA	ΔCT for unmethylated DNA	ΔCT for *TSH2B* (methylated human DNA)	ΔCT for *GAPDH* promoter (human unmethylated DNA)
1	T21	-	-	-	-
2	T21	0.59	-6.58	1.5	-8.37
3	T21	1.67	-5.33	2.3	-9.09
4	T21	4.07	-0.31	4.48	-3.41
5	T21	1.29	-1.49	2.39	-4.49
6	T21	0.52	-7.01	1.29	-10.17
7	N	-	-	-	-
8	N	0.66	-7.41	2.02	-9.89
9	N	1	-6.3	2.57	-10.64
10	N	0.13	-6.87	2.07	-9.33
11	T21	0.18	-6.74	1.4	-7.9
12	N	-0.52	-7.23	1.5	-8.14

T21 – trisomy 21; N – normal pregnancy

Analysis of imunoprecipitation efficiency for a given locus was calculated using the following formula:

% (meDNA-IP/ Total input) = 2^[(CT(10%input) - 3.32) - CT(meDNA-IP)]x 100%

where 2 is the AE (amplification efficiency), CT (meDNA-IP) and CT (10% input) are threshold values obtained from exponential phase of qPCR for the methyl DNA sample and input sample respectively; the compensatory factor (3.32) is used to take into account the dilution 1:10 of the input.

To achieve qPCR for highlighting regions on chromosome 21 in the fetal methylated DNA were used eight pairs of specific primers for chromosome 21, primers for hypermethylated region on chromosome 13 and hypomethylated region on chromosome 22, according Papageorgiou et al. [420]. For EP4 and EP6 primers was used a concentration of 900nM, for EP5 primer was used a concentration of 450nM, for EP7 primer was used at a concentration of 750nM, while the other primer's concentrations were 300nM.

The reaction for PCR was carried out using a device PalmCyclerTM (Corbett, USA) and the parameters of amplification were: pre-incubation (95°C/7 min), followed by 40 cycles of amplification (95°C/15 sec with ramp rate of 4.4°/sec and 60°/1 min with ramp rate 2.2°/sec; fluorescent acquisition was done at 465-510), melting (95°C/15 sec with ramp rate 4.4°/sec, 65°/1 min with ramp rate 1º/sec, fluorescence acquisition was made at 465-510), cool (40°/30 sec, ramp rate of 2.2°/ sec).

The amplification program used a temperature of 60°C for hybridization for all mixtures. In the mix of EP 4 at this temperature were obtained primer dimer thereby making it difficult to interpret. For mix EP4, application of a hybridization temperature of 64ºC eliminated the nonspecific products, so for all mixes we got a single melting peak, which allowed the comparison samples. Samples 1 and 7 were not considered because was not obtained amplification for Input nor controls or for sets of primers used for the analysis of differentially methylated regions on chromosome 21.

II.3.4.3. RESULTS

For assessment of the results were calculated the following parameters: ΔCT for normal and trisomic pregnancies, normalized ΔCT for normal and trisomic pregnancies, the ratio value per sample and ratio value per DMR, and the amount of discrimination D. For this we use the formulas:

$$\Delta CT^{PB\ Normal} = CT^{PB\ Normal\ IMP} - CT^{PB\ Normal\ IP}$$

$$\Delta CT^{PB\ T21} = CT^{PB\ T21\ IMP} - CT^{PB\ T21\ IP}.$$

(where PB=Peripheral Blood, T21= Trisomy 21, IMP – Input; IP= Immunoprecipitated)

$$\text{Norm } \Delta CT \text{ value}^{\ PB\ Normal} = E^{\ \Delta CTPB\ Normal}$$

(where Norm= Normalized)

$$\text{Norm } \Delta CT \text{ value}^{\ PB\ T21} = E^{\ \Delta CT\ PB\ T21},$$

(where $E = 10^{[-1/slope]}$ = efficiency of the primer)

$$\text{Ratio Value}^{\ Sample;\ DMR} = \text{Norm } \Delta CT^{PB\ Sample\ (Normal\ or\ T21)} / \text{Median } (\text{Norm } \Delta CT^{PB\ Normal})$$

$$D = -6{,}331 + 0{,}959\ X_{EP4} + 1{,}188\ X_{EP5} + 0{,}424\ X_{EP6} + 0{,}621\ X_{EP7} + 0{,}028\ X_{EP8} + 0{,}387\ X_{EP10} - 0{,}683\ X_{EP11} + 0{,}897\ X_{EP12},$$

(where X_{EPi}= fraction value for the 8 markers used: 4, 5, 6, 7, 8, 10, 11 şi 12).

Applying the above formulas we obtained the results shown in tables II.25., II.26. and II.27.

Table II.25. The values of ΔCT for markers of chromosome 21

		ΔCT							
Probe	Pregnacy	EP4	EP5	EP6	EP7	EP8	EP10	EP11	EP12
1	T21	-	-	-	-	-	-	-	-
2	T21	-2.7	-3.2	-7.1	-7	-5	-4.3	-7.6	-7
3	T21	-2.8	2.7-	-8.7	-8.3	-4.1	-5.1	-7.5	-7.1
4	T21	-1.8	-2.1	-7	-3.1	-3.6	-2.2	-6.7	-5.4
5	T21	-3.5	-3.7	-7.9	-7.5	-4.6	-4.6	-8.8	-5.4
6	T21	-4	-4.1	-9.8	-7.3	-4.5	-5.1	-7.8	-7.4
7	N	-	-	-	-	-	-	-	-
8	N	-4.6	-4.7	-9.1	-5.2	-5.2	-3.6	-7.6	-6.4
9	N	-4.3	-4.1	-8.5	-6.2	-4.7	-4.1	-8.2	-6.1
10	N	-5.2	-4.7	-8.1	-3.5	-7.3	-5.4	-7.7	-7.3
11	T21	-3	-2.5	-9.3	-7.8	-7.1	-5.8	-7.7	-7.9
12	N	-4.5	-4.2	-8.4	-5.1	-6.5	-5.1	-4.9	-6.5

T21 – trisomy 21; N – normal

Table II.26. The values of normalized ΔCT and for median of normalized ΔCT for markers of chromosome 21

		Norm ΔCT							
Probe	Pregnacy	EP4	EP5	EP6	EP7	EP8	EP10	EP11	EP12
1	T21	-	-	-	-	-	-	-	-
2	T21	0.15389	0.10881	0.00728	0.00781	0.03125	0.05076	0.00515	0.00781
3	T21	0.14358	0.15389	0.00240	0.00317	0.05831	0.02915	0.00552	0.00728
4	T21	0.287174	0.23325	0.00781	0.11662	0.08246	0.21763	0.00961	0.02368
5	T21	0.08838	0.07694	0.00418	0.00552	0.04123	0.04123	0.00224	0.02368
6	T21	0.0625	0.05831	0.00112	0.00634	0.04419	0.02915	0.00448	0.00592
7	N	-	-	-	-	-	-	-	-
8	N	0.04123	0.03847	0.00182	0.02720	0.02720	0.08246	0.00515	0.01184
9	N	0.05076	0.05831	0.00276	0.01360	0.03847	0.05831	0.00340	0.01457
10	N	0.02720	0.03847	0.00364	0.08838	0.00634	0.02368	0.00480	0.00634
11	T21	0.125	0.17677	0.00158	0.00448	0.00728	0.01794	0.00592	0.00418
12	N	0.04419	0.0544	0.00296	0.02915	0.01104	0.02915	0.03349	0.01104
Median for normalized ΔCT									
		0.042714	0.06464	0.00286	0.02818	0.01912	0.04373	0.00498	0.01144

T21 – trisomy 21; N – normal

For better highlight the results we achieved the figure II.35., which reflects the graphical representation of value D for the 10 cases analysed.

All pregnancies without trisomy 21 were correctly identified by obtaining a value of negative discrimination, the value of D ranging between -1.74 and -6.32. In five of the six samples with trisomy 21 considered in the final analysis, we discovered a correlation between the presence of trisomy and positive value of factor D: sample 2 (D = 1.56); sample 3 (D = 1.41); sample 4 (D = 12.40); sample 5 (D = 0.34), sample 11 (D = 1.02).

The only case in which we found negative values of factor D was sample 6 (D = -2.96), which was inconsistent with the presence of trisomy.

II.3.4.4. DISCUTION

The results confirmed the high specificity of this new method for testing the presence of 21 trisomy by a non-invasive diagnosis. Thus, we obtained a good correlation between the results of analysis of methylation of free foetal DNA from maternal blood and the results of

prenatal diagnosis. In all pregnancies with normal foetus, we obtained a negative value for discrimination factor D, concordant with presence of a pair of chromosomes 21. On the other hand, the analysis of methylation of foetal DNA showed positive values for D factor in 5 from 6 probes from pregnancies with trisomy 21. These results are concordant with an excess of hypermethylated foetal DNA, characteristic to aneuploidy.

Table II.27. The values of the ratio for each probe, the median for normal probe and the median for trisomic probes for markers of chromosome 21

		D	Ratio							
Probe	Pregnancy		EP4	EP5	EP6	EP7	EP8	EP10	EP11	EP12
1	T21		-	-	-	-	-	-	-	-
2	T21	1.56	3.60283	2.34314	2.54759	0.27722	1.63384	1.16073	1.03464	0.68261
3	T21	1.41	3.36156	3.31370	0.84039	0.11258	3.04886	0.66666	1.10890	0.63689
4	T21	12.4	6.72313	5.02264	2.73044	4.13857	4.31175	4.97617	1.93071	2.06928
5	T21	0.34	2.06928	1.65685	1.46320	0.19602	2.15587	0.94280	0.45035	2.06928
6	T21	-2.96	1.46320	1.25566	0.39205	0.22517	2.31061	0.66666	0.90070	0.51732
7	N		-	-	-	-	-	-	-	-
8	N	-2.56	0.96535	0.82842	0.63689	0.96537	1.42234	1.88561	1.03464	1.03464
9	N	-1.74	1.18849	1.25566	0.96535	0.48267	2.01150	1.33333	0.68261	1.27379
10	N	-2.19	0.63689	0.82842	1.27379	3.13645	0.33177	0.54150	0.96535	0.55445
11	T21	1.02	2.92641	3.80645	0.55445	0.15922	0.38110	0.41038	1.18849	0.36580
12	N	-6.32	1.03464	1.17157	1.03464	1.03464	0.57765	0.66666	6.72313	0.96535
Median ratio normal										
			1	1	1	1	1	1	1	1
Median ratio trisomy 21										
			3.14399	2.82842	1.15179	0.21060	2.23324	0.80473	1.07177	0.65975

T21 – trisomy 21; N – normal

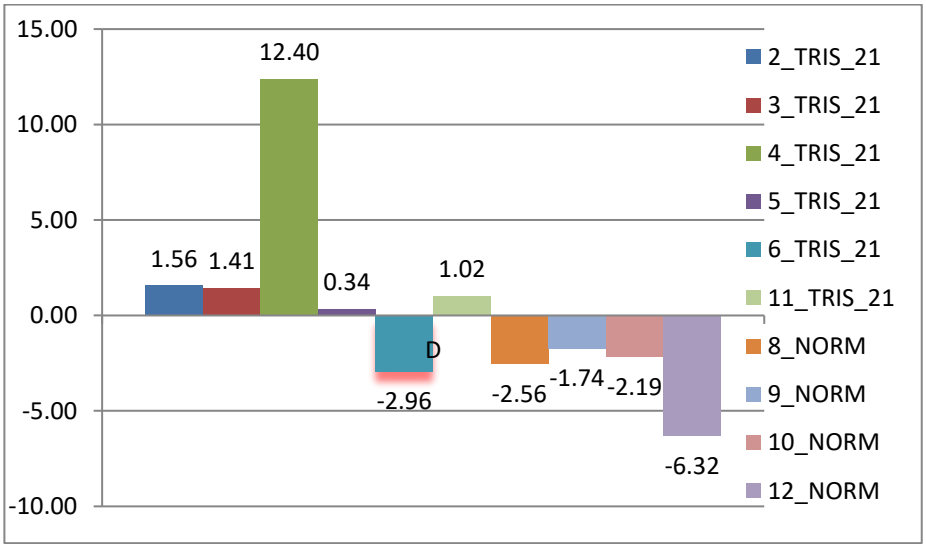


Figure II.35. Graphical representation of the D-factor values for the 10 cases analysed

Our study indicated that analysis of methylation status of foetal DNA from maternal blood could have a practical value, allowing a good discrimination between a normal pregnancy and a pregnancy with 21 trisomy's foetus. These data are concordant with studies

of Papageorgiou et al. [419, 420]. Although our study considered only ten probes we found two positive aspects. First, in normal pregnancies the values of D factor were negatives (concordant with absence of an excess of hypermethylated foetal DNA) and this indicates the absence of false positive results with a 100% specificity.

The second aspect was the good detection of trisomy 21 – five from six cases with trisomy 21 were confirmed by analysis of methylation. Thus, although the group is not statistically significant, we can say that the sensitivity of the method is quite large, correlated with the presence of a single false negative result. The presence of a sensibility less than 100% was observed also by Tong et al, that indicated a negative value of D factor in two of three cases of 21 trisomy analysed by same method [558].

The main aim of our study was to verify the reliability of method and we had some limitations correlated with type of sampling collection. Thus, we perform the noninvasive prenatal test on blood samples providing from women with knowing foetal chromosomal formula: 7 with trisomy 21 and 5 with normal foetus.

The cohort is very small and the obtaining values for sensibility and sensitivity of method may differ for real values. Thus, it is impossible to estimate a positive or negative predictive value of the test, but such problem is present in all methods for detection of foetal DNA in maternal blood [375, 399, 433].

II.3.4.5. CONCLUSIONS

Our study confirmed the reliability of noninvasive prenatal diagnostic method based on the comparison of the methylation status of maternal versus foetal DNA, which could be a future alternative to current invasive prenatal diagnostic methods marked by various incidents, the most serious being miscarriage and obstetric hemorrhagia.

II.3.5. PRENATAL DIAGNOSIS BY FISH METHOD

II.3.5.1. BACKGROUND

Chromosomal diseases, caused by the presence of chromosomal abnormalities, are an important component of genetic pathology. They are public health problems because they have a high overall incidence, are serious, are chronic, debilitating and preventable. A method for studying fetal and embionic cells is the fluorescent in situ hybridization (FISH) based on complementarity between a specific sequence of the analyzed chromosome (target DNA) and a DNA probe [205, 496].

II.3.5.2. GROUP AND METHOD

We made a retrospective analysis of prenatal diagnosis of chromosomal numerical anomalies by FISH technique. Retrospective study is based on a sample of 233 pregnant women investigated during January 2004 - March 2007. In all patients was performed amniocentesis followed by chromosome study by FISH in prenatal Cytogenetic laboratory of Maternity "Cuza Voda" Iaşi.

Amniocentesis was performed under ultrasound guidance. From every pregnant woman was collected 2.5 to 5 ml of amniotic fluid that was centrifuged and the cells from supernatant were hybridized. Hybridization was performed with centromeric Aneuvision® probes CEP 18, CEP X, CEP Y for chromosomes 18, X and Y, respectively locus specific probes LSI 13, LSI 21 for chromosomes 13 and 21. Analyse of hybridization was performed with a Zeiss fluorescence Axiomot 2 microscope, and image processing was performed by Isis software. In each case was analyzed at least 50 cells. The results were communicated in 2-3 days, allowing the adoption of appropriate management in pathological cases.

II.3.5.3. RESULTS

Amniocentesis was performed in 233 pregnant women. Abnormal results - trisomy 21, trisomy 18, trisomy 13 or monosomy X - were identified in 38 cases (16.30% of total): 17 cases of trisomy 21, 16 cases of trisomy 18, 3 cases of monosomy X, and 2 cases with trisomy 13 (figure II.36.).

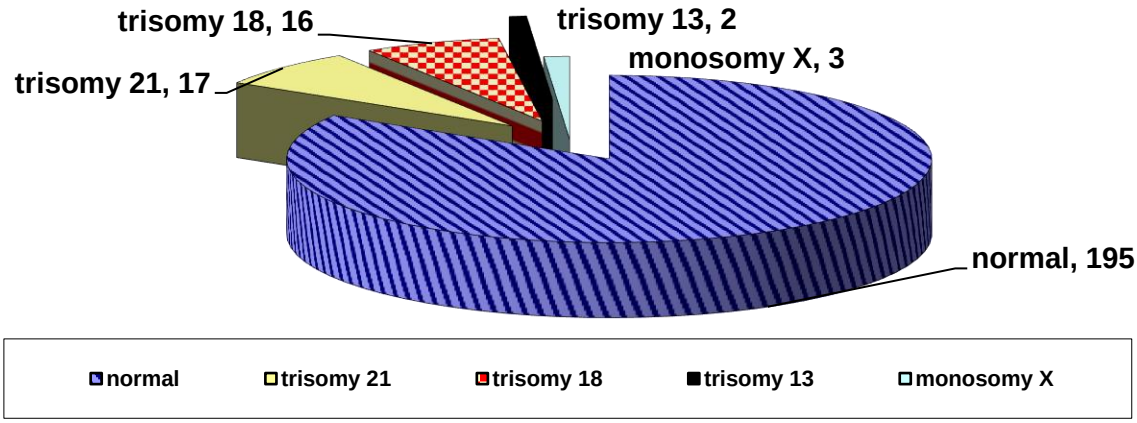


Figure II.36. Types of chromosomal abnormalities

Gestational age at the time of the procedure ranged between 12-32 weeks: 21 amniocenteses (9.01%) before 16 weeks of amenorrhea, 74 amniocentesis (31.75%) between 16-20 weeks of gestation, 87 amniocentesis (37.33%) between 20-24 weeks of gestation and 45 amniocentesis (9.01%) after 24 weeks of gestation. In 2004, 65.62% of the amniocentesis was performed after 20 weeks of pregnancy, while in 2007 only 40.74% of the procedure was made after 20 weeks of pregnancy. This is encouraging because on the slides provided from a small gestational age the cell number is higher, and the percentage of cells that provided hybridization is higher.

The prenatal diagnosis was imposed by the following circumstances: advanced maternal age, abnormal ultrasound, abnormal biochemical screening, previous aneuploidy and other reasons (table II.28.).

Table II.28. Circumstances of performing amniocentesis on calendar years

	Number of analysis (% from total)				
Year	Advanced maternal age	Abnormal biochemical screening	Sonographic anomalies	Previously aneuploidy	Other reasons
2004	9 (28.12)	4 (12.5)	16 (50)	3 (9.37)	0 (0)
2005	25 (36.76)	5 (7.35)	29 (42.64)	8 (11.76)	1 (1.47)
2006	34 (35.78)	10 (10.52)	42 (44.21)	7 (7.36)	2 (2.10)
2007	20 (52.63)	9 (23.68)	8 (21.05)	0 (0)	1 (2.63)

Maternal age over 35 years at conception was the reason for performing prenatal diagnosis in 88 pregnant women (37.77% of cases) and we detected 11 abnormal cases (12.5%): 9 of trisomy 21 and 2 of trisomy 18.

Nuchal fold greater than 3 mm, abnormalities of brain, renal abnormalities, cardiac abnormalities, single umbilical artery or multiple congenital anomalies associated with poly- or oligohydramnios were the reasons for perform amniocentesis in 95 women (40.77% of cases) and we found 25 abnormal cases (26.15%). In 3 of 6 cases with nuchal fold greater than

3 mm we found homogeneous 21 trisomy. Nine of 43 cases with brain abnormalities were abnormal (20.93%): trisomy 21 (2 cases) and trisomy 18 (7 cases). Two from 6 cases with cardiac anomalies presented trisomy 18. In 7 of 8 cases with cystic hygroma we detected trisomy 21 (1 case), trisomy 18 (4 cases) or monosomy X (2 cases). In 5 of 14 cases with multiple congenital anomalies associated with poly- or oligohydramnios we found: 2 cases with trisomy 13, 1 case with trisomy 18 and 2 cases with trisomy 21. All other pregnancies with congenital anomalies were without aneuploidy.

Of the 28 cases with abnormal triple test, only 2 were abnormal (7.14%): a trisomy 18 and a monosomy X.

From 18 cases with a previously child with Down syndrome, none was pathological. A similar situation we identified in the 4 pregancies in which amniocentesis was performed for other reasons.

II.3.5.4. DISCUTION

We detected 38 pathological cases (16.30%), 35 presenting an autosomal trisomy (21, 18 or 13) that generates severe plurimalformative syndromes. The percentage of abnormal cases obtained by us is higher than that obtained by other researchers (table II.29.).

Table II.29. Reported abnormal cases in different studies

Study	Number of cases	Abnormal cases confirmed by FISH	% of abnormal cases
Jobanputra (2002) [268]	81	5	6,17%
Weremowicz (2001) [601]	911	75	8,23%
Lim (2002) [326]	130	5	3,84%
Pergament (2000) [426]	2336	87	3,72%
Tepperberg (2001) [550]	5197	570	10,96%
Eiben (1999) [153]	3150	125	3,96%
Our study	**233**	**38**	**16,30%**

A possible explanation for these discrepancies is that most of the studies were conducted in countries where prenatal diagnosis is mandatory in women who are older than 35 (38) years, while we made a selection of women at risk. The main reasons for amniocentesis were: ultrasound abnormalities (40.77%) and advanced maternal age at conception (37.77%). These data are similar to those reported by other researchers (table II.30.) [153, 268,326, 426, 550, 601].

The result of amniocentesis imposed by the presence of aneuploidies in history or other reasons was always normal. Our results are similar to those obtained by other authors that identified aneuploidy in less than 5% of women investigated for such reasons [153, 268, 326, 426, 550, 601].

In patients with abnormal biochemical screening result was abnormal in only 2 cases (7.14%). Our results are less than those quoted in the literature, which indicates that 10-15% of cases with abnormal biochemical screening present an aneuploidy. This finding indicates the need to improve the method of triple test.

In cases of women with increasing age at conception, we found 11 abnormal cases (12.5%), similar to the literature values that indicating a presence of aneuploidy in 10-15% of pregnancies tested for advanced maternal age [153, 268, 326, 426, 550, 601].

In cases with abnormalities of fetal morphology, we identified an aneuploidy in 25 of the 95 cases (26.15%). Abnormalities most commonly associated with aneuploidy were cystic hygroma - 7 cases of 8, nuchal fold> 3 mm - 3 cases of 6, multiple congenital anomalies - 5

cases of 14, congenital heart abnormalities - 2 cases of 6 and brain abnormalities - 9 cases of 43.

Table II.30. Comparison of clinical motivation to perform amniocentesis

	Number of analysis (% from total)				
Studiu	Advanced maternal age	Abnormal biochemical screening	Sonographic anomalies	Previously aneuploidies	Other reasons
Jobanputra (2002) [268]	27 (33.33)	22 (27.16)	23 (28.39)	3 (3.70)	6 (7.40)
Weremowicz (2001) [601]	198 (21.73)	253 (27.77)	429 (4709)	0 (0)	31 (3.40)
Lim (2002) [326]	10 (7.60)	65 (50)	49 (37.90)	4 (3)	2 (1.50)
Pergament (2000) [426]	1642 (70.29)	510 (21.83)	152 (6.50)	32 (1.36)	0 (0)
Tepperberg (2001) [550]	1301 (27.21%)	907 (18.91)	1469 (30.72)	0 (0)	1104 (23.09)
Eiben (1999) [153]	1449 (46)	1276 (40)	425 (14)	0 (0)	0 (0)
Our study	**88 (37.77%)**	**28 (12.01%)**	**95 (40.77%)**	**18 (7.72%)**	**4 (1.71%)**

Based on these data we believe that the abnormal ultrasound could be a reliable indicator to perform prenatal chromosomal analysis. These results are similar to those cited in the literature, indicating that in 25-30% of cases with abnormal ultrasound could be identify a numerical chromosomal abnormality [153, 268, 326, 426, 550, 601]

II.3.5.5. CONCLUSIONS

Our study revealed the usefulness of FISH technique in prenatal diagnosis of aneuploidy, aspect certified by identification of 38 pathological cases (16.30%). Our data showed that the main indications to perform amniocentesis followed by application of FISH technique are advanced maternal age and a congenital anomaly detected by ultrasound. Another important aspect is that prenatal diagnosis by FISH technique ensures rapid results, allowing the choice of reproductive options in pathological cases.

II.3.6. PRENATAL DIAGNOSIS OF GONOSOMAL ANOMALIES BY FISH METHOD

II.3.6.1. BACKGROUND

Chromosomal diseases are caused by genomic or chromosomal mutations, with an approximately 1% incidence of newborns, high severity, chronic and disabling evolution, but they can be prevented by prenatal cytogenetic diagnosis. It requires obtaining embryonic or fetal cells with a minimally invasive method and it is based on expensive and laborious methods, requiring a rigorous selection of cases.

The indications of prenatal diagnosis of chromosomes abnormalities are: visceral anomalies during ultrasound examination, advanced maternal age, positive biochemical screening (double or triple test abnormal), family history of chromosomes disease and the existence of a balanced structural chromosomal abnormalities for a parent. [204, 205]

Sex chromosome abnormalities are common genetic diseases, affecting 1/400 - 1/500 live births. Increased frequency is correlated with minimal phenotypic changes. Gonosomal abnormalities detected by prenatal diagnosis create difficulties in genetic counseling related to the impossibility of estimating the severity of clinical features, with the decision-making process becoming a complex medical act [21]. Thus, the parents must choose between

recognizing the severity of the genetic disease, understanding and accepting the chronic illness, without a pathogenic treatment, or terminating the pregnancy by therapeutic abortion.

Severe chromosomal diseases are not marked by these dilemmas, because keeping the pregnancy is not put into question. Instead, gonosomal anomalies allow in most cases a fairly normal life. Thus, for gonosomal anomalies detected prenatal, the genetic counseling must be completed by appropriate support, helping the parents to deal with psychological problems.

II.3.6.2. MATERIAL AND METHODS

A retrospective study of nine years (2004 – 2012) targeted the gonosomal anomalies identified in the Department of Prenatal Diagnosis of the "Cuza Voda" Maternity Iasi. Prenatal diagnosis was performed by interphasic FISH method on amniocytes for 1685 pregnant women.

Amniocentesis was done between 16 and 27 weeks of amenorrhea (WA). FISH method used fluorescent labeled DNA probes corresponding for chromosomes 13, 18, 21, X and Y (AneuVysion Multicolor DNA Probe Kit Vysis®).

The lot of patients includes 15 cases that were detected with gonosomal abnormalities or there were difficulties in interpreting the number of fluorescent signals corresponding to the two sex chromosomes: X and Y (table II.31.).

Table II.31. Group study particularities

Nr.	Initials	Analysis	Gestational age	Results	Way to end the
1	FE	children with	20	46,XX/46,XY denied by	healthy newborn
2	TI	CPC	24	46,XX/46,XY denied by	healthy newborn
3	BN	CH, AF	17	45,X	TA
4	LM	ATT	17	45,X	newborn ♀ ST
5	GV	HC	16	45,X	TA
6	GI	ATT	18	47,XYY	newborn ♂ clinically
7	DT	CPC	26	47,XXY	newborn ♂ abnormal,
8	JI	ADT	17	45,X	SA, fetus with CH
9	BI	ATT	16	47,XXY	newborn ♂ without KS
10	VG	ATT	19	46,XX/46,XY	SA, foetus with IC
11	CG	ADT	18	69,XXX	TA fetal malformations
12	SE	DH, OH, SUA	27	69,XXX	TA fetal malformations
13	AE	ADT	18	45,X/46,XX	SA
14	RȘ	VSD, ASCA	22	45,X	SA
15	AG	ATT, AMA	18	47,XXY	newborn ♂ without KS

DS –Down syndrome; **CPC** – choroid plexus cyst; **CH** – cystic hygroma; **AF** - fetal anasarca; **ATT** – abnormal triple test; **AMA** – advanced maternal age; **ADT** – abnormal double test; **DH** – diaphragmatic hernia; **OH** – oligohydramnios; **SUA** – single umbilical artery; **VSD** – ventricular septal defect; **ASCA** – aberrant subclavian artery; **TA** – therapeutic abortion; **TS** –Turner syndrome; **CCM** – complex chromosomal mosaic; **SA** – spontaneous abortion; **KS** –Klinefelter syndrome; **IC** –intersex condition;

II.3.6.3. RESULTS

Six of our cases were X monosomy. In one case pregnancy completed with the birth of a girl with Turner syndrome (confirmed by postnatal karyotype). Three cases completed with miscarriage (one with cystic hygroma) and two cases completed with therapeutic abortion due to cystic hygroma. In three cases we found discordant results: cells with two X chromosomes and cells with chromosomes X and Y. The false positive mosaic 46,XX/46,XY was denied by repeating the analysis in two cases, stating that the first amniotic fluid samples was contaminated with maternal blood. In both pregnancies normal male babies were born. In the last case, repeated analysis confirmed the mosaic 46,XX/46,XY, indicating a true

hermaphroditism. The intersex condition was established by anatomopathological examination, the pregnancy ending with a miscarriage.

In three cases we found a trisomy XXY. In two cases the pregnancy resulted in the birth of two boys, postnatal karyotype confirming the trisomy XXY.

In another case we faced one of the limits of FISH technique in prenatal diagnosis. Thus, although in most fetal cells we detected three fluorescent signals corresponding to the gonosomes, we also identified cells with extra small green fluorescent signals. Due to the small number, these cells were interpreted as artefacts. Although the fetus presented choroid plexus cysts, there were no reasons for therapeutic abortion and pregnancy ended with the birth of a boy weighing 2750 g and measuring 47 cm in height. Clinical examination showed microcephaly with scafocefalia, hypertelorism, epicantus, bilateral cryptorchidism and hypotonia, aspect that are conserved at the age of 2.

The postnatal karyotype reveals a complex mosaic: 48,XY,+mar,+mar/47,XY,+mar/48,XY,r(X),+mar/49,XY,r(X),+mar,+mar/46,XY/50,XY,r(X),r(X),+mar,+mar. The six cell lines proportion was: [29]/[14]/[10]/[3]/[3]/[3].

In one case we found a trisomy XYY and the pregnancy ending with the birth of a normal boy (confirmed by postnatal karyotype).

In two cases we identified three green signals corresponding to X chromosome, but also three chromosomal fluorescent signals for 13, 18 and 21, proving a triploidy: 69, XXX. Both pregnancies were terminated, and the anatomopathological examination revealed foetuses with multiple congenital anomalies and severe intrauterine growth retardation.

II.3.6.4. DISCUSSION

Globally, gonosomal abnormalities have a high incidence, being the most common chromosomal pathology, affecting 1/500 live births. The most common abnormalities are monosomy X, trisomy X, trisomy XXY and trisomy XYY [182, 395].

Monosomy X causes Turner syndrome and affects 1/2500 women. Phenotype is usually characterized by short stature, primary amenorrhea, infertility and normal intellectual development. 90% of fetuses with monosomy X shows fetal anasarca and are eliminated by spontaneous abortion [182, 376]. The prenatal detection of X monosomy is usually accidental and not represents an indication of therapeutic abortion, except for the cases with cystic hygroma [73]. Monosomy X was the most common gonosomal abnormality in our group. One pregnancy had a normal evolution and the girl has a favorable outcome. The other five cases finished by abortion.

XYY trisomy affects 1/1000 boys and is diagnosed fortuitously, because no particular clinical signs are present [182, 534]. In our case the family decided to keep the pregnancy, which was completed by the birth of a clinically normal boy.

Trisomy XXY causes Klinefelter syndrome, a common cause of male infertility. The disease affects 1/1000 boys and is characterized by hypogonadotropic hypogonadism, but close to normal intellectual development. Two of the XXY trisomy cases detected by us were homogeneous, and at the time of birth the children showed no clinical manifestations. In the third case we identified a complex mosaic with six cell lines, with additional X chromosome fragments (X chromosome ring and marker chromosomes). Diagnosis of such case is impossible by using only the FISH technique, the anomaly being in mosaic and chromosomal marker representing X chromosome fragments that do not contain centromeric sequences. XY polysomy with more than two X chromosomes generates mental retardation and birth defects, features identified in our case. Also, additional X ring chromosome induces microcephaly, growth retardation, genital abnormalities and mental retardation [182, 350].

Triploidy generates a severely altered phenotype, which usually induces miscarriage [197]. In our cases we identified three fluorescent signals corresponding to X gonosomes, and three signals for chromosomes 13, 18 and 21. Both pregnancies ended by miscarriages, and the fetuses had multiple congenital anomalies.

In all cases there were some genetic counseling issues and difficulties in providing psychological support. Communication of an abnormal result of prenatal diagnosis is a difficult process, inducing various reactions from the parents: confusion, guilt, anxiety related to child's development and integration into society, helplessness, rejection of the baby. In the first instance, most couples chose termination of a pregnancy with gonosomal abnormalities, even if the development is usually normal.

Reaction to diagnosis depends on culture, education, religion and economic status of the couple. Overcoming obstacles imposed discussions with parents so that they understood the significance of the diagnosis and took an informed decision about the pregnancy [1, 7, 219, 616].

The pregnancies with gonosomal anomalies could be divided into two groups. In first group are the cases with trisomy XXY, trisomy XYY and X monosomy without cystic hygroma, characterized by a positive development and satisfactory integration in society. In these cases, during the genetic counseling it is explained to the family that there is no reason for therapeutic abortion [7, 105, 219, 616]. This strategy was followed by us in our eight cases of such type.

In the second group we classified cases of triploidy and X monosomy with cystic hygroma. In both situations, the pregnancy has a negative prognosis, ending in a miscarriage, pregnant women require exceptional attention, particularly when the pregnancy is obtained after several attempts. The severe evolution of the disease should be explained, the best option being the therapeutic abortion [80]. In our cases parental couples have chosen therapeutic abortion.

In cases with false positive mosaic 46,XX/46,XY there were genetic counseling difficulties only in first instance, but repeating tests that confirmed amniotic fluid sample contamination with maternal blood eliminated any dilemma. Instead, in cases with true 46,XX/46,XY mosaicism is necessary that parental couple to understand the implications of a hermaphroditism [342].

Another problem we faced is the legitimacy of abortion. In Europe there are countries that do not allow abortion (Malta, Ireland), countries that support therapeutic abortion when the fetus has non-viable birth defects or the pregnancy is dangerous for the mother (this approach is applied in Cyprus to 28 weeks WA, 24 weeks WA in Finland and United Kingdom and 22 weeks WA in Spain) and countries that allow abortion on-demand (mostly to 12 weeks WA, except Romania 14 weeks WA, 18 weeks WA in Sweden and 10 weeks WA in Slovenia). In all therapeutic abortion cases, pre-and post-abortion counseling support is necessary [1].

II.3.6.5. CONCLUSIONS

Our study highlights the limits of FISH technique in prenatal diagnosis of gonosomal anomalies and shows the genetic counseling difficulties. The main limitations are related to the impossibility of correct quantifying of mosaic gonosomal anomalies and the interpretation difficulties for some mosaics,.

Genetic counseling in gonosomal anomalies has difficulty in convincing the couple that the fetus has a chromosomal abnormality with minor changes in phenotype that allows a normal integration into society, so that they choose to maintain the pregnancy.

On the other hand, in cases with non-viable abnormalities or with minimal development potential, the results should be presented objectively showing the negative impact of maintaining the pregnancy.

II.3.7. POLYPLOIDY IN PRENATAL PERIOD

II.3.7.1. BACKGROUND

Polyploidies are rare lethal numerical chromosomal abnormalities characterized by the presence of additional haploid sets of chromosomes [516].

Triploidy is characterized by the presence of one extra haploid set of chromosomes (3n) and has a prevalence of 1:3,500 at the end of first trimester, and 1:30,000 at 16 weeks of amenorrhea [517].

Triploidy is present in 1-3% of embryos, but more than 99% of cases are spontaneously aborted [365, 464]. Triploidy was found in 0.6% of stillborn and in only 0.002% of new-born [621]. Cases that develop up to the second trimester and rarely into the third trimester have though been reported [517].

Tetraploidy is a severe unbalanced chromosomal abnormality characterized by the presence of two additional haploid sets of chromosomes (4n). Tetraploidy is extremely rare in newborn, only few cases being reported [65]. However, the anomaly is relatively common in aborted embryos, accounting for 5% in the first trimester of miscarriages [556].

II.3.7.2. METHODS

The retrospective study includes cases diagnosed during 11 years, from January 2008 to December 2018, in three Romanian medical genetics laboratories from Timisoara (center A) Iasi (center B) and Craiova (center C).

All three laboratories offered standard karyotype testing. Additionally, each provided alternative or complementary testing. In Center A, diagnosis was made by chromosomal analysis. In Center B, both FISH and karyotype were performed. In Center C, both QF-PCR and karyotype were performed.

All patients provided written informed consent and genetic counseling was offered for each couple. During the pre-testing counseling, family history (pedigree), maternal age, gestational age and ultrasound results were recorded. The post-test genetic counseling session dealt with the results of the genetic testing and the recurrence risk of the chromosomal aberration.

Cytogenetic analyse was done by standard chromosome protocol, followed by GTG banding technique. At least 20 metaphases from each sample were analyzed. Mosaicism was confirmed by identifying the same cell lines in a separate, independent culture.

Molecular analysis was performed by quantitative fluorescent polymerase chain reaction (QF-PCR), which evaluates several stable short-tandem repeats (STRs) for each of the tested chromosomes, to infer the quantity of genetic material. IVD QF-PCR Devyser (Devyser AB, Stockholm, Sweden) and Elucigene (Tepnel Diagnostics, Manchester, England) kits test for aneuploidies of chromosomes 21, 18, 13, X and Y were used, as well as extended kits for chromosomes 15, 16, and 22. DNA purification was performed using Promega Wizard™ Genomic (Promega, Madison, WI, USA).

The quantitative PCR reaction was performed using commercially available kits. The amplicons were migrated on the ABI3730xl platform (Applied Biosystems, USA), and data was analyzed using GeneMarker v 2.2 software.

FISH method was applied on uncultivated amniocytes. Amniocytes were hybridized with fluorescent probes for chromosomes 21, 13 (locus specific probes) 18, X and Y (centromeric probes).

The analysis of fluorescent signals was enabled by a Zeiss Axiomot 2 epifluorescent microscope, and images were processed using Isis software. We analyzed in each case a minimum of 100 cells. In all cases, FISH analysis was doubled by G banding karyotype following 10-14 days of amniocyte culture.

II.3.7.3. RESULTS

Our retrospective study concerns 603 products of abortion and 4590 amniocenteses. Polyploidies were identified in 39 cases of products of conception (6.46%) and 17 cases of prenatal testing (0.37%), as stated in Table II.32.

Table II.32. Structure of samples from the three regions of Romania

Genetic Center	Number of miscarriage sample				Number of amniocentesis		
	Total No	Polyploidy	Triploidy	Tetraploidy	Total No	Abnormal cases	Triploidy
Center A	337	25 (7.41%)	16 (4.7%)	9 (2.67)	1301	60 (4.61%)	3 (0.23%)
Center B	0	0	0	0	2659	191 (7.18%)	14 (0.52%)
Center C	266	14 (5.26%)	10 (3.75%)	4 (1.5%)	630	34 (5.4%)	0
Total:	603	39 (6.46%)	26 (4.31%)	13 (2.15%)	4590	285 (6.2%)	17 (0.37%)

The results of chromosomal analysis in products of spontaneous abortion with polyploidy are presented in Table II.33. and Table II.34.

We found 26 cases with triploidy: 13 cases with 69,XXY (Figure II.37.); 11 cases with 69,XXX ; a single case with 69,XYY and one case with mixoploidy 69,XXY/46,XX (3n/2n).

We report 11 cases of homogenous tetraploidy (5 cases with 92,XXYY and 6 cases with 92,XXXX) and two cases with mosaic 4n/2n. One of cases with mosaic presented a mixoploidy 92,XXXX (50%) / 46,XY (50%). A particular case with tetraploidy and two pericentrical inversion of 9 chromosome is shown in Figure II.38.

Most pregnancies with triploidy ending up as miscarriages progressed up to around 9 weeks, with limits between 4 and 13 weeks of pregnancy (mean pregnancy of 9.2 weeks). All pregnancies with tetraploidy stopped in the first trimester of pregnancy with mean gestational age of 7.92 weeks, ranging between 6-12 weeks. It is noticeable that tetraploidy leads to earlier termination of pregnancy than triploidy.

Table II.33. Triploid cases obtained from miscarriage sample in the first trimester of pregnancy

Karyotype	**No cases**	**%**
69,XXX	11	42.30
69,XXY	12	46.15
69,XXY,inv(9)(p12q13)	1	3.84
69,XXY(50%) / 46,XX(50%)	1	3.84
69,XYY	1	3.84
Total	26	100%

Table II.34. Tetraploid cases obtained from the miscarriage sample in the first trimester of pregnancy

Karyotype	**No cases**	**%**
92,XXYY	5	38,46
92,XXYY(80%)/ 46,XY(20%)	1	7,69
92,XXXX	4	30,76
92,XXXX,t(1;11)(	1	7,69
92,XXXX,inv(9)(p24;q22),inv(9)(p24;q22)	1	7,69
92,XXXX(50%)/ 46,XY(50%)	1	7,69
Total	13	100%

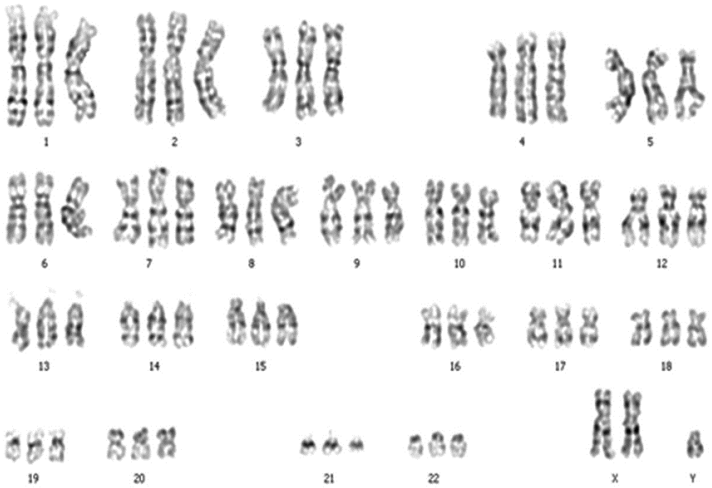


Figure II.37. A triploid case 69,XXY

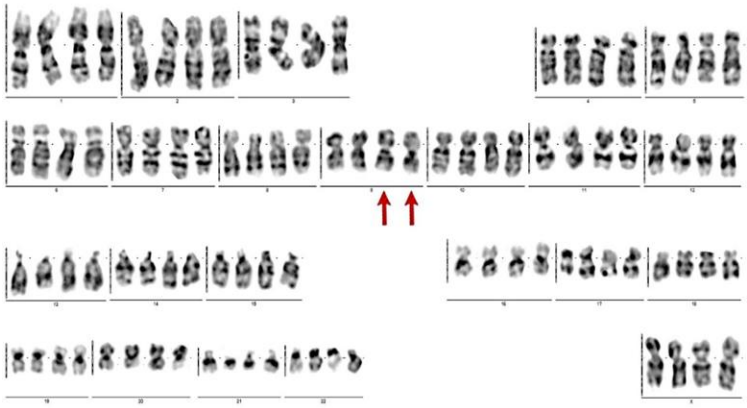
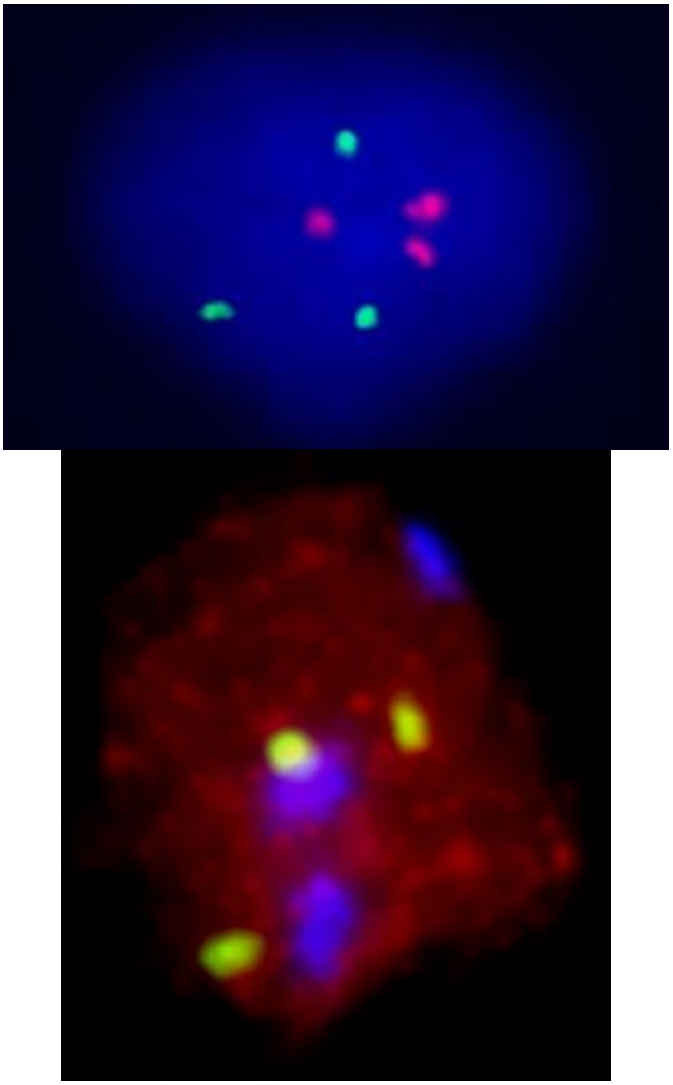


Figure II.38. – Karyotype 92,XXXX,inv(9)(p24;q22),inv(9)(p24;q22).
The two copies of chromosome 9 that have a pericentric inversion are marked by arrows.

For all miscarriages, average maternal age was 32.29, with the range between 20 - 43 years old. Mean maternal age for triploidy was 30.16 years, with the range between 20-41 years. Maternal age was below 35 years in 84.6% cases (N = 22/26), and over 35 years is 15.38% cases (N = 4/26). For tetraploidies the average maternal age was 36.92 years with the range between 25-43 years. Maternal age was below 35 years in 53.84% cases (n = 7/13), and over 35 years is 46.15% cases (n = 6/13).

Amniocentesis was performed in 4590 pregnancies, at age from 15 to 27 weeks of pregnancy, with a mean of 18.47 weeks. Average maternal age was 30.94 years, ranging between 17-39 years. Recommendations for prenatal chromosomal diagnosis were: abnormal ultrasound features and/or biochemical screening (double test or triple test), advanced maternal age and history of chromosomal disease. Chromosomal abnormalities were found in 285 cases from the three centers. Out of these cases, 17 had triploidy (Figures II.39-II.41).

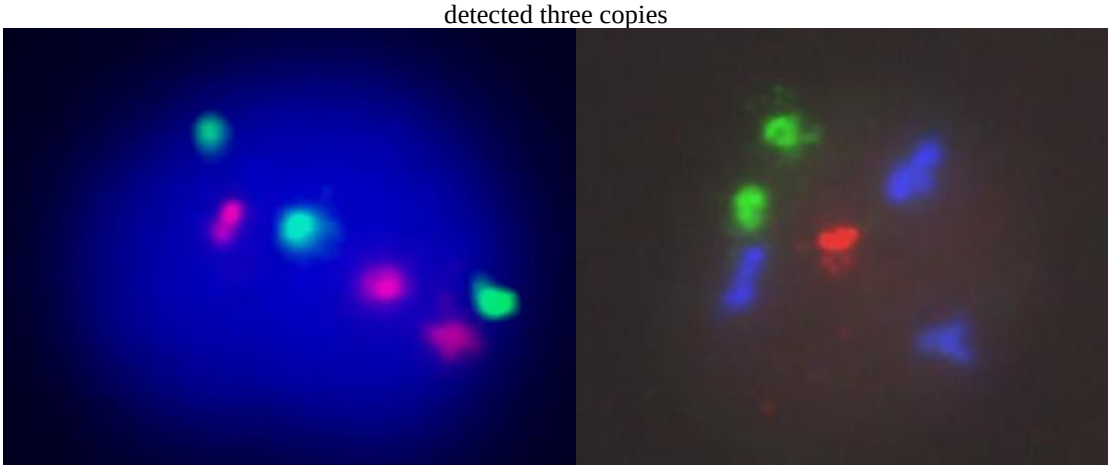
The patterns of the markers in Figure II.41. show trisomies of chromosomes 13, 18, 21, X identified by QF-PCR and exclude sample contamination. A similar image was found for STRs corresponding to chromosomes 15, 16, 22 (not shown). Altogether, this is highly suggestive for a triploid case. The diagnosis was confirmed by the cultured cells, revealing a karyotype 69,XXY. There are several non-informative markers (eg.13A). Typical marker aspects for trisomy are markers with a 2:1 ratio (eg marker 13B), or markers showing three alleles of equal areas under the peaks 1:1:1 (eg. marker 13K). In this case, genetic gender is male (the presence of SRY). AMELXY ratio is skewed due to the presence of an additional X chromosome.



a b

Figure II.39. Triploidy 69,XXX detected by FISH

a. LSI signals corresponding to chromosome 13 (yellow) and 21 (red) – for each chromosome were detected three copies; b CEP signals corresponding to chrosome 18 (blue) and X (yellow) – for each chromosome were detected three copies



a b

Figure II.40. Triploidy 69,XXY detected by FISH

a LSI signals corresponding to chromosome 13 (yellow) and 21 (red) – for each chromosome were detected three copies; b CEP signals corresponding to chrosome 18 (blue) X (yellow) and Y (red) – were detected three copies of chromosome 18 two copies of chromosome X and one copy of chromosome Y

Marker	# Allele	Peak Area	Peak Ratio
13A	2	5464:5832	0.94
13B	3 (2:1)	6615:3046	2.17
13C	1	7970	
13D	1	8057	
13K	3 (1:1:1)	8589:7162:7855	
18B	3 (1:1:1)	6891:6867:5364	
18C	3 (2:1)	6164:2857	2.16
18D	3 (1:1:1)	5163:5104:4785	
18J	3 (2:1)	10922:5380	2.03
18M	3 (1:1:1)	10414:9565:8079	
21A	3 (1:1:1)	2496:2445:2224	
21B	3 (1:2)	3003:6021	0.50
21C	3 (2:1)	11551:5774	2.00
21D	3 (2:1)	6136:2551	2.41
21H	1	11435	
21I	3 (1:1:1)	10207:11716:10290	
AMELXY	2	15305:9539	1.60
SRY	1	6174	
T1	2	18530:11275	1.64
T3	2	8621:5029	1.71
X1	2	12645:11093	1.14
X3	2	6874:6185	1.11
X9	2	5944:5924	1.00
XY2	3 (1:1:1)	3440:3822:3156	
XY3	3 (2:1)	10282:4704	2.19
ZFYX	3 (1:2)	22915:46573	0.49

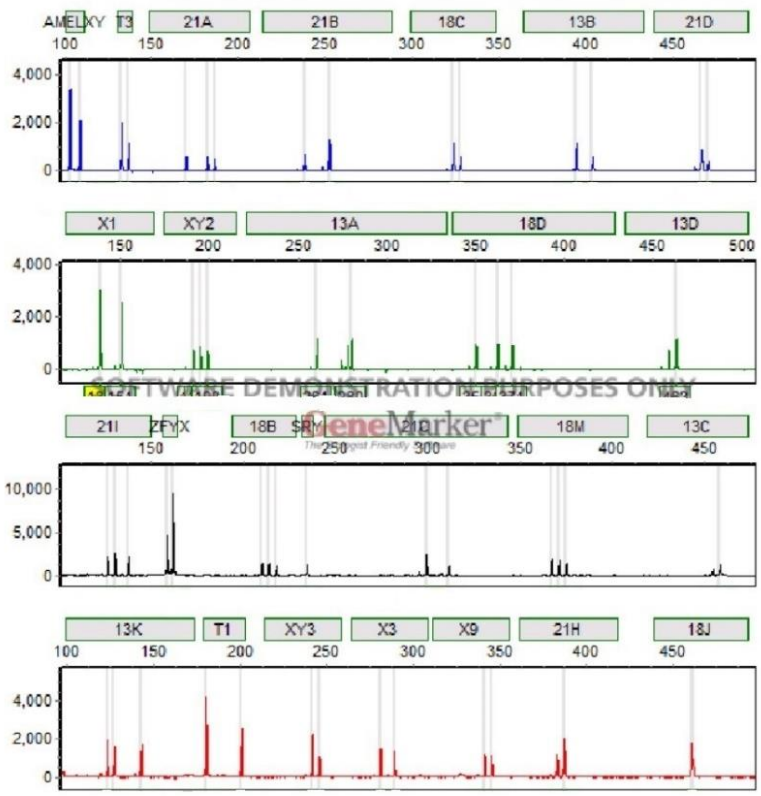


Figure II.41. Triploidy 69,XXY detected by QF-PCR in an aborted foetus

QF-PCR report masked for identifiers. Left side - the table indicates evaluated markers, the number of alleles detected, the area under these peaks and the ratio of the areas. Right side - the electropherograms show for each fluorophore the relative lengths of each allele detected (X-axis) and relative fluorescence (Y-axis). The boxes above the electropherogram strips indicate the expected length of the evaluated marker.

After genetic counseling, in all cases with amniocentesis, parents chose pregnancy termination. Pathological examination of placenta revealed molar pregnancy in all cases. In few cases parents agreed to post mortem examination and below we present a case-study.

In table II.35. are summarized the features of triploid cases identified after amniocentesis. Maternal age ranked from 17 to 39 years, with a mean age of 29.05 years. Gestational age ranked from 15 to 27 weeks of amenorrhea, with a mean age of 18.47 weeks of amenorrhea. Ultrasound examination (before amniocentesis) in cases with triploidy showed different congenital anomalies, the most frequent being: intrauterine growth restriction (IUGR) – 10 cases – and oligohydramnios – 6 cases. Some of these women applied a biochemical prenatal screening – double or triple test – and in five cases was present a high risk for different aneuploidies (trisom 18 or trisomy 21).

II.3.7.4. DISCUSSION

Chromosomal abnormalities have been reported in 50% of spontaneously aborted fetuses of clinically recognized pregnancies. Triploid cases are relatively common in miscarriage, representing 1:100 cases, but are rare in the second trimester when they represent 1: 3300 cases [265].

Triploidy (3n) occurs in 1% of all conceptions and is one of the most common chromosome abnormalities in humans regardless of whether the pregnancy were spontaneously obtained or through an in vitro fertilization process (IVF) [478]. A triploid embrio has three sets of chromosomes, and triploidy accounts for 10% of all spontaneous abortions [519].

Table II.35. Clinical and laboratory findings for patients with triploidy discovered after amniocentesis

No	Maternal age	Weeks of amenorrhea	Ultrasound Findings	Biochemical screening	Karyotype	FISH
1	29	22	IUGR, severe oligohydramnios, large placenta	TT=1/50 risk trisomy 18	69,XXX	
2	29	16	IUGR, oligohydramnios, large placenta with cysts	DT=1/664 risk trisomy 21	69,XXX	
3	32	19	Oligohydramnios, microcephaly, slow movements,	TT=1/50 risk trisomy 18	69,XXY	
4	32	17	IUGR, oligohydramnios, unilateral renal agenesia,		69,XXX	69,XXX
5	32	17	Omphalocel		69,XXX	69,XXX
6	21	26	Spina bifida, left hydrothorax,		69,XXY	69,XXY
7	24	15	IUGR, holoprosencephaly		69,XXY	69,XXY
8	39	18	IUGR	TT 1/50 risk trisomy 18	69,XXY	69,XXY
9	25	17	Ventriculomegaly		69,XXX	69,XXX
10	39	17	IUGR	DT 1/80 risk trisomy 21	69,XXY	69,XXY
11	30	15	Hydrops fetalis, nuchal translucency (5 mm)		69,XXX	69,XXX
12	31	27	Oligohydramnios, diaphragmatic hernia, single umbilical artery		69,XXX	69,XXX
13	21	22	IUGR, oligohydramnios, atrioventricular septal defect		69,XXX	69,XXX
14	25	19	IUGR		69,XXX	69,XXX
15	17	15	IUGR, asystolia		69,XXX	69,XXX
16	32	16	IUGR, hydrops fetalis,		69,XXX	69,XXX
17	36	16	IUGR, holoprosencephaly, single umbilical artery		69,XXX	69,XXX

IUGR= intrauterine growth restriction, DT= double test, TT= triple test, FISH=fluorescence in situ hibridisation

Our study, reported triploidy in 4.31% of the cases investigated in the first trimester and in 0.37% of cases analyzed second trimester. In both case, our values are lower than those reported in previous studies. Regarding second trimester triploidy cases in which amniocentesis was performed, we noticed a sex ratio ♂:♀ of 4:13, different to the ratio previously reported [294, 519]. In our study was no correlation between triploidy and advanced maternal age and mean maternal age in triploid cases was 30.16 years in miscarriage and 29.05 years at amniocentesis. Maternal age was below 35 years in 84.6% of cases, observation consistent with other studies [617].

Two types of triploidy are known depending on the parental origin of the extra chromosome set. In type I, the additional chromosome set has paternal origin and placenta is enlarged and partially multicystic whereas the foetus is relatively well-grown with either proportionate head size or slight microcephaly. In the cases with a partial hydatiform mole, recurrence risk has been reported to be 1-1.5%, therefore parents should be informed about detailed ultrasound scan and prenatal cytogenetic diagnosis in future pregnancies [49]. Type II, which is the most common, is characterized by an extra set of the chromosome with maternal origin and has a normal aspect of the placenta, but a severely growth restricted foetus [519].

Correlations between the karyotype and the biochemical marker profile were made in order to make early prenatal diagnosis of triploidy. It is considered that diandric triploidy is characterized by a combination of increased free βhCG and moderately decreased PAPP-A,

similar to the biochemical profile of cases of trisomy 21 [156, 563]. In our study, case number 2 (table II.35.) showed 4.54 MoM for PAPP-A and 9.13 MoM for fβ-hCG, values concordant with a moderate risk for trisomy 21. Another case with high biochemical risk of trisomy 21 (1/80) presented a 69,XXY chromosomal formula, and we suppose a diandric origin (impossible to prove because the fetus was aborted outside of hospital). In cases of digynic triploidy, the biochemistry demonstrates markedly decreased of free βhCG and PAPP-A with MoMs similar to the biochemical profile of cases of trisomy 18. In our study there were three cases with biochemical risk of trisomy 18 one 69,XXX and two 69,XXY, in all case we presumed a digynia. These observations on biochemical markers in the screening program for common aneuploidy are useful for detecting the rate of triploidy using these screening strategies, widespread today [151].

Placental mosaicism in miscarriages was noticed in special in case of diploid/triploid mixoploidy. We identified a such case 69,XXY/46,XX in abortion product of a young woman (aged 26) whose pregnancy stopped at 9 weeks. Studies concerning mixoploidy did not showed any patterns regarding parental and gestational age. Diploid/triploid mixoploidy is a unique form of triploid mosaicism that requires the aberrant segregation of entire parental genomes into distinct blastomere lineages (heterogenic cell division) at the earliest zygotic divisions [92].

Intrauterine growth restriction (IUGR) or placentomegaly are the typical findings that recommend evaluation of triploidy, although oligohydramnios may be the first sign in some cases. In our study the most common abnormalities observed in triploid fetuses at ultrasonography were: IUGR in 58.82% cases and oligohydramnios in 35.29% cases. The presence of triploidy generates many malformations and usually fetuses have IUGR beginning early in the pregnancy, and does not affect the head as severely as the body. Although increased nuchal translucency is relatively frequently reported in triploid fetuses, we have seen only one reported case in the first trimester of pregnancy [494].

In triploids, central nervous system (CNS) defects are common. We found a diversity of CNS: holoprosencephaly, ventriculomegaly, myelomeningocel, spina bifida but also microcephaly which together account for 35.3% of cases. Congenital heart defects are also common and we found an atrioventricular septal defect and an asystolia [294].

Lethality is a general rule in polyploidies, although rarely some triploid pregnancies can continue until the later stages of pregnancy. In our study the most advanced pregnancy age for amniocentesis was 27 weeks (case 12, table II.35.). However, if triploidy is diagnosed during the pregnancy, termination must be the better option due to the additional health risks for the mother (pre-eclampsia or choriocarcinoma) [151].

Tetraploidy was reported with 1.4% - 5% frequency of miscarriages and it is considered a rare anomaly [519, 556]. In our study, we found an average frequency of 2.15%. The average maternal age was 36.92 years with the range between 25-43 years. We could not oberve a significant difference between the below 35 age group (53.84% cases with tetraploidy) and over 35 (46.15%). One of the causes of tetraploidy is failure of cytokinesis. Homogenous tetraploidy is a severe anomaly and all such pregnancies terminate in the first trimester, aspect seen also in our study. We detected two cases of mixoploidy (4n/2n) both terminated at 12 weeks. Such anomaly could be viable and the diagnosis is subsequently made by performing blood and fibroblast karyotype in individuals with suggestive phenotype for mental retardation with asymmetric growth deficiency and pigmentary skin variation [150]. Sometime, when mixoploidy is characterized by different sexual lines, it generates a mixed gonadal dysgenesia and a plurimalformative syndrome. Rarely, tetraploidy could survive after the first trimester of pregnancy and are detected by amniocentesis. In our study we did found such situation. However, was reported also neonates with tetraploidy that presented multiple birth defects such

as: brain defects, abnormal facial features (microphthalmia, microretrognathia, abnormal ears) associated with heart defects, abnormal genitals and clubfoot [519].

In Romania, conventional karyotyping is currently considered the gold standard of products of conception testing and offers the possibility of detection of mixoploidy [86].

QF-PCR is a fast molecular diagnostic technique that allows detection of aneuploidies. The most important advanteges of this technique are short period to obtain results and the applicability on different types of biological samples. Other benefit of testing is possibility to detect maternal contamination of the fetal sample [500].

Also FISH method may identify polyploidy, by count of fluorescent chromosomal signals for chromosomes 13, 18, 21, X and Y. The method offers quick results and does not requires the amniocytes cultivation [315].

Prenatal screening of polyploidy is impossible. Abnormal levels of specific maternal blood proteins such as alpha-fetoprotein, human chorionic gonadotropin, estriol and pregnancy-assisted plasma protein-A have been detected in some cases with polyploidy, but the abnormal pattern was similar those of trisomy 18, or 21. The non-invasive prenatal testing using cell-free fetal (cff) DNA in the diagnosis of triploidy requests supplementary experiment because it's accuracy is still reduced. Pre- and post-test genetic counseling is considered to be an important component of the management of polyploidy cases [245].

II.3.7.5. CONCLUSIONS

This is the first study on polyploidy developed over a period of more than 10 years in patients from three regions of Romania, and the results are informative and statistically significant.

The analysis of chromosomal abnormalities in products of conception is useful to determine the causes of miscarriage, and to provide information for genetic counseling.

Overall, although there is questionable advantage in screening for triploidy from the view of birth defect prevention, early prediction and diagnosis of polyploid pregnacies would provide to pregnant women an opportunity to early termination of an affected pregnancy.

Chromosomal analyze continues to be the gold-standard in the detection of polyploidies, but molecular approaches may provide additional valuable information for the management of the case. The results of the present study may provide useful information for prenatal genetic counseling.

II.3.8. SONOGRAPHIC PRENATAL SCREENING IN CASES WITH NORMAL KARYOTYPE

II.3.8.1. BACKGROUND

Although the thickened skin of nuchal region is a feature of Down syndrome, only the development of ultrasound scanning allowed specialists to detect the nuchal edema presented by certain fetuses.

Once a standardized assessment to measure nuchal translucency (NT) was developed, [390] an increasing number of reports substantiated the value of this ultrasound marker for screening for aneuploidy during the first trimester.

Increased NT has been associated with structural fetal abnormalities, cardiac malformations, high risk of miscarriage, and intrauterine death. However, some reports demonstrated that a substantial number of fetuses with increased NT during the first trimester have no anomalies and favorable neonatal outcomes.[25, 570]

To assess this ultrasound marker, our study investigated the presence of increased NT in fetuses without chromosomal aneuploidies in an attempt to identify the proportions of other pathologies and normal fetuses associated with this marker.

II.3.8.2. MATERIALS AND METHODS

This study was conducted retrospectively on cases investigated by the Genetics Department of Iasi University of Medicine and Pharmacy Gr. T. Popa, Romania, between 2005 and 2014. During this period, 2007 amniocenteses were performed for prenatal diagnosis due to various indications. In particular, increased NT was an indication for amniocentesis in 71cases. NT was measured according to the criteria proposed by Nicolaides and reviewed by the Fetal Medical Foundation, with the 99^{th} percentile defined to be 3.5 mm.

The inclusion criteria for the study were: a measurement of >3.5 mm for NT, considered by initial standards as pathological; a complete obstetrical record of the patient and the pregnancy that includes maternal age, gestational age, abnormalities discovered by ultrasound, and serological markers for aneuploidy or a description of the karyotype, by the FISH method, completed with cell culture karyotype - the pregnancy outcome.

We excluded cases that did not satisfy all of theafore mentioned criteria. In addition, we excluded cases for which data regarding the offspring outcome were unavailable.

The genetic analysis was conducted via FISH for chromosomes 21, 13, 18, X and Y, and via karyotype cell culture.

The results were entered into a database and analyzed in accordance with typical statistical assessment practices (Microsoft Excel, T Test evaluation).

In the 71 examined cases, the median maternal age was 30.5 years (range, 19–40 years), and the median gestational age at the time of amniocentesis was 17 weeks.

Most of the selected cases (55 case, representing 78% of the total) had no aneuploidy with a normal 46,XX (33 cases) or 46,XY karyotype (22 cases). We could emphasize this as a comforting element for patients who could be stressed by the finding of an increased NT. On the other hand, there were 13 cases of trisomy 21 (T21), 2 cases of trisomy 18 (T18), and 2 cases of triploidy.

In most of the 71 examined cases (81%), the indication for amniocentesis was increased NT alone; additional indications observed in the remaining cases included advanced maternal age (5.5%), abnormal serologic markers (10%), and other ultrasound findings (3.5%) (2 cases: heart abnormalities, fetal hydrops).

Pregnancy outcomes were evaluated for the 55 cases with normal karyotypes. A favourable outcome at term was obtained in 40 cases (72%); nonlethal cardiac or kidney conditions at birth or complications related to prematurity were observed in 6 cases, and miscarriages occurred in the remaining 6 cases. Three cases were lost at follow up and considered abnormal but excluded from diagnosed pathology.

II.3.8.3. DISCUSSION

NT should be measured between 11 and 14 weeks of pregnancy (or for a crown-rump length of 45 to 84 mm), as recommended by Nicolaides et al [390] in 2002. When the standards for NT measurement are appropriately followed, this marker could reduce the incidence of T21 at birth by 78 %.

However, T21 is not the only aneuploidy associated with increased NT; for instance, Turner syndrome is also associated with increased NT. Different mechanisms appear to be involved; in T21, cardiac failure is thought to be the main driver of increased NT, whereas in Turner syndrome, increased NT results from abnormal or delayed lymphatic system development. In trisomic foetuses, the increased secretion of brain and atrial natriuretic hormone is another mechanism that could contribute to increase NT. In T18, a diaphragmatic hernia may impair venous return, resulting in increased NT. In our study, in addition to T21, 2 cases of T18 and 2 cases of triploidy were observed. Small chromosomal abnormalities at the subtelomeric level, impossible to identify by karyotype, can be present in certain cases [211].

Euploid fetuses with increased NT may present structural anomalies, including cardiac defects, diaphragmatic hernias, exomphalos, body stalk anomalies, and skeletal defects; certain genetic syndromes, such as congenital adrenal hyperplasia, fetal akinesia, or Noonan syndrome, have been cited as possible causes [126]. A diaphragmatic hernia can occur also in fetus with a normal karyotype [128]. Other authors have reported a significant correlation between increased NT and a higher risk of orofacial cleft defects [554].

An increased NT has also been associated with a high risk of miscarriage or fetal death. This risk increases with increasing NT thickness, and miscarriage or fetal death may be preceded by cardiac failure symptoms such as fetal hydrops. According to Goetzl [196], such miscarriages typically occur within the first 20 weeks of pregnancy.

In our study, indications for amniocentesis were either increased NT alone or increased NT in combination with advanced maternal age, abnormal serum markers, or other ultrasound abnormalities. These indications have also been described in the literature, although prior studies have included additional analyses of increased NT. For instance, relative to female foetuses, male foetuses are more prone to increased NT but also have more favourable outcomes in cases involving increased NT (with an RR of 0.47 for adverse outcomes for males) [553]. This interesting finding is consistent with the results from our series, where less favourable outcomes for euploid foetuses were more frequently observed among females than among males.

In addition, several studies emphasize the possibility that foetuses with NT>95th percentile may still have normal outcomes. The question is whether other factors, such as procedures of assisted reproductive technique, like the intracytoplasmatic sperm injection (ICSI), are associated with a higher risk of increased NT but no other anomalies [396].

In a large follow-up study, Vieira et al [584] analyzed the outcomes of foetuses with increased NT and found that 54.4% of such foetuses were euploid and that 93% of these foetuses had normal births and immediate postnatal development, with only 3 cases involving miscarriage or neonatal death. In our study, outcomes were normal status at birth for 54% of foetuses, including 40 out of the 58 euploid cases (69%), and perinatal complications in 17% of cases. Regarding cases involving miscarriages or complications in the euploid group, there were 18 cases involving cardiac abnormalities, kidney failure, or complications due to prematurity (17% of all cases and 21% of euploid foetuses) and 6 miscarriages. Adverse outcomes were observed for 31% of euploid foetuses in our study, value similar to other study [55].

In our study, we were unable to follow the neurodevelopment of euploid children in cases involving increased NT. However, the neurodevelopmental pathologies in children with increased NT and normal karyotype, did not appear to be higher than that reported for the general population [522]. De Domenico et al [128] indicated that the probabilities of delivering a baby with no major abnormalities are 70% for NT of 3.5 to 4.4 mm, 50% for NT of 4.5 to 5.4 mm, 30% for NT of 5.5 to 6.4 mm, and 15% for NT of 6.5 or more.

Our study has certain weaknesses related to the fact that prenatal scans and NT measurements were performed by different specialists during a time period when no clear standards such as those later provided by the FMF recommendations had been proposed. To address these issues, we incorporated a cut off value of 3.5 mm, which is accepted even by the FMF as indicative of a high probability of a true positive result. Also, our study used mainly FISH and cell culture karyotype to identify aneuploidies, as other methods were not available at that moment, for example array-CGH.

II.3.8.4. CONCLUSION

If aneuploidies are excluded, the management of increased NT in euploid foetuses should not greatly differ from the management of foetuses with normal NT. A thorough

ultrasound examination performed at 20 to 24 weeks that should be interpreted in conjunction with other elements (clinical history and serum markers) is required.

II.4. RARE DISEASES

The researches concerning rare diseases were published in the following papers
Pânzaru M., Rusu C., Voloşciuc M., Braha E., Butnariu L., Ivanov I., Grămescu M., Popescu R., Caba L., Sireteanu A., Macovei M., Covic M., **Gorduza E.V.,** *Optimizarea strategiei de diagnostic genetic în sindromul velo-cardio-facial,* Rev. Med. Chir. Soc. Med. Nat. Iaşi, 2011, 115(3) 756-761
Socolov D., Terinte C., **Gorduza E.V.,** Socolov R., Puiu J.M., *Limb Body Wall Complex – case presentation and literature review*, Rom. J. Leg. Med., 2009, XVII (2), 133-138
Arghir A, Popescu R, Resmerita I, Budisteanu M, Butnariu LI, **Gorduza EV**, Gramescu M, Panzaru MC, Papuc SM, Sireteanu A, Tutulan-Cunita A, Rusu C, *Pallister–Killian Syndrome versus Trisomy 12p—A Clinical Study of 5 New Cases and a Literature Review*, Genes 2021, 12, 811.
Momanu A, Caba L, Gorduza NC, Arhire OE, Popa AD, Ianole V, **Gorduza EV,** *Gorham-Stout Disease with Multiple Bone Involvement—Challenging Diagnosis of a Rare Disease and Literature Review.* Medicina 2021, 57, 681.
Spiridon M.R., Petris A.O., **Gorduza E.V.,** Petras A.S., Popescu R., Caba L., *Holt-Oram Syndrome With Multiple Cardiac Abnormalities*, Cardiol Res. 2018;9(5):324-329
Gug C., Caba L., Mozos I., Stoian D., Atasie D., Gug M., **Gorduza E.V.,** *Rare splicing mutation in COL1A1 gene identified by whole exomes sequencing in a patient with Osteogenesis imperfecta type I followed by prenatal diagnosis: a case report and review of the literature*, Gene, 2020, PII: S0378-1119(20)30234-1
Gug, C., **Gorduza, E. V.,** Lăcătuşu, A., Vaida, M. A., Bîrsăşteanu, F., Puiu, M., Stoicănescu, D., *CHARGE syndrome associated with de novo (I1460Rfs*15) frameshift mutation of CHD7 gene in a patient with arteria lusoria and horseshoe kidney*. Experimental and Therapeutic Medicine 2020, 20: 479-485. https://doi.org/10.3892/etm.2020.8683, https://www.spandidos-publications.com/10.3892/etm.2020.8683, IF – 1,785
Caba, L., Gug, C., **Gorduza, E.V.** , *Heterogeneity in combined immunodeficiencies with associated or syndromic features (Review).* Experimental and Therapeutic Medicine, 2021, 21, 84. doi.org/10.3892/etm.2020.9517, https://www.spandidos-publications.com/10.3892/etm.2020.9517/abstract, IF – 1,785
Florea L, Caba L, **Gorduza EV,** *Bardet-Biedl Syndrome—Multiple Kaleidoscope Images: Insight into Mechanisms of Genotype-Phenotype Correlations*. Genes 2021, 12, 1353. https://doi.org/10.3390/genes12091353, https://www.mdpi.com/2073-4425/12/9/1353, IF – 4,096
Gavril EC, Luca AC, Curpan AS, Popescu R, Resmerita I, Panzaru MC, Butnariu LI, **Gorduza EV**, Gramescu M, Rusu C, *Wolf-Hirschhorn Syndrome: Clinical and Genetic Study of 7 New Cases, and Mini Review*. Children 2021, 8, 751. https://doi.org/10.3390/children8090751, https://www.mdpi.com/2227-9067/8/9/751/pdf, IF – 2,078
Butnariu LI, Țarcă E, Cojocaru E, Rusu C, Moisă ȘM, Leon Constantin M-M, **Gorduza EV**, Trandafir LM. *Genetic Modifying Factors of Cystic Fibrosis Phenotype: A Challenge for Modern Medicine*. J Clin Med. 2021; 10(24):5821. https://www.mdpi.com/2077-0383/10/24/5821, https://doi.org/10.3390/jcm10245821, IF – 4,242

II.4.1. INTRODUCTION

A rare disease is a disorders that interest less than 1/2,000 individuals. The number of these diseases is important, at this moment being count around 8,000 entities. Thus, a large number of individuals – between 6 and 8% of population – have a rare disease.

Aproximative 80% of rare diseases present a genetic etiology. In this period, the rare diseases became a public health issue and the study of these disorders is in the center of mondial medical researches [200].

II.4.2. STUDY OF VELO-CARDIO-FACIAL SYNDROME

II.4.2.1. BACKGROUND

Velo-cardio-facial syndrome (SVCF) is a variable phenotypic spectrum that includes velo-palatal abnormalities, cardiac malformations (CM), suggestive facial dysmorphia (long face, asymmetric during crying, malar hypoplasia, bulbous nose with hypoplasia of nasal wings, low-set ears, short filter, microstoma), hypoplasia of thymus and parathyroid glands, learning difficulties and mental disorders. SVCF is produced by microdeletion 22q11.2 [81]. The severity spectrum of SVCF varies widely from severe life-threatening to mild form with facial dysmorphia, nasal speech or hypoparathyroidism [509]. The clinical variability, including within the family, does not depend on the size of microdeletions [472].

Initially, the clinical manifestations characteristic to SVCF were defined as distinct diseases under various names, but after identification of the genetic cause it chosed the name of monosomy 22q11.2, DiGeorge, Velocardiofacial or cardio-facial syndrome [83, 190, 606]. Velo-cardio-facial syndrome is a common chromosomal disorders, with a prevalence of 1:2,000- 1:4,000 people [9, 81]. Frequently microdeletion 22q11.2 appears de novo, but it can be inherited in 6-28% of the cases [197]. *TBX1* gene that encodes a transcription factor involved in the regulation of embryonic development of branchial arches and heart development field plays a key role and it's haploinsufficiency explain most of the characteristic signs. Other candidate genes are *PRODH, COMT* and *RTN4R* [197, 282, 551, 613]. Correct diagnosis of velo-cardio-facial syndrome is made by molecular cytogenetic techniques: FISH, MLPA or array-CGH [87]. Because molecular tests are expensive, it is necessary a phenotypic selection of cases using a clinical algorithm. Early diagnosis is helpful in establishing adequate management, preventing complications and providing correct genetic counselling [291, 362, 493, 557].

II.4.2.2. MATERIAL AND METHOD

The retrospective study was conducted on 21 patients clinically diagnosed with SVCF in Iasi MGC, from 2003 to 2010. Chromosome preparations were obtained by standard methods, using lymphocyte cultures stimulated with phytohemagglutinin. For FISH technique we used TUPLE1 LSI probe (HIRA) located in the region frequently affected by microdeletion and LSI ARSA control probe located in the 22q13 region (Abbott Molecular, Vysis). We divided patients into 2 groups: group A - patients with velo-cardio-facial syndrome confirmed by FISH technique - and group B with clinical features of VCFS but infirmed by FISH method. Group A comprises 6 patients, two females and four males, aged between 1 month and 33 years. Group B includes 15 patients, five female and 10 male, aged 1 month to 16 years. Clinical signs were compared to the algorithms proposed by Tobias et al. and McDonald-McGinn et al [472, 493].

II.4.2.3. RESULTS

The main phenotypic features of patients with velo-cardio-facial syndrome are presented in table II.36.

In group A, with confirmed diagnosis, four clinical signs were present in all cases: facial dysmorphia, palatine insufficiency/nasal voice, learning difficulties and long, tapered fingers. Cardiac malformation (CM) was identified in 83% of cases. In group B (infirmed by FISH), the most common clinical signs were CM and learning difficulties in 80% of cases and facial dysmorphia (66% of cases).

Using Tobias algorithm the FISH investigation in VCFS will be applied, if present, one A criteria or two B criteria or one B criteria and two (or more) C criteria [557]. In the case of Mc Donald-McGinn score the application of FISH analysis is imposed in the presence of

minimum three points [362]. Our results correlated with Tobias algorithm and Mc Donald-McGinn score are presented in table II.37. For both cohorts our scores were superior to the threshold than the molecular analysis imposes.

Table II.36. Clinical features in patients withVCFS

Signs	**Group A - FISH+**	**Group B - FISH-**
Palatal anomalies	6/6	2/15
Heart congenital anomalies	5/6	12/15
Facial dysmorphia	6/6	10/15
Long, tapered fingers	6/6	5/15
Repeated infections	2/6	3/15
Learning difficulties	6/6	12/15
Hypocalcemia	0/6	5/15

Table II.37. Diagnosis algorithm in VCFS

Clinical feature	Tobias Algorithm [557]	Mc Donald-McGinn Score [362]	Cohort A	Cohort B
Conotruncal CM	Class A	1 point	0/6	3/15
One parent with SVCF	Class A	-	1/6	0/15
Facial dysmorphia	Class B	¼ point	6/6	10/15
Non-conotruncal CM	Class B	1p	5/6	9/15
Learning difficulties/ mental retardation	Class B	1/2 point	6/6	12/15
Velopalatine insufficciency	Class B	1 point	5/6	2/15
Hypocalcemia	Class B	1 point	0/6	5/15
Immunodefficciency	Class B	1 point	2/6	3/15
Finger's anomalies	Class C	½ point	6/6	5/15
Renale anomalies	Class C	½ point	1/6	1/15
Hypotonia	Class C	-	0/6	1/15
Shortstature	Class C	½ point	0/6	6/15
Psychic troubles	Class C	½ point	?	?
Familial MCC	Class C	-	2/6	7/15
Nasal voice	-	¼ point	6/6	2/15
Indication of FISH	one A criteria or two B criteria or one B criteria and two C criteria	Minimum 3 points		

II.4.2.4. DISCUSSION

Phenotypic spectrum of velo-cardio-facial syndrome is variable and this variability is associated with: age, modifying genes and phenocopies [84, 277, 379, 510]. Symptomatology includes palatal abnormalities, facial dysmorphia and conotruncal CM. In group A we found the palate abnormalities, facial dysmorphia and learning difficulties. Velopalatine abnormalities, albeit with a lesser degree of severity, are shown in group A in all cases, as opposed to group B in which, although the severity was increased, the number of cases was small (2/15). In this context, nasal voice that betrays a poor functioning of the palatine veil was a valuable diagnostic element (6/6 in group A).

CM were present in similar proportions in both groups (A - 83%; B - 80%) (concordant with data of literature - 80%) [362]. The presence of conotruncal CM in group B (3/15) suggests that, although is the most frequent type of CM in SVCF (7-50%) [291, 362], it is necessary to be associated with other suggestive elements for applying molecular techniques. We noticed that a positive family history for CHD was present in a high number of cases in group B compared to group A, which casts doubt on the value of this criterion, given the high frequency of familial cases of CM in the general population.

Facial dysmorphia proved to be a valuable criterion, but unfortunately is a subjective assessment and it becomes evident only with age. Among the facial dysmorphic elements useful have been shown: long face (5/6 in group A compared with 5/15 in group B), hypoplasia of zygomatic bone (5/6 vs. 3/15), tubular bulbous nose (6/6 vs. 6/15), and hypoplasia of nasal wings (5/6 vs. 4/15). Other signs without differences between the 2 groups were low-set ears (4/6 vs. 13/15) or helix abnormalities (4/6 vs. 10/15).

Learning difficulties is an important criterion for clinical diagnosis of SVCF. Many patients showed only slight learning difficulty and MR was identified only in group B. Although in literature hypocalcemia is considered a valuable criterion for the SVCF diagnosis (present in 17-60% of patients) [557], assessing the significance of this symptom in our patients was difficult because they were sporadic and incomplete tested during neonatal period. Similar data concern immunodeficiency that was considered only declarative (number of repeated infections). The long and tapered fingers could be a valuable diagnostic element. It is present in all confirmed cases, but only in 33% of patients from group B. The data of our study indicate that skeletal abnormalities (3 cases) shortstature (absent in all confirmed cases) renal anomalies (2 cases) genital abnormalities (3 patients) central nervous system malformations, hypotonia, and seizures were not useful criteria guidance.

For clinical examination of our patients we used a scale developed in Iaşi GMC (Table II.38.).

Table II.38. Iaşi MGC algorithm to calculate clinical scores in SVCF for testing by FISH method

Anomalies	**Types/observations**	**Score**
MCC	Conotruncal/ septal defects	1 point
Velopalatine anomalies	Cleft, velopalatine insufficciency, nasal voice	1 point
Hypocalcemia	Ca^{2+} or parathormone	1 point
Mental retardation/ learning difficulties/ psychological issues	Specific psychic profile (attention deficit, hyperactivity, schizophrenia, bipolar disorders)	1 point
Facial dysmorphia	Long face hypoplasia of malar bone, bulbous/tubular nose, hypoplasia of nasal wings, low-set ears with anomalies of helix	1 point
Anomalies of fingers	Long, tapered fingers	1 point
Immunodefficiency/ hypoplasia of thymus	Low number of T lymphocytes, repetead infections	1 point
Renal anomalies	Hypo/aplasia	0.5 point

Thus, FISH testing is recommended in newborn if the clinical score is at least two points, and during childhood and teenage, if the score is more than 3 points. The sensitivity of the score (30%) is superior to sensitivity obtained in similar studies [557] and can be explained by the fact that study was made on selected patients. Given that 6-28% of SVCF cases are inherited and intrafamilial variability could be important, family screening is indispensable for providing correct genetic counselling [124]. It is obvious that the selection of patients remains the cornerstone of any strategy for the diagnosis, even with the most powerful and inexpensive diagnostic test, the global testing of the entire population is not possible. The improvement strategy of genetic diagnosis in DiGeorge syndrome at the population level can be achieved in two ways: by increasing the number of patients included in the diagnostic process (with stringent selection criterions) or by relaxing the stringency of the criteria applied for the selection of patients to be tested. Given that clinical genetic consultations are the "second line" consultations, to increase the number of patients studied we need a good cooperation with other specialists, as well as their awareness of the clinical and genetic aspects of this syndrome. The reduction of clinical score also enable the diagnosis of more than 22q11.2 microdeletions, in particular the ones with a discrete phenotype.

Since diagnostic test used in this study - FISH test - has sensitivity and specificity of 100%, the only way to increase the number of tests at a constant budget is to reduce the cost of analysis. This can be accomplished by the use of interphase FISH technique, reduced cost analysis is obtained by removing the cell culture step required for the production of mitosis or by reducing the reaction volume to reduce the cost of chemicals needed for the analysis.

II.4.2.5. CONCLUSIONS

We found only 6 patients with 22q11.2 microdeletion in seven years. Because the incidence at birth of SVCF is 1/2,000-1/4,000 neonates, it is evident that patients diagnosed are only the "tip of the iceberg". The clinical spectrum of DiGeorge syndrome is highly variable and accurate diagnosis can be made only by molecular cytogenetic techniques, by selecting cases with phenotypic evocative elements. To increase the efficiency of the selection is necessary a clinical score and the strategy used in this study were focused on maximizing the sensitivity of the clinical score.

II.4.3. A CASE REPORT OF LIMB BODY WALL COMPLEX

II.4.3.1. BACKGROUND

Limb Body Wall Complex (LBWC) is a fetal malformative syndrome which consists of neural tub defects, body wall disruption and limb abnormalities. The diagnosis is confirmed by the presence of at least two of the above features [442, 572, 586]. The particularity of our case consists in the association of the LBWC with the polycystic disease of the liver and kidneys.

II.4.3.2. CASE REPORT

A 31 year old woman was referred at 36 weeks gestational age for antenatal ultrasnography because of a plurimalformative syndrome. The scan revealed a large, ill defined abdominal wall defect through which the abdominal contents herniated into the extraembryonic celom (figure II.42.). The protruded organs formed a whole with bizarre appearing and entangled with membranes. The diaphragm was intact. We found scoliosis and spina bifida occulta. The superior limbs were normal. The inferior left limb presented club foot, while the right inferior limb was completely absent. The color flow Doppler showed a single umbilical artery with a short umbilical cord. A provisional diagnosis of LBWC was made based on the ultrasound description.

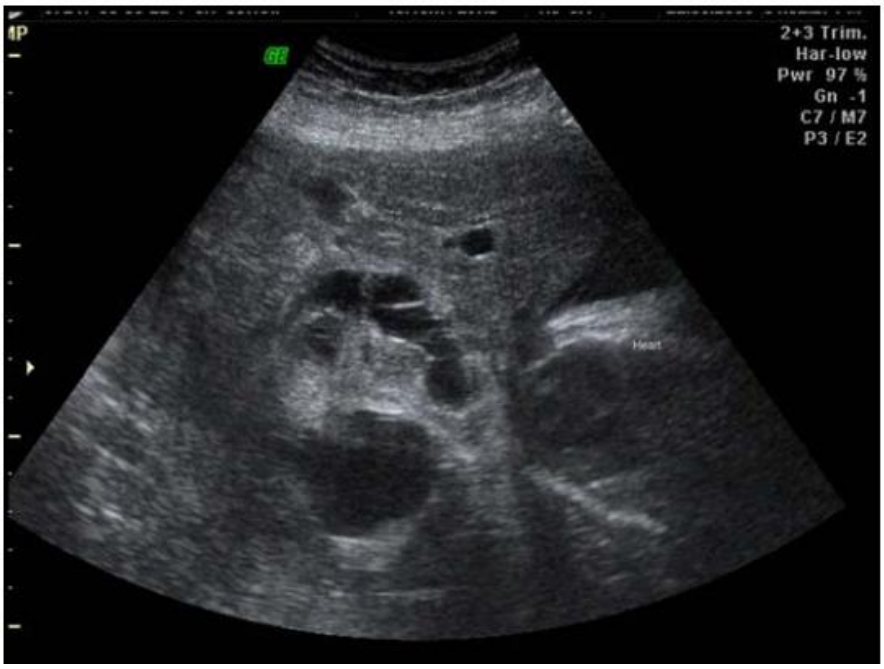


Figure II.42. Ultrasound aspect with liver and bowel protrusion through anterior abdominal defect. The heart is inside the thorax (on the right)

The patient was informed of the poor prognosis and after counselling she accepted the termination of the pregnancy. Following the induction of labour she delivered a still-born fetus of 3000g.

The necropsy report confirmed the diagnosis of LBWC (figure II.43.) showing herniation of the abdominal content through a large abdominal wall defect, exteriorisation of the liver, and of the small and large bowel content, kidneys, and stomach, cloaca, entangled with membranes, bilateral cardiac ventriculomegalia with arterial duct persistence, a type II polycystic kidney (the adult form) (figure II.44.).

Figure II.43. General Appearance

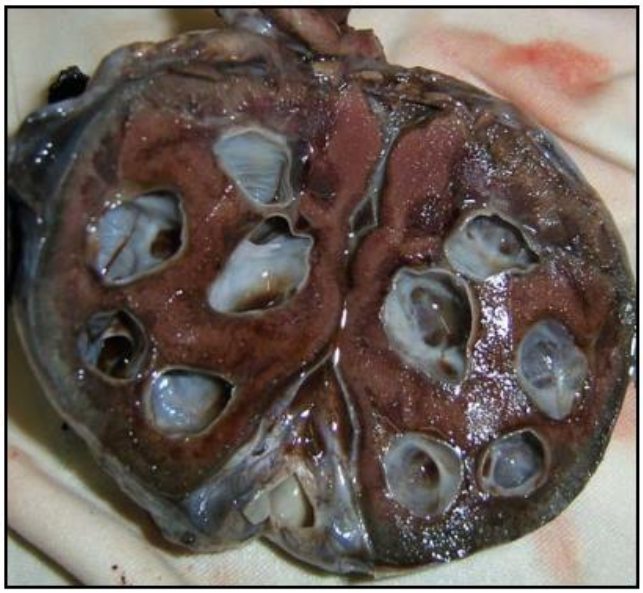


Figure II.44. Macroscopic appearance of polycystic kidney

One kidney was enlarged (4/6 cm), with a large pielon situated at its inferior pole; the other one was small (0.4 cm), localised on the surface of the big kidney. The large bowel ended in a "*cul-de-sac"* manner, into a 3/4 cm pool with thick wall: the cloaca. Other abnormalities were: pancreas agenesia, ambiguous external genital organs, imperforated anus, *spina bifida occulta*.

The microscopical examination found multiple intrahepatic cysts (0.5-0.8 cm) with serosangvinolent content, cysts of 0.8/1/1.5cm, disposed in the renal cortex and medulla, separated by a fibrous tissue and a short umbilical cord (17 cm), with two vessels (one artery and one vein separated by a segment of 2 cm) which confirmed also the ultrasound description.

The karyotype effectuated from the cord blood immediately after delivery was normal – 46,XY.

II.4.3.3. DISCUSSION

LBWC is a rare, polimalformative fetal syndrome, appearing in 0.21-0.31/10,000 deliveries, with only about 245 cases described in the literature [138, 515].

Van Allen [607], mentioned 3 essential features: 1. exencephaly/ encephalocele with facial clefts; 2. toraco- and abdominoschisis; 3 limb defect. The diagnosis needs 2 of the above features. There is no correlation with fetal gender, parent's age or karyotype anomalies, as in our study too. The disease is invariably fatal [122, 138, 440, 442, 515, 572, 586].

Serum alpha-fetoprotein analyse and ultrasonographic examination is the key of prenatal diagnosis [349]. The ultrasound milestone of LBWC consists of thoracic-abdominal defect, spinal and cord anomalies, limb deformities and anomalies of umbilical cord and membranes [122, 138, 440, 442, 515, 572, 586].

The pathogenesis of LBWC is unclear. Four pathogenic mechanisms are proposed to be involved: germinal disk defect with early embryonic maldevelopment, the early amnion rupture theory [430, 431, 495, 562], the vascular disruption theory [17, 100, 572], and the embryonic dysgenesis theory [75, 222, 476].

Different authors [139, 471] described two different phenotypes: placenta-cranial and placenta-caudal types. The first type shows craniofacial defects and amniotic bands and/or adhesion related to early vascular disruption. The second phenotypes present urogenital anomalies, anal atresia, short umbilical cord, abdominal placental attachment and persistence

of an extraembrionic celom. It is correlated with embryonic maldevelopment. Our case seems to be explained by the second mechanism.

LBWC must be differentiated from other abdominal wall defects classified according to their localisation: gastroschisis, omphalocele, ectopia cordis, cloacal dystrophy or urachal cyst [228, 273].

The specific signs for LBWC are: presence of membranes covering the contents of the herniated sac, bowel abnomalities, presence or absence of urinary bladder, scoliosis and limb defects.

II.4.3.4. CONCLUSIONS

LBWC is a rare polimalformative syndrome that could be detected by antenatal ultrasound examination. It presents two phenotypes: placento-cranial (amniotic bands syndrome) and placento-caudal (body stalk syndrome). The syndrome is invariably fatal, but there is no risk for recurrence. The prenatal diagnosis is confirmed by autopsy, accompanied by microscopic pathological examination.

II.4.4. STUDY OF PALLISTER - KILLIAN SYNDROME

II.4.4.1. BACKGROUND

Pallister–Killian syndrome (PKS, OMIM #601803) is a rare, sporadic genetic disorder defined by the association of a characteristic dysmorphic face with pigmentary skin anomalies, profound intellectual disability, hypotonia, and seizures [275, 479, 480]. The disorder was described first in adults by Pallister [417] and later in children by Killian and Tescler-Nicola [285] , and shows a moderate preponderance of females. It has a prevalence of 1/20,000 and is due to mosaic 12p tetrasomy resulting from a supernumerary isochromosome i(12p) [253, 416] and only rarely due to mosaic 12p hexasomy with two isochromosomes i(12p) [416, 587]. Tissue-specific mosaicism is characteristic for this condition – the detection rate of 12p tetrasomy is 0–2% in lymphocytes, 50–100% in fibroblasts and chorionic villi, and 100 % in amniocytes and bone marrow cells [525]. The low detection rate of the mosaicism in lymphocytes can be explained by reduced response to the PHA stimulation used for cell culture [453], or a selection against i(12p) positive cells [523]. New diagnostic methods applied in PKS are interphase FISH on lymphocytes, skin fibroblasts, or a buccal smear [402, 523]; chromosomal microarray analysis [50, 111]; or droplet digital PCR, which detects low levels of mosaicism [16 - 25]. The SNP array also evaluates meiotic origin [111], whereas MLPA is a simple, low-cost, multiplex diagnostic method with high accuracy in copy number estimation [115]. In most cases, the isochromosome is inherited from the mother [111, 113] in correlation with advanced maternal age [600]. The critical region seems to be 12p13.31, with the strongest candidate genes being *ING4*, *CHD4*, and *MAGP2* [50, 257].

Typical facial characteristics include frontal bossing and high frontal hairline, temporofrontal balding, sparse eyebrows and lashes, hypertelorism, small and flat nose, full cheeks, long and simple philtrum, large mouth with downturned corners, thin upper lip and distinct cupid bow (“Pallister lip”), micrognathia, and low-set dysplastic ears [101, 240, 264, 316, 486, 524, 604]. Orodental features also include macroglossia, alveolar ridge or gingival overgrowth, delayed teeth eruption, and missing or double teeth [23]. As the child grows older, baldness diminishes (wiry hair or hair that grows only a few centimeters long covers the frontotemporal regions), the face becomes coarser, the eyebrows become thick, macroglossia develops, and the lips and chin become prominent [230, 240, 477]. Skin depigmentation is usually patchy, suggesting the presence of a mosaic, but is not always visible. Some cases have limb shortening, lymphedema, and increased soft tissues of the extremities, but commonly palms and fingers are short and broad, with transverse flexion creases and clinodactyly of 5th finger [59, 486]. The most frequent malformations associated are supernumerary nipples,

congenital heart defects, congenital diaphragmatic hernia, and anal defects. Renal, genital, and ocular malformations are rarely associated [413, 486]. Marked hypotonia and profound intellectual disability are present from birth onwards, with developing seizures and contractures. However, mild–moderate ID cases have been cited, as well as self-stimulatory and self-injurious behavior [59]. Seizures are common, have variable age of onset (typically occur in the first four years of life), and are frequently associated with non-epileptic paroxysmal events, which require combined therapies [193, 604]. Brain MRI reveals brain atrophy, corpus callosum dysgenesis, polymicrogyria, and spot calcifications in the perisylvian region [325]. Sensory impairment (deafness and blindness) is frequently associated [192, 490]. PKS probably includes autonomic dysfunction, anhidrosis or hypohidrosis, and episodes of hyperventilation interspersed with breath-holding being relatively common. Growth is excessive prenatally (with macrosomia at birth) and reduced velocity postnatal. Anterior fontanel closure is delayed. Puberty is within the normal age ranges in girls and delayed in boys [59]. The radiological examination may reveal ovoid vertebral bodies, delayed axial and pubic bone maturation, as well as a metaphyseal flare of long bones. Differential diagnosis should consider mainly Fryns syndrome, Trisomy 12p, and SifrimHitz-Weiss syndrome [480].

Almost all cases in the literature have been sporadic, with the recurrence risk being very low [325]. Fetal movements are decreased or late. PKS fetuses have increased biparietal diameter (BP) and head circumference (HC) above the 90th percentile associated with significant femoral growth delay under the 10th percentile [479]. Prenatal sonographic features include polyhydramnios, increased nuchal translucency, extremely flat facial profile, congenital diaphragmatic hernia, small stomach, short limbs with small or abnormal extremities [525]. Array CGH on genomic DNA extraction from uncultured amniocytes may help to obtain a final diagnosis [316].

The goal of this report is to present three new cases of PKS and two cases of trisomy 12p to analyze the differences between entities, to illustrate and discuss some features rarely cited in the literature, and also to present some particularities not cited yet. Moreover, we present alternative methods of diagnosis that could be easily used in daily practice.

II.4.4.2. MATERIALS AND METHODS

The study includes 5 patients: 3 with PKS due to mosaic tetrasomy 12p (as the result of a supernumerary i(12p)) and 2 with trisomy 12p. For each of them, the diagnostic odyssey was different, and the methods used are described in the case presentation.

The informed consent was signed by the parents according to the SPO-MED-52/2013 and PO-MED-17/2013 procedures, approved by the Ethics Commission of Obregia Clinical Hospital of Psychiatry and the Ethics Commission of Saint Mary's Emergency Children's Hospital Iasi, Romania.

II.4.4.3. RESULTS

3.1. Case 1

The first case is a female aged 3 years (y) and 7 months (mo), the second child of a young, unrelated, apparently healthy couple. Pregnancy was uneventful, however fetal sonographic evaluation (2^{nd} trimester) revealed enlarged cerebral ventricles. The child presented at birth overgrowth of weight 5310 g (+5.3 SD), length of 57 cm (+4.3 SD), head circumference of 38 cm (+3.3 SD), and Apgar score of 8, with difficult respiratory adaptation. She presented hypotonia and marked developmental delay (at 3 y 7 mo of age she could raise the head with difficulty, was able to sit with support, but spoke no intelligible words). Myoclonic jerks were noted from the first day of life and intensified after 1 y of age. More complex seizures were noted after 3 years.

The child presents bilateral moderate sensorineural hearing loss. A hearing aid was inserted at 8 mo and frequent otitis media were recorded in time. No visual reaction was noted at 2 mo, but ophthalmologic examination was relatively normal (cortical blindness). The child was diagnosed at 2 mo with a congenital heart defect. Oxygen desaturation during the night was observed after 1 y of age. Recurrent infections in different locations were diagnosed over time (otitis, urinary tract infection, pneumonia). Left hip dysplasia was diagnosed at 1 mo of age. Delayed and abnormal teeth eruption (but all teeth buds were present upon radiography) was noted, as well as slow growth of hair and nails.

The mother noted slow bowel movements after 7 mo of age and days with long periods of very deep sleep after 3 y of age. First clinical genetic examination was performed at 2 mo of age and revealed overgrowth, followed by growth delay, fine, depigmented hair; light complexion; marked excess of hair on the forehead and ears (figure II.45.); sparse or fine eyebrows and lashes; hypertelorism; particular iris characteristics (dark blue, except the part around the pupil, which is light blue; pointed pupil with prompt reaction to light); short or broad nose with a depigmented area of skin on the lateral side; long or featureless philtrum; downturned mouth with thin upper lip and everted lower lip; micrognathia; full cheeks; low-set ears; short neck; prominent abdomen with umbilical hernia; hypoplastic external genitalia and anterior displacement of anus; relatively short limbs with acromicria; soft, puffy skin; small aplasia cutis in perineal region; hypotonia; absence of osteotendinous reflexes.

Later reevaluations revealed marked developmental delay and no reaction to visual stimuli, sounds, or smell. Temporofrontal baldness and coarse face became evident after 5 mo of age.

Various investigations were performed (hematology: mild microcytic anemia; biochemistry: lactic dehydrogenase increased repeatedly; ECG: incomplete right bundle branch block; echocardiogram: atrial septal defect, mild pulmonary valve stenosis; EEG: isolated epileptic spasms when the child is awake, associated with slow biphasic waves; cerebral MRI: thin corpus callosum with delayed myelination, anterior fossa not completely formed and disproportionately small; small pineal gland; eye examination: iris stromal atrophy, mild myopia; ASSR: moderate hearing loss; endocrine: low IGF1, high ACTH, normal cortisol; neonatal metabolic screening: normal).

A standard G-banded karyotype performed on peripheral lymphocytes at 3 mo of age revealed no abnormalities after evaluating 150 metaphases (450 bands).

Array CGH analysis of genomic DNA extracted from a buccal swab was conducted using a CGX-HD oligonucleotide array (Perkin Elmer, Turku, Findland). Data were analyzed using CytoGenomics 2.5 (Agilent, Santa Clara, CA, USA) and Genoglyphix 3.0 (Perkin Elmer, Turku, Findland) software with annotations of Genome Build 37. Array CGH analysis revealed a gain of 33.89 Mb on chromosomal region 12p: arr[GRCh37] 12p13.33-p11.1(190,462−34,078,153) × 3~4 (ISCN 2020), i.e., tetrasomy 12p comprising 218 OMIM-annotated genes (figure II.46.).

Having the clinical suspicion of PKS confirmed by array CGH, we extracted DNA from other biological samples (blood, buccal swab, and urine) and performed MLPA using a SALSA MLPA P230 Human Telomere-7 kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. The number of DNA copies was estimated using the Coffalyser.Net software, which calculates the ratio of peak areas in test samples over those of normal controls for each target sequence. We detected the 12p copy number gain (30–50% increased relative peak area of the amplification products of all probes) only in the buccal swab sample (figure II.47.). In the clinical context presented above and due to the different results for different tissues, we interpreted this result as being mosaic tetrasomy 12p, however a FISH test with probes for 12p would be necessary for a definitive diagnosis.

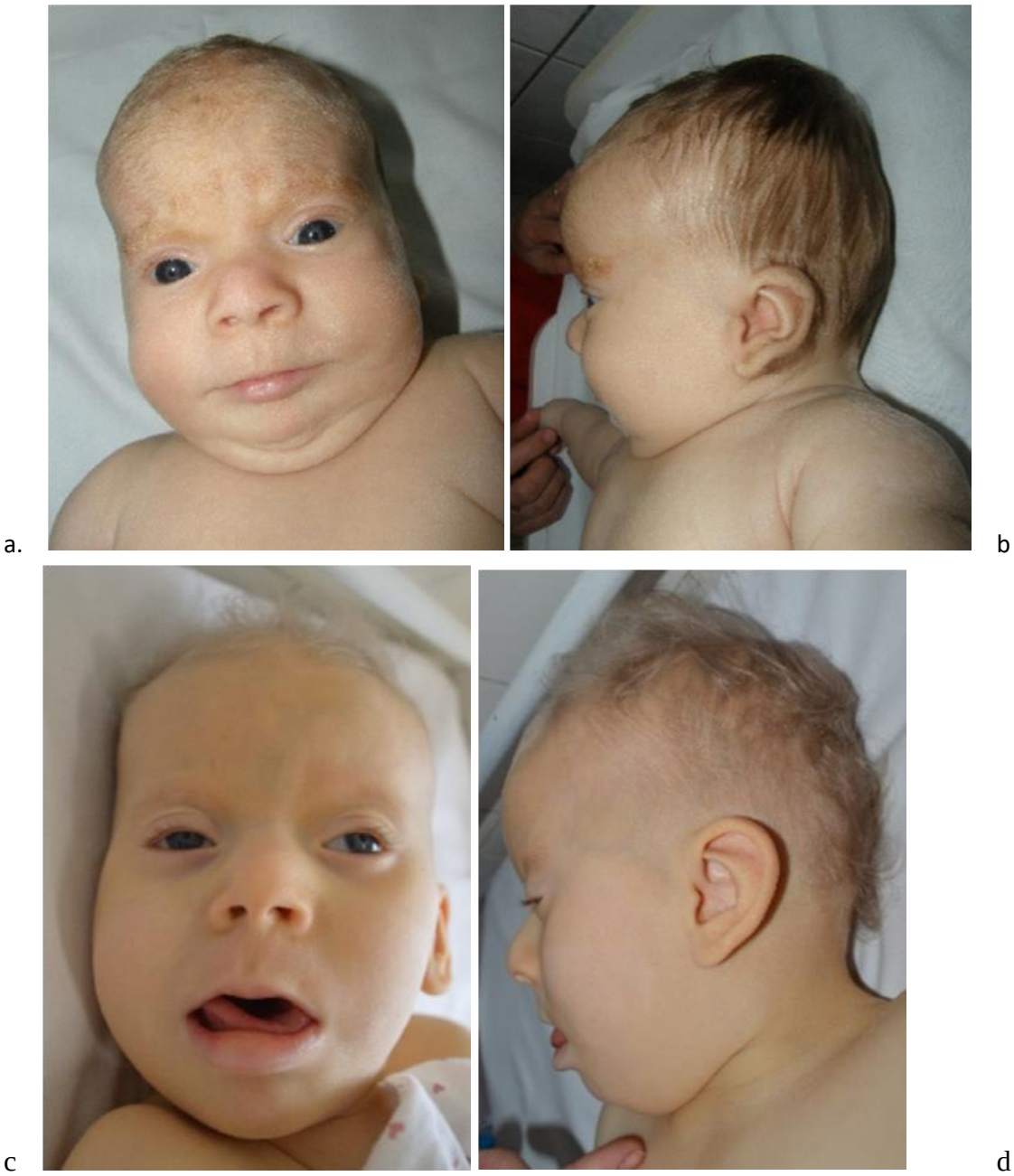


Figure II.45. Facial characteristics of patient 1
a, b - 2 mo of age; c, d - 3 y and 7 mo of age.

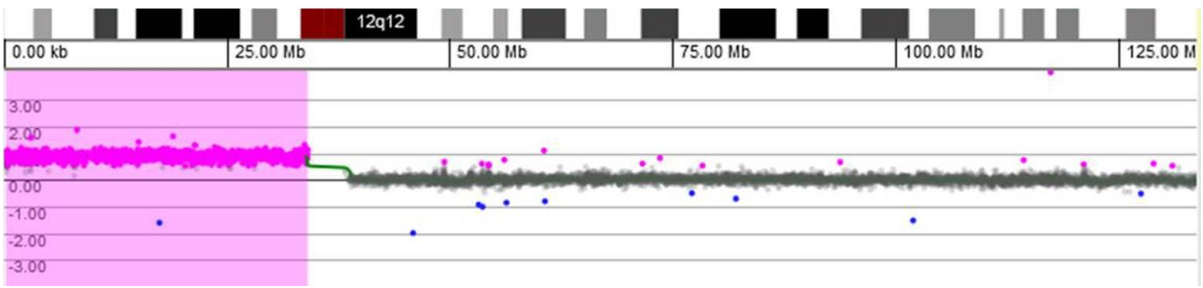


Figure II.46. Array CGH result for patient 1—DNA extracted from a buccal swab.

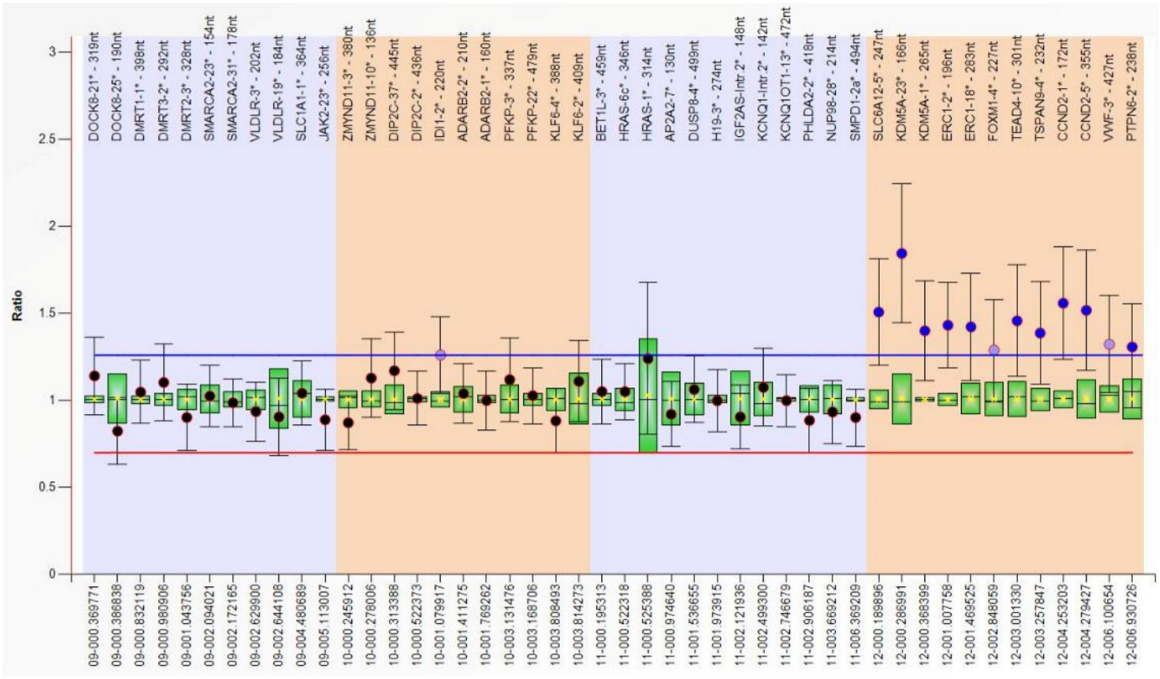


Figure II.47. MLPA result for patient 1—DNA extracted from buccal swab

3.2. Case 2

The second case is a male aged 16 y, the first child of a young, unrelated, apparently healthy couple. Pregnancy evolved with HTA, threatening miscarriage at 14 weeks. The child was born by C-section at 9 mo, with a weight of 3800 g (+1.2 SD), length of 51 cm (+0.5 SD), head circumference of 37 cm (+1.6 SD), Apgar score of 8. He was diagnosed at birth with hypospadias and bilateral talus valgus. He had frequent unexplained episodes of fever, frequent respiratory infections until age 7 y, temporary teeth extraction at 4 y, and at 8 y of age had adenoidectomy (anesthesia incident noticed). Seizures started after 3.5 mo of age. Marked developmental delay occurred (raised head at 4 y, not able to sit or walk). We first evaluated him at 1.5 mo, when we noticed: normal size; translucid skin with visible collateral circulation on head and abdomen; dry skin on the head and dorsal region; abnormal hair distribution with marked frontotemporal baldness; dysmorphic face (tall, broad, prominent forehead; sparse eyebrows and lashes; small cornea, no reaction to visual stimuli; short nose with anteverted nares; long and smooth philtrum; macrostomia with thin upper lip and everted lower lip; microretrognathia; minor ear defects); short neck; supernumerary nipple; upper limbs with rhizomelic shortening; hands with ulnar deviation of fingers, camptodactyly, flexed fist; deep palmar and plantar creases; bilateral talus valgus; hypospadias, bifid scrotum, large testes; marked hypotonia. At 14 mo, we noticed: brachycephaly; thick alveolar ridges; mispositioned teeth; deep palmar and plantar creases; shawl scrotum; marked hypotonia; and developmental delay. The last reevaluation was at 16 y, when he had: microbrachycephaly; mild frontotemporal baldness; dysmorphic face (sparse eyebrows and lashes, relatively small cornea, “pointed pupil”, flat zygomatic area, normal nose, short and prominent philtrum, macrostomia, minor ear defects); upper and lower limb spasticity; severe intellectual disability (figure II.48.).

Brain CT showed symmetric dilatation of basal cisterns and subarachnoidian space, moderate dilatation of the ventricular system. Babygram (1.5 mo old): hypoplastic humerus, delayed ossification of the cranial vault.

The first karyotype (from blood, performed at age 1 y) raised the suspicion of an addition on 22q, and in 1 out of 36 metaphases analyzed an i(12p) was identified (interpreted

as an artifact), however no further investigations were possible at that moment. The karyotype from skin fibroblasts (figure II.49.) was repeated at 6 y and established the diagnosis of PKS (result: mos47,XY,+i(12p); ish12pter (telpterx4) (40%)/46, XY (60%)). When we reevaluated the child at age 16 y, all genetic investigations (blood karyotype and MLPA, as well as MLPA using DNA samples extracted from buccal swab, hair root, and urine) were normal.

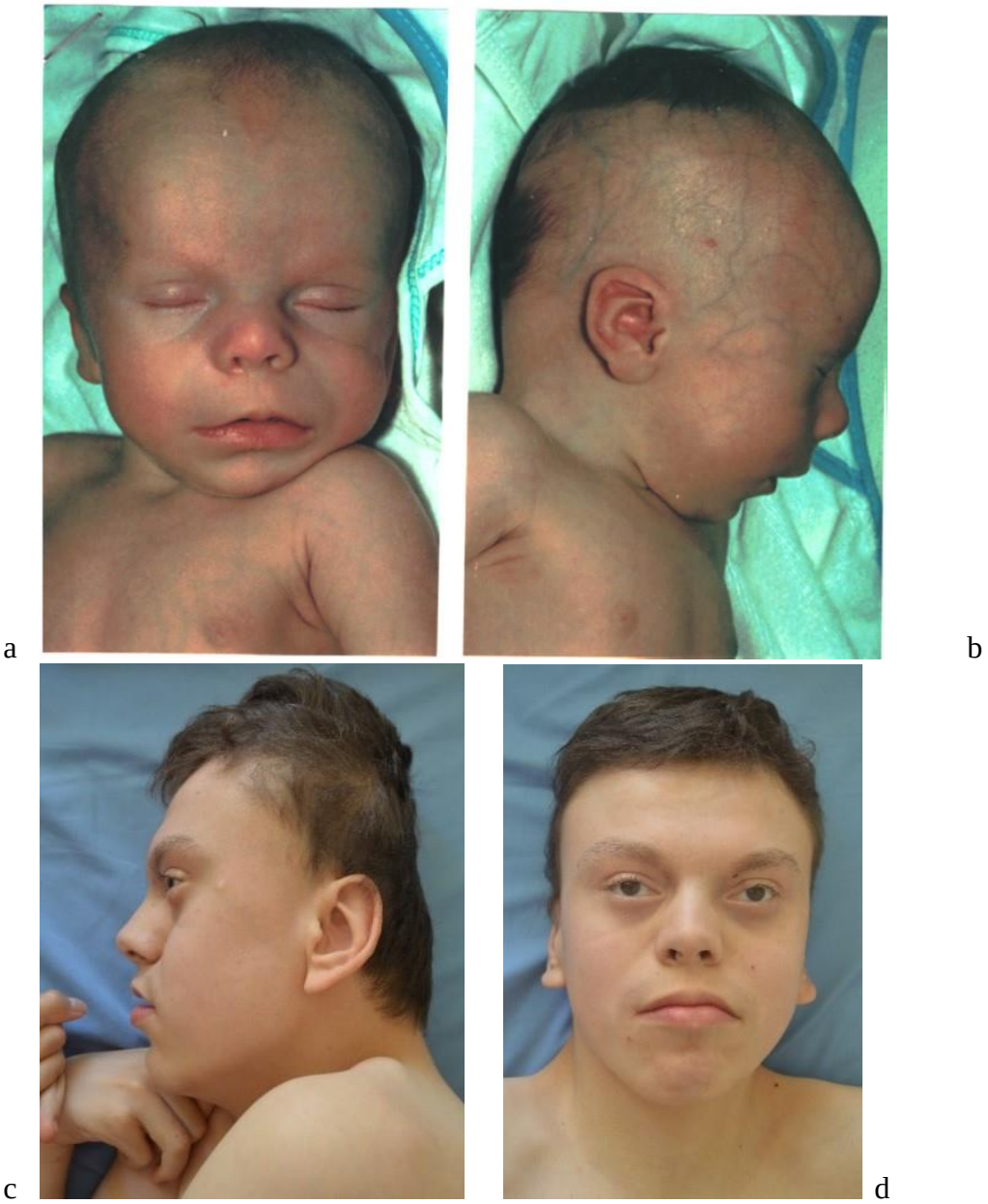


Figure II.48. Clinical features of patient 2:
a - 1.5 mo old; b - 16 y old.

The third patient is a girl aged 6 mo, the first child of healthy, unrelated parents, born at 36 weeks by C-section due to polyhydramnios, with a weight of 2840 g (−1.2 SD), length of 49 cm (0.0 SD), head circumference of 36 cm (+1.5 SD), Apgar score of 4/6/8. Physical examination revealed: normal growth, and dysmorphic face (prominent forehead, frontotemporal baldness, sparse eyebrows, hypertelorism, down slanting palpebral fissures, ptosis, epicanthal folds, broad nasal bridge, long philtrum, thin upper lip, everted lower lip, micrognathia, low-set malformed ears); marked hypotonia; delayed motor development with no head control, no syllables, no prehension; and a mental age around 2 mo. She presented epileptic seizures and infantile spasms. EEG revealed epileptiform discharges.

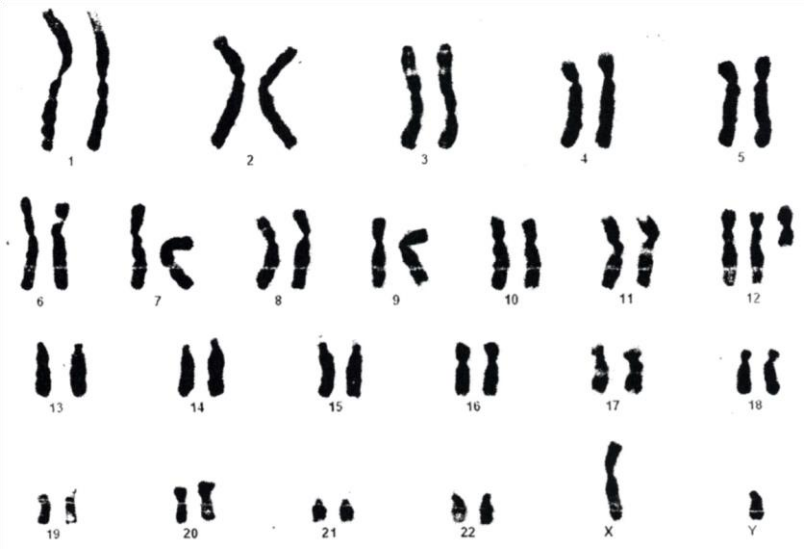


Figure II.49. Karyotype for patient 2.

3.3. Case 3

Patient genomic DNA extracted from peripheral blood was investigated by array CGH using commercial female DNA (Agilent) as a reference and an 8 × 60K human oligonucleotide array platform (Agilent Technologies). Data analysis was performed with Agilent Cytogenomics software using the reference Genome Build 37. Array CGH analysis revealed a gain of 12p13.33–p11.1, with a size of 34.52 Mb: arr[GRCh37] 12p13.33p11.1 (230,421_34,756,209) × 2~4 (ISCN2020). This gain was interpreted as a mosaic tetrasomy of 12p, thus confirming the PKS diagnosis. The G-banded karyotype performed on peripheral blood revealed no cytogenetic changes. Interphase FISH with 12p telomeric probes confirmed the presence of mosaic 12p tetrasomy in peripheral blood nuclei (figure II.50., II.51.).

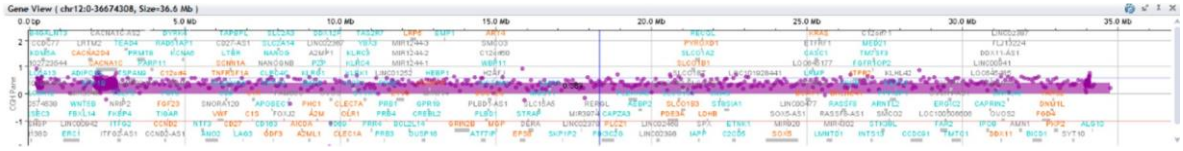


Figure II.50. Genetic tests for patient 3

array CGH result—DNA extracted from a peripheral blood;

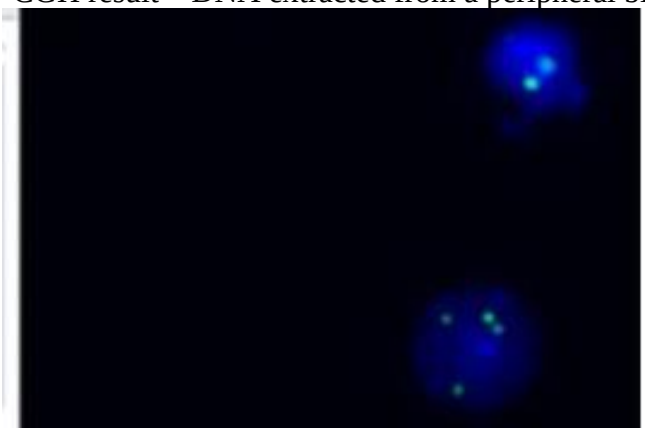


Figure II.51. Genetic tests for patient 3

interphase FISH—peripheral blood.

3.4. Case 4

The fourth patient is a girl aged 7 mo, the first child of healthy, unrelated parents, born at 32 weeks by C-section, with a weight of 2000 g (+0.3 SD), length of 42 cm (0.0 SD), head circumference of 31.5 cm (+1.6 SD), Apgar score of 7, but with good postnatal adaptation. Physical examination revealed: length of 65 cm (−0.63 SD), weight of 8950 kg (+2.0 SD), HC of 45.5 cm (+2.5 SD), dysmorphic face (prominent forehead, hypertelorism, downslanting palpebral fissures, partial palpebral ptosis of the left eye, broad nasal bridge, long philtrum, thin upper lip, everted lower lip, micrognathia, low-set malformed ears), muscle hypotonia, delayed motor development (she cannot roll over or sit; she says no syllables; she has no prehension, no smile, no interest in toys or objects); her mental age is around 2 mo. The girl also presented gastroesophageal reflux; her abdominal ultrasound was normal.

Array CGH analysis was performed using patient genomic DNA extracted from peripheral blood and genomic profile analysis revealed a deletion of 12p13.33 (0.174 Mb) and an interstitial gain of 12p13.33-p11.22 (28.28 Mb): arr[GRCh37] 12p13.33 (230,421_404,743) × 1, 12p13.33p11.22 (574,727_28,854,069) × 3 (ISCN2020) interpretated as duplication of 12p13.33-p11.22 flanked by a small deletion (figure II.52.). The deletion did not encompass genes considered relevant for the phenotype, while the partial trisomy overlaps 12p13.31 region, and among the 233 OMIM genes includes the candidate genes for the PKS phenotype [600]. The G-banded karyotype performed on peripheral blood confirmed the 12p duplication in this patient.

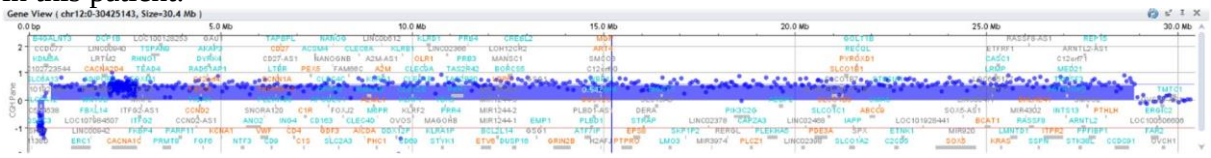


Figure II.52. Array CGH for patient 4 — DNA extracted from a peripheral blood.

3.5. Case 5

The fifth patient is a boy aged 2 y, the first child of healthy, unrelated parents. The pregnancy and birth were uneventful. He has delayed psychomotor development - he raised his head at 6 mo, sat at 14 mo, walked at 2 y, and said his first syllables at 18 mo. Physical examination revealed: a height of 91 cm (+1.8 SD), weight of 14 kg (+2.0 SD), HC of 49 cm (0 SD), dysmorphic face (prominent forehead, hypertelorism, down slanting palpebral fissures, broad nasal bridge, anteverted nostrils, long philtrum, thin upper lip, prominent and everted lower lip, dysplasia of dental enamel, low-set malformed ears, abnormally folded helix, preauricular pit), 5th finger clinodactyly, wide space between first and second toe, pectus carinatum, and cryptorchidism. Neurological examination showed hypotonia, delayed motor development, speech delay, and severe intellectual disability (a mental age of 8 mo). The psychological evaluation revealed a DQ of 34. The boy also presented recurrent wheezing, gastroesophageal reflux, and atopic dermatitis. Cerebral CT showed mild cortical atrophy.

Karyotype showed duplication with inversion of 12p12.3-12p13.3 [46,XY,dup(12)(p13.3p12.3)] , result confirmed by FISH with probes for chromosome 12 (Aquarius, Cytocell).

II.4.4.4. DISCUSSION

All patient data are described in detail for every patient, and both the literature data and patient information are summarized in Table II.39. We identified some novel and rarely cited clinical features concerning facial hair distribution, eye structure, skin and connective tissue,

immune defense, autonomic and sensory dysfunction, and seizures particularities that will be discussed in detail below.

Table II.39. Literature data (columns 2–4 for differential diagnosis) and patient data (columns 5–9).

Clinical Features	**Pallister – Killian syndrome**	**Trisomy 12p**	**Fryns syndrome**	**Sifrim–Hitz–Weiss**	**Case 1**	**Case 2**	**Case 3**	**Case 4**	**Case 5**
Frontotemporal baldness	+	−	−	−	+	+	+	−	−
Coarse face	++	−	+	+	+	−	+	−	−
Prominent forehead	+	+	−	+	+	+	+	+	+
Flat occiput	+	−	−	−	−→+	+	−	−	−
Long philtrum	+	+	−	+	+	+→−	+	+	+
Macroglossia	+	+	−	−	+	−	+	−	−
Cleft lip/palate	+/−	+	+	+	−	−	−	−	−
Everted lower lip	+	+	−	−	+	+→−	+	+	+
Accessory nipples	+	+	−	−	−	+	−	−	−
Focal aplasia cutis	+	−	−	−	+	−	−	−	−
Anormal pigmentation	+	−	−	−	+	−	−	−	−
Congenital diaphragmatic hernia	+	−	+	+	−	−	−	−	−
Deafness	+	+	−	+	+	−	−	−	−

Only features that make a difference between syndromes are presented; + present; − not present

The striking facial aspect noticed at the first clinical genetic evaluation of case 1 (2 mo of age) consisted of a marked excess of hair on the forehead and ears (figure II.45.), features that disappeared by the second evaluation at 5 mo. This could represent a particular variant of lanugo, as it was not generalized and disappeared in the first months of life. However, classical lanugo is found in premature babies and could be a sign of severe malnutrition, which is not the case in our patient. Moreover, hirsute face and distal digital hypoplasia are features rarely associated in Fryns syndrome and not in PKS [480]. This observation raises the question of whether PKS and PKS-like disorders in fact represent a spectrum ranging from Fryns syndrome (severe end) to PKS (moderate), trisomy 12p (moderate–mild), and Sifrim-Hitz-Weiss syndrome (mild end). Further studies are needed.

The eye malformation identified in case 1 consisted of the particular color of the iris (dark blue, except the part around the pupil, which is light blue) and miosis (“pointed pupil”), however with prompt reaction to light (figure II.45.). This aspect has been constant over time and is due to the stromal atrophy of the iris, as described by the patient’s ophthalmologist. The “pointed pupil” symptom was also noticed in case 2 but not in case 3. Iris atrophy has rarely been described [258, 552], however it probably should be investigated in children with suspicion of PKS.

Other craniofacial changes with age were provided by case 2, who had 16 years of follow-up: the skull (normal initially) became markedly brachycephalic in time, frontotemporal baldness diminished with age, zygomatic areas became very flat in time, and the nose changed totally (from flat and broad with anteverted nostrils to a normal, pointed nose). The perioral

region and ears remained unchanged. Occipital and zygomatic flattening was also noticed in case 1 (figure II.45.). A possible explanation for occipital flattening over time could be prolonged periods spent lying in bed in the same position due to severe hypotonia. Studies in the literature mention that with age, the face becomes coarser and the chin becomes more prominent. In our cases, face coarseness did not change over time (present in case 1 , absent in case 2), however prognathism was noticed in case 2 at an older age. Malar flattening is cited in the literature [480], however very few authors mention it. Because we have noticed this in two out of three PKS cases, we suggest the use of dedicated software for photo analysis could provide the entire description of phenotype changes over time.

Patient 1's skin is soft and puffy, which is the reason why the child was suspected and investigated for hypothyroidism. Thyroid investigations have been repeatedly normal. However, such soft, doughy skin (which later became translucent, with visible blood vessels) is associated with iris stromal atrophy, umbilical hernia, hip dislocation, and delayed tooth eruption (case 1). Case 2 presents translucid skin with visible collateral circulation, which made us think of a possible connective tissue defect, however further studies are necessary to confirm this hypothesis. Moreover, ectodermal derivatives are also abnormal (lanugo; temporofrontal baldness; fine or sparse eyebrows and lashes; slow-growing, fine, depigmented hair; slow-growing nails; abnormal tooth eruption), with some of these features having not yet been reported in the literature.

Immune defects are not documented in the literature on PKS so far. Patient's 1 and 2 have experienced repeated episodes of infection. However, these infections are difficult to identify, as child 1 developed no fever during infection and child 2 had frequent unexplained episodes of fever, suggesting hypothalamic dysfunction. As our initial immunological tests were normal, lymphocyte subpopulation analysis should be performed. The autonomic dysfunction is also supported by the presence of days with long periods of very deep sleep, which in case 1 was noticed by the mother. The immune phenotype seems to be associated with a particular immune reaction, and such particularities have not yet been described in PKS and probably should be investigated in confirmed cases.

Patients 1–3 present the typical growth pattern cited in the literature for some of the PKS cases [50, 115], including initial macrosomia, followed by postnatal growth deficit evident by one year of age. The explanation is that PKS individuals have elevated IGFBP2 that binds IGF1. The reduced IGF1 available will lead to growth deficit [258]. We were not able to evaluate IGFBP2 in case 1, but because we found low IGF1, we expect that our patient has elevated IGFBP2, explaining the noticed postnatal growth deficit. Moreover, we found a small pineal gland in brain MRI and high ACTH. We appreciate that more in-depth endocrine investigations should be performed to elucidate the entire mechanism and provide eventual therapeutic options. Periods of lethargy noticed by the mother in case 1 could be related to the dysfunctional brain, however also to episodes of hypoglicemia, a reason why we plan to monitor blood sugar levels in our patient.

Classical sensory impairments described in PKS are deafness and (central) blindness [64]. Both are present and relatively severe in patient 1, contributing to the severe developmental delay of the child. Moreover, we have tried to test her ability to smell and found no reaction, however we think this is a very difficult test for the age of 1 y and 5 mo and we should test her again when she has grown older. Cases 2–3 do not seem to have sensory impairments but were not tested in detail.

Seizures have been a constant feature in all of our PKS cases. Myoclonic jerks have been noted in case 1 from the first day of life, which intensified after 1 y of age. In PKS seizures are extremely rare neonatally and they become evident later in life [170]. Detailed anamnesis referring to mild types of seizures (e.g., myoclonic jerks) should be performed in any confirmed PKS case. Contractures have been present from an early age in case 2, which progressed to

marked spasticity of upper and lower limbs. These features have rarely been described in the literature.

Regarding diagnostic laboratory methods, the initial karyotype (from cultured peripheral blood) was normal for case 1, however due to the suggestive PKS features, we performed array CGH using DNA extracted from a buccal swab. This test confirmed the presence of 12p tetrasomy (figure II.52.).

However, we performed telomeric MLPA on DNA samples from different sources (blood, buccal swab, urine) to prove the existence of 12p tetrasomy using an alternative, cheaper method. Surprisingly, 12p tetrasomy was identified only in the buccal swab. We offer two possible explanations for this result: either the 12p tetrasomy is only present in some organs, or because we performed this test when the child was already 1 y and 5 mo old of age, by this time abnormal cells could have disappeared due to the specific selection against i(12p) positive cells.

We suggest MLPA (follow-up kit P230-B1) should be done in children under suspicion of PKS using samples from different tissues (buccal swab should definitely be one of them). In case 2, the first karyotype identified the isochromosome, but because it was found only in 1 out of 36 cells read, it was considered an artifact.

Thinking retrospectively, using interphasic FISH with 12p probes would probably have provided the diagnosis earlier.

Moreover, a comprehensive clinical evaluation is essential for infants with multiple congenital anomalies and severe developmental delay. For such cases, blood analysis may in fact be enough to diagnose PKS within the first months of life.

In our experience, the association of dysmorphic face with marked developmental delay and seizures is highly suggestive for the diagnosis of PKS, and MLPA should be performed as a first intention test using DNA samples from blood and a buccal swab. An alternative method is array CGH using a DNA sample extracted form a buccal swab. In confirmed cases, sensorial dysfunction should be investigated.

Trisomy 12p is defined by similar facial characteristics to PKS (but without frontotemporal baldness), associated with hypotonia and developmental delay, however no other major anomalies are associated. The cases presented here are typical and the diagnoses have been clearly established using array CGH. However, because array CGH is a laborious and relatively expensive method, we recommend using subtelomeric MLPA first (especially because some cases of trisomy 12p provide for a balanced anomaly in one of the parents, and the 12p duplication could be associated with other partial monosomy). For confirmed cases, as the trisomy could be inherited, a karyotype is indicated for both parents.

Differential diagnosis between PKS, Fryns, and Sifrim-Hitz-Weiss syndrome (table II.39.) is an important issue, as the syndromes are clinically very similar, however the genetic causes are totally different. We suggest that these aspects should be evaluated before proceeding to genetic testing.

II.4.4.5. CONCLUSIONS

We have identified features that have not yet been or that are rarely reported in PKS studies i.e., marked excess of hair on the forehead and ears in the first months of life, a particular eye disorder (abnormal iris color with pointed pupil), connective tissue defects, repeated episodes of infection and autonomic dysfunction, endocrine malfunction as a possible cause of postnatal growth deficit, more complex sensory impairments, and mild early myoclonic jerks. MLPA (follow-up kit P230-B1) and array CGH using DNA extracted from a buccal swab are reliable methods of diagnosis in PKS and we recommend them as the first intention diagnostic tests.

II.4.5. STUDY OF GORHAM-STOUT DISEASE

II.4.5.1. BACKGROUND

Gorham-Stout disease (GSD) is a very rare condition characterized by massive osteolysis and lymphangiomatosis. Approximately 300 such cases were described in the literature [441]. GSD represents one of the idiopathic osteolysis according to Hardegger's classification [220]. Recently, the classification of The International Society for the Study of Vascular Anomalies considers GSD a simple vascular malformations IIa—Lymphatic malformations among other diseases [256]. GSD is a multifactorial disease caused by genetic predisposing factors or mosaicism for a somatic mutation [136]. Among the genes involved in various stages of pathogenesis are: *PTEN*, *TNFRSF11A* and *TREM2* [104].

Symptoms depend on the location of the osteolytic process and present as: localized pain, swelling, weakness and functional impairment of affected limbs, respiratory distress and failure, neurological defects, deformity, paralysis, and death [136].

II.4.5.2. CASE REPORT

An 18-year-old Caucasian female, with no family history of bone disease, was admitted to the infectious diseases unit with weakness, myalgia, significant bone pain, and febrile syndrome. A lumbar puncture excluded meningeal syndrome. After five days, an incomplete motor deficit was installed in the upper limbs and the patient was transferred to the neurosurgery service. Subsequently, the patient had multiple hospital admissions in different medical departments and was evaluated by a multidisciplinary team in order to establish a definite diagnosis, neuromotor recovery, and neurosurgical treatments.

The chest X-ray requested by the neurosurgery department showed osteolytic lesions in C6-C7, T1, and the posterior arch of the first left rib and left clavicle. Computed tomography (CT) and cervical-dorsal magnetic resonance imaging (MRI) examination revealed C6-C7-T1-T2 osteolytic lesions and C7 pathological fracture (Figure II.53.).

Some tumor markers (CA 15-3, CA 19-9, CA 125, carcinoembryonic antigen) had normal values. Normal values of creatine kinase-MB, rheumatoid factor, antithyroglobulin antibody, and circulating immune complexes, excluded autoimmunity. The laboratory tests showed no increase in inflammation markers at onset, but subsequently a nonspecific inflammatory syndrome was present with a high erythrocyte sedimentation rate of 80 mm/hour, and C-reactive protein, and fibrinogen at levels of 61.8 mg/L and 527 mg/dL, respectively.

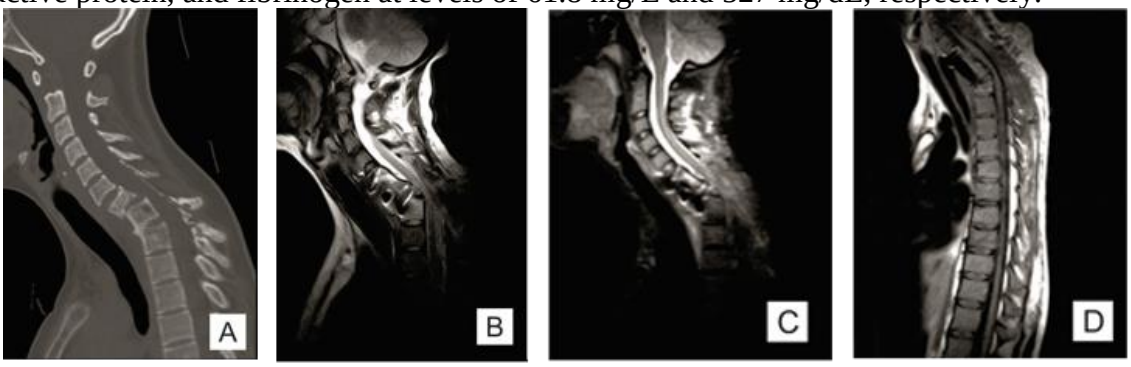


Figure II.53.MRI aspect of spine
A – osteolitic lesions C6-C7, T1-T2, pathologic fracture C7 vertebrae; B sagittal T2 MRI; C sagittal STIR MRI; and D sagittal T1 MRI: important spinal canal stenosis at C6–C7 level up to an anteroposterior diameter of 5 mm, the medullary cord presents in this area discreetly T2-STIR hypersignal.

The patient had normal fasting bone turnover markers with only the beta isomer of C terminal telopeptide of type 1 collagen slightly increased (0.68 ng/mL, normal range ≤ 0.573

ng/mL). Bone mineral metabolism markers, ionized calcium, magnesium, phosphorus, and PTH, were normal except for 25-hydroxy vitamin D, which was low (15.4 ng/mL).

A bone marrow biopsy showed osteolysis, replacement of bone tissue with connective tissue, and chronic non-specific inflammation (Figure II.54.).

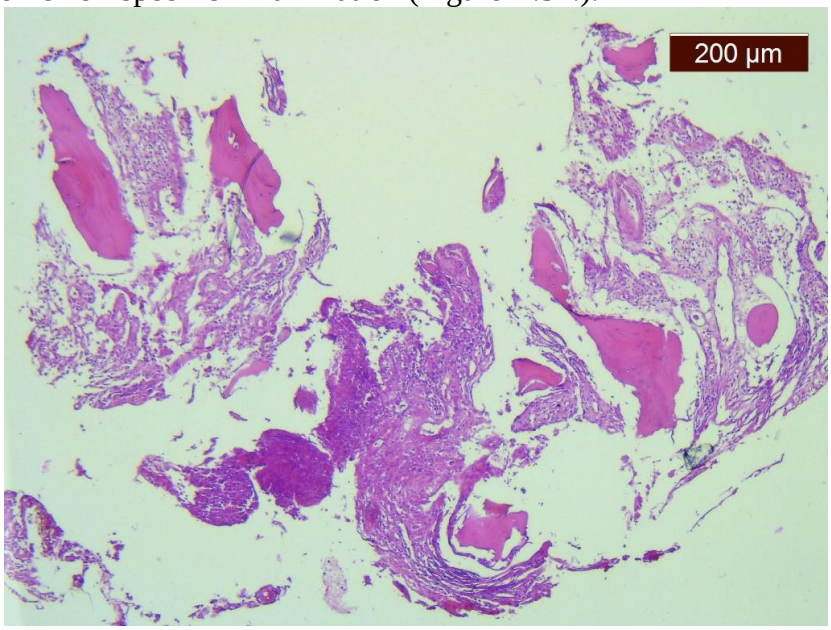


Figure II.54. Bone marrow biopsy - fibrosis, foci of osteolysis, and moderate inflammatory infiltrate with lymphocytes and plasma cells (HE, ×50).

The diagnosis of Gorham-Stout disease was established based on the clinical and imaging elements, and exclusion of thyroid or parathyroid disease, lymphoma, neoplasia, and autoimmune disorders.

The evolution was unfavorable and rapidly progressive with a severe motor deficit in the lower limbs and cervicothoracic pain. The CT scan and the MRI examination showed the mechanical deterioration of the previous fixation system, cervical spine dislocation with maximum angulation angle at the C6-C7 level, and spinal stenosis at the C6-C7 level (Figure II.53. B–D and Figure II.55.).

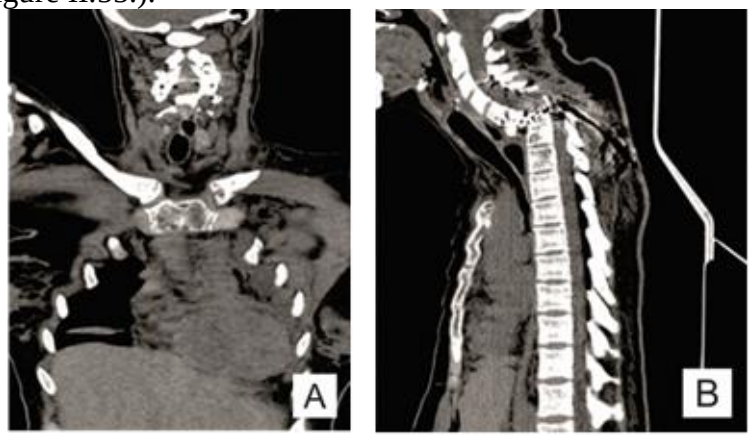


Figure II.55. Thoracic CT
A – pseudocomplete osteolisis of left clavicle with absence of acromial end; B - cervical spine dislocation with maximum angulation angle at the C6–C7 level, spinal cord compression at this level, and bone lysis in the C6 and C7 bodies

During evolution, osteolysis interests other areas: lumbar vertebrae, pelvic bones, and femur. An abdominal CT showed a normal sized spleen, but with multiple splenic nodules.

The treatment was neurosurgical, but only paleative with ablation and reconstruction of most damaged vertebraes.

The main complications included rapid and severe neurological deficits, chylothorax and mechanical damage to the anterior fixation system, and, over time, damage of the osteosynthesis material. Pleural effusion appeared in the first months and gradually increased in quantity, and laboratory tests confirmed presence of chylothorax. The neurological deficit was progressive to spastic paraplegia, an inability to walk and stand. The patient received recovery therapy during hospitalizations in the neuromotor recovery clinic and pharmacological treatment, but with modest results.

Unfortunately, death occurred due to neurological complications in association with the presence of chylothorax that generated an impairment of the respiratory function.

II.4.5.3. DISCUSSION

GSD is a rare disease and has no gender or race predilection [213]. Rare diseases affect less than 5 individuals per 10,000 people [109].

There are no specific tests or biomarkers for the diagnosis of GSD. The positive diagnosis imposes the exclusion of thyroid or parathyroid disorders, cancer, lymphoma, autoimmune disorders, and others syndromes with massive osteolysis. Numerous hospital admissions in different departments were necessary in order to complete the diagnosis. Different tests were performed and a multidisciplinary approach was applied. The diagnosis was established seven months after the onset.

Heffez established a set of diagnostic criteria for GSD: (1) biopsy showing angiomatous tissue or fibrous connective tissue; (2) absence of cellular atypia; (3) minimal or no osteoblastic response and absence of dystrophic calcifications; (4) evidence of progressive local bone resorption; (5) the lesion is not ulcerative and does not provoke cortical expansion; (6) absence of visceral involvement; (7) osteolytic radio-graphic pattern; and (8) negative hereditary, metabolic, neoplastic, immunological, and infectious etiology [104, 226].

In our case, the first two criteria were met because the bone marrow biopsy showed osteolysis, replacement of bone tissue with connective tissue, and no sign of cellular atypia. The patient showed minimal osteoblastic response because she had normal fasting bone turnover markers with only β-CTX slightly increased. Normal bone mineral metabolism markers and the absence of dystrophic calcifications are the evidence for the third criterion. Local bone resorption was progressive. The onset of lytic lesions were described in the left clavicle and, in just a few months, the imaging examinations showed almost complete osteolysis of the left clavicle with an absence of the acromial end. The cortex of the bone was affected by destruction and resorption, without cortical expansion, which is consistent with criterion 5. There was no visceral damage, except the splenic nodules seen on the abdominal CT scan. The association between characteristic progressive bone lesions and splenic and soft tissue lesions is pathognomonic for GSD [305].

Four stages were described in terms of radiological appearance. In our patient's case, within a few months of the onset, there were radiological lesions in all stages: stages I-III (spine), stages I-II (ribs, pelvic bones, and femur), and stage IV (clavicle). The negative family history and the normal results of the laboratory tests excluded other pathologies (thyroid or parathyroid disease, lymphoma, neoplasia, and autoimmune disorders), thus fulfilling the last criterion proposed by Heffez [106, 226, 305, 333, 619].

Clinical Features

The clinical picture is heterogeneous in GSD and depends on the involved structures and the progression of osteolysis [331]. The average age of onset is 25 years, but age extremes can be reached (from 1 month to 75 years of age) [68]. In some cases, a previous trauma has been

described [470]. In our case, the onset of disease was before the age of 20, and there was no history of trauma.

Symptoms presented in GSD include localized pain, swelling, weakness and functional impairment of affected limbs, respiratory distress and failure, neurological defects, deformity, and paralysis. The clinical features of osteolysis are non-specific, and often GSD patients present pathological vertebral fracture or a symptomatic vertebral deformity [146]. At the time of onset, our patient had a pathological fracture of the C7 vertebra and the manifestations were significant: bone pain, weakness, myalgia, febrile syndrome, and an incomplete motor deficit in the upper limbs.

Osteolysis can affect both the axial skeleton and the appendicular skeleton. Theoretically any bone can be affected, but it seems that the most commonly involved are: maxillofacial region in 30% of cases (especially the mandible), clavicle, ribs, cervical vertebrae, pelvis and femur [136, 331].

A comparative study between GSD and GLA showed that the appendicular skeleton is involved in only 26.3% of patients with GSD compared to patients with GLA (87.5%). The ribs were the bones most frequently involved in both situations, followed by the skull, clavicle, and cervical spine in GSD, while in GLA bone involved are thoracic spine, humerus, and femur [313, 334, 400]. In GSD pathological fractures are more frequent than in GLA [411]. In GSD one or more bones are affected with progressive cortical osteolysis, visceral thoracic, or abdominal damage, and pleural effusions [596]. In our patient's case, osteolysis foci were multiple, both in the axial (vertebrae and ribs) and appendicular (clavicle, pelvic bones, and femur) skeleton.

Pathogenesis

GSD is characterized by massive progressive osteolysis and vascular proliferation [397]. Several main hypotheses were proposed in the etiopathogenesis of GSD: local hypoxia/acidosis, endothelial dysplasia, increased osteoclastic activity or sensitivity of osteoclast precursors to humoral factors, lack of thyroid C cells and calcitonin, and lymphatic vessel proliferation [104].

Rossi et al. observed that 75% of osteoclasts of patients with GSD presented suggestive elements for a more motile phenotype (lamellipodia, stress fibers, and membrane ruffling) and an increased activity of more than 4.5 times. Transcriptomic analysis showed enrichment of some pathways involved in osteoclasts differentiation and function (angiotensin II-stimulated signaling through G proteins and beta-arrestin, the PI3 kinase pathway, and the EGF receptor signaling pathway). At the level of osteoblasts in patients with GSD, it is an increase of gene expression for *MMP13* and *RXFP1*; its overexpression in fibrocartilaginous cells increased matrix metalloproteinase-13 and 9) [468].

There are no biomarkers available for GSD, but in some studies miRNAs and serum biomarkers were reported as possible biomarkers. There are studies that show changes in miRNAs in GSD, suggesting that these molecules could be used as potential biomarkers in GSD. Some of these molecules of miRNAs (miR-1246, miR-137, and miR-1) are correlated with angiomatous proliferation and regulation of osteoclastogenesis and altered osteoclast morphology resulting in a more motile phenotype. Other miRNA molecules (miR-204-5p, miR-378a-3p, miR-615-3p, and miR-204-5p) play a role in inhibiting osteoblast differentiation and are elevated in GSD [469].

The characteristics of osteolysis are spontaneous, idiopathic, and progressive without new bone production [60]. Histopathological lesions are represented by massive loss of the bone matrix, and proliferation of thin-walled capillary-sized vascular channels or fibrous connective tissue [104]. Not always the progressive bone resorption is followed by significant vascular proliferation. However, lesions are progressive and can spread to nearby bones or adjacent structures [199]. Bone resorption can stop spontaneously and this it is the most common

evolution. There are some cases where lesions have remained stable for decades without any reossification [68].

Treatment

There are no standardized treatment protocols, but there were trials of pharmacological therapy, surgery, radiotherapy or combinations therein [154]. Pharmacological treatments used included: interferon, bisphosphonates, calcium salts and vitamin D; interferon and zoledronic acid; cyclophosphamide and fluorouracil; and salmon calcitonin, alendronate sodium, and sirolimus [154, 609]. Sirolimus is a mammalian target of rapamycin (mTOR) inhibitor, which has antiangiogenic activity and the effect of improving clinical symptoms and quality of life in GSD patients [412]. Probably the best pharmacological treatments should include an anti-osteoclast drug, anabolic drug, and angiogenesis inhibitors [468]. The surgical techniques are usually associated with complications such as infections, nerve and vascular damage, recurrence of osteolysis, and graft resorption. Chylothorax requests thoracic duct ligation, pleurodesis, and chest drainage [249]. Surgical treatment and radiation therapy are preferred in cases with extensive lesions and functional instability [286, 397].

In our case, the treatment was a combination of surgery, pharmacological, and recovery therapy. Initially, a corpectomy and reconstruction was performed with autologous graft and fixation with a cervical-thoracic hybrid system, but in time mechanical damage appeared to the anterior fixation system and, later, damage of the osteosynthesis material. Recovery therapy (physiotherapy, massage, and kinetotherapy) and pharmacological treatment brought only mediocre short-term improvements. Chest drainage was performed for chylothorax.

Life Expectancy, Complications, Mortality

In most cases the progression of the disease is slow, but the prognosis remains unpredictable [60]. The involvement of certain bones such as ribs, scapula, and thoracic vertebrae can cause chylothorax and nerve root compression with paraplegia, and are considered negative prognostic factors because life-threatening complications can occur [68, 331]. Chylothorax has been identified in 17% of cases with thoracic involvement and could be the consequence of extension of the disease into the pleural cavity or thoracic duct involvement [305]. Overall mortality was estimated at 13%, but mortality increased to 30% when osteolysis involved the spine, with spinal cord compression or transection of the spine. Mortality increased to 43.6–50% in cases of chylothorax [68].

The unfavorable prognostic factors in our case were the involvement of cervical and thoracic vertebrae, ribs that produced chylothorax, nerve root compression with paraplegia, and, finally, respiratory failure and death.

II.4.5.4. CONCLUSIONS

Gorham-Stout disease is a multifactorial one, which may have a poor prognosis and should be considered after the exclusion of other pathologies with massive osteolysis. Osteolytic lesions of the spine and pleura are poor prognostic factors because of the compression of spinal nerves and the presence of chylothorax.

II.4.6. STUDY OF HOLT – ORAM SYNDROME

II.4.6.1. BACKGROUND

Holt-Oram syndrome (HOS) is a rare monogenic disorder characterized by upper limb abnormalities, congenital heart defects (CHD) and/or conduction abnormalities [32]. HOS is the most common heart-hand syndrome and usually associated with an atrial septal defect (ASD). [622]. HOS was described by Mary Clayton Holt and Samuel Oram in 1960, when they reported members of a family with thumb abnormalities and ASD. The birth prevalence of HOS is estimated to be about 0.7 in 100,000 births without gender differences [409].

HOS is an autosomal dominant condition caused by a mutation in *TBX5* gene. Majority of the mutations are *de novo*. *TBX5* is expressed in heart, forelimb, lungs and eyes [189]. *TBX5* gene consists in nine coding exons and belongs to T-box domain, transcription factors involved in development regulation [436]. The cardiac expression of *TBX5* gene is produced in heart tube, sinus venosus, atria, atrioventricular canal, left ventricle (LV), and right ventricle trabeculae [386, 436]. In heart development, many gene networks are involved and *TBX5* interacts with *GATA4* gene and presents a synergistic action with *Nkx2-5* gene in coactivation of *ANF* gene [231]. Also, in heart development is produced a *TBX5*-nucleosome remodeling with consequences in cardiac septation. *TBX5* gene is involved in forelimb specification and outgrowth and plays also a role in dorsoventral patterning of the eye [579, 590].
Mutations in TBX5 gene were identified in more than 70% of cases with clinical signs of HOS. More than 70 mutations (missense or nonsense substitutions, large deletion of multiple exons, frameshift mutation, intragenic duplications) have been described in HOS. These mutations generate a haploinsufficiency, with a low DNA binding activity, or have a dominant negative effect on downstream targets [360].
The disease is a pleiotropic disorder with complete penetrance, but with a variable expressivity, even in the same family. Clinical diagnostic criteria of HOS include CHD and/or conduction defects and preaxial radial anomalies in at least one upper limb. The abnormalities may vary in presentation, from structural cardiac anomalies to conduction disorders, respectively from an abnormal carpal bone or triphalangeal thumb to bilateral phocomelia. Sometime, upper limb abnormalities can be very subtle, with only a delayed carpal bone age on wrist radiographs [36].

II.4.6.2. CASE REPORT

A young male presented surgical scar of the thumb (after pediatric history of polydactyly of the right hand that was operated at the age of 4 years), dilated cardiomyopathy and severe mitral regurgitation. Other cardiac changes were valvular changes (thickened aortic valve, mitral valve (MV) prolapse) and ostium secundum ASD with left to right shunt diagnosed at age of 3 years. However, the patient had no cardiac symptoms and a normal effort tolerance. Clinically, he presented a perioral cyanosis and a systolic 2/6 murmur at the apex.

The electrocardiogram (ECG) reveals a HR of 85 bpm, a heart axis of +60°, peaked P waves (P pulmonale) in II, III, aVF and LV hypertrophy (Sokolow Lyon criteria).

The transthoracic echocardiogram (TTE) shows a mildly dilated LV (adjusted for body surface area, 3.2 cm/m^2), but with the rest of cardiac chambers within normal limits, a thickened MV, mainly the anterior MV, with an adjacent calcified cord. The MV closes with grade 2 - 3 mitral regurgitation with eccentric jet pointing to the posterior wall of the left atrium, but without the appearance of MV prolapse. The aortic valve appears tricuspid, thickened. An ostium secundum ASD is noted, with left to right shunt. The TTE also reveals trabeculations of the LV lateral wall, septal and anterior wall hypokinesis, and global LV ejection fraction of 50%.

Due to the multiple changes that have been found on TTE, we turned to transesophageal echocardiography (TEE) for a better assessment of associated lesions and to visualize a possible MV prolapse as etiology of the mitral regurgitation. Thus, it has been noticed that the aortic valve was actually bicuspid, the right coronary cusp and the noncoronary cusp being welded together by a hyperechoic raphe (Figure II.56.). No MV prolapse aspect was noticed. Bubble contrast echocardiogram was performed with the detection of left to right shunt by negative contrast effect (Figure II.57.).

Starting from association between cardiac abnormalities and polydactyly, we presumed a genetic disorder that affects both heart and hand. Radiologic investigation of upper limbs showed a delayed bone age (16 years age), small sized bilateral trapezium and trapezoid bones

that are partly overlapped, hypertrophic bilateral pisiform bones and a metacarpophalangeal angle over 5° at the third and forth digits (Figure II.58).

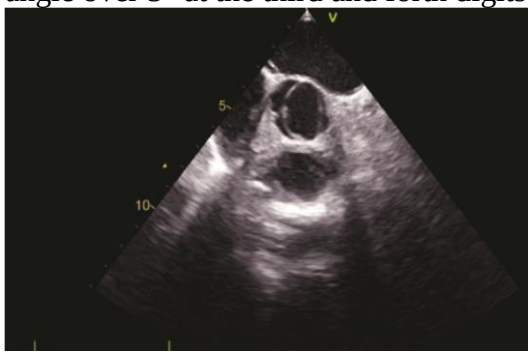


Figure II.56. TEE - bicuspid aortic valve.

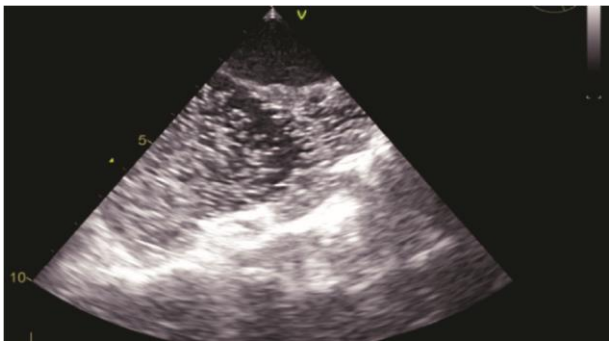


Figure II.57. TEE - negative contrast effect showing the left to right shunt in ASD.

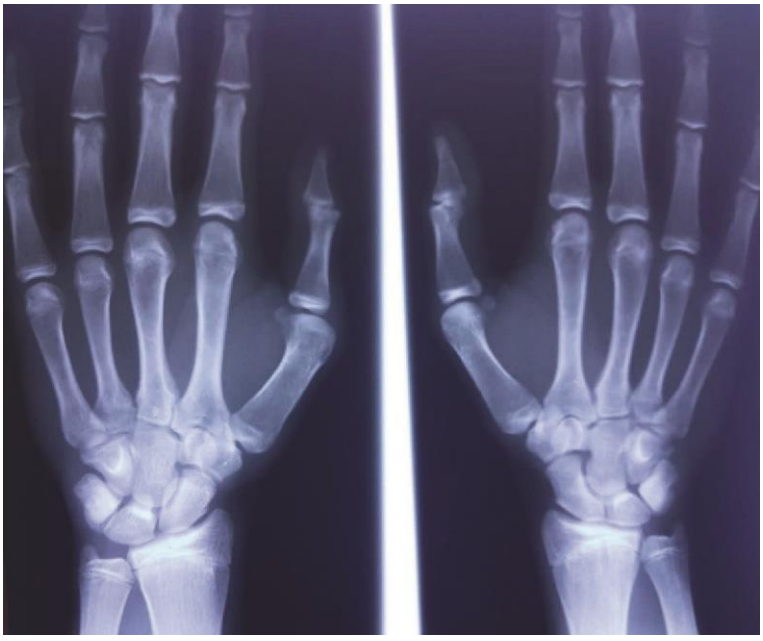


Figure II.58. Posteroanterior hand radiograph

Genetic testing was done by MLPA using the SALSA MLPA P180 Limb malformations-2 probe mix (MRC-Holland, Amsterdam, Netherlands) and result was normal.

The family history for HOS was negative. Two unsymptomatic family members (mother and sister's proband) are screened by clinical exam, ECG, TTE and posteroanterior radiograph of hands, and we not found pathological changes. Also, the father and a brother were without clinical manifestations.

II.4.6.3. DISCUSSION

The management of patients with HOS involves a multidisciplinary team consisting of a specialist in cardiology, orthopedics, and medical geneticist.

Radial defect is the most common deficiency of the upper limb. It is characterized by modifications of radius (hypo- or aplasia), carpal bones (scaphoid and trapezium), thumb and metacarpal bone of the thumb. Manifestations are bilaterally in the vast majority of cases, and the left hand is severely impaired compared to the right one [414].

In our case it seems to be polydactyly type IV (Wassel classification) with complete duplication of both phalanges but with only one metacarpal bone. The abnormalities of carpal bones are bilateral, but supplementary thumb was present only on the right side. The carpal bones changes were: bilateral hypertrophic pisiform, small bilateral trapezium and trapezoid

bones and delayed bone age. An abnormal large pisiform was described in some cases of HOS [438]. Delayed bone age was a radiological sign in others patients with HOS [274].

CHD are one of the most frequent congenital abnormalities in 1% to 2-3% newborns. The incidence of CHD in HOS is high (75%). The clinical effects could be severe. In complex CHD the mortality remained high, despite the progresses of cardiac surgery. More defects were identified in our patient: ASD, bicuspid aortic valve, LV noncompaction. ASD is the most common CHD in HOS. A few cases of LV non-compaction in HOS have been reported in the literature, apparently not determined determined by *TBX5* mutation, but further studies are needed [466]. The risk consists in the development of subsequent cardiomyopathy, arrhythmias and thromboembolism [427]. Patients are at risk for cardiac conductive defects, first-degree atrioventricular block or right bundle branch block. In our case, we noted intraventricular conduction disorder manifesting as slurring of QRS in aVL and V3. Atrial fibrillation, atrial flutter or sudden cardiac deaths have also been reported [438].

HOS is inherited in an autosomal dominant manner, with only 15% of cases familial. Sometime, is present an apparently negative familial history by failure to recognize the signs and symptoms of HOS, early death of a person, and mild phenotype. In our case, family history was negative for HOS,

The genetic tests are recommended to all first-degree relatives if the mutation is known in proband, otherwise the tests like ECG, TTE and wrist radiograph are recommended for the screening of clinical changes. Posteroanterior wrist radiograph is very important because the carpal bones abnormalities are present in 100% of the cases and sometimes are the unique sign of this disorder [360]. Mother and sister are normal in our cases, and father and brother will be investigated.

Genetic testing by MLPA was normal in our patient, but this result does not rule out the HOS because this technique does highlight only *TBX5* gene deletions/duplications and on the other hand the pathogenic variant was identified just in 74% of cases with strict clinical criteria [359].

II.4.6.4. CONCLUSIONS

HOS is a pleiotropic disorder, in which the main anatomic regions implicated are the heart and the upper limbs. Because of variable expressivity the diagnosis is sometimes delayed. An early diagnosis is necessary for preventing complications in patients and for identifying other family members at risk.

II.4.7. STUDY OF OSTEOGENESIS IMPERFECTA

II.4.7.1. BACKGROUND

Osteogenesis imperfecta (OI) represents a heterogeneous group of connective tissue diseases that have major clinical signs of bone fragility that cause fractures, bone deformities and small stature. Prevalence is estimated at between 1/10,000 and 1/20,000 [247, 248].

Initially, OI classified into four entities on the basis of clinical, radiological and transmission patterns, most of which being caused by mutations in the *COL1A1* and *COL1A2* genes. The discovery of other genes involved in the pathogenesis of the disease allows a change of classification for OI, today being recognized 19 types of OI (table II.40.). [327, 423].

OI types I-IV are determined by mutations in the genes *COL1A1* and *COL1A2*. The transmission pattern is an autosomal dominant one for OI type I-V, autosomal recessive for OI type VI-XVIII and X-linked recessive for OI type XIX. The pathophysiological mechanisms in which the products of the genes involved interfere are: impairment of collagen synthesis and structure (*COL1A1, COL1A2)*, compromised bone mineralization (*IFITM5, SERPINF1*), abnormal collagen post-translational modification (*CRTAP, P3H1, PPIB*), compromised collagen processing and crosslinking (*SERPINH1, FKBP10, SP7, PLOD2*), altered osteoblast

differentiation and function (*BMP1, TMEM38B, WNT1, CREB3L1, SPARC, TENT5A, MBTPS2*) (table II.40.) [175, 327].

Table II.40. OI classification – genetic and clinical characteristics [175, 247, 248, 327, 353, 571]

OI type	***OMIM***	***Gene***	***Mechanism***	***Inheritance***	***Bone deformity***	***Sclerae***	***Hearing loss***	***DI***
I	166200	*COL1A1/ COL1A2*	Impairment of collagen synthesis and structure	AD	Rare to very severe	N, grey to dark blue	Absent to common	Absent to common
II	166210	*COL1A1/ COL1A2*		AD	Rare to very severe	N, grey to dark blue	Absent to common	Absent to common
III	259420	*COL1A1/ COL1A2*		AD	Rare to very severe	N, grey to dark blue	Absent to common	Absent to common
IV	166220	*COL1A1/ COL1A2*		AD	Rare to very severe	N, grey to dark blue	Absent to common	Absent to common
V	610967	*IFITM5*	Compromised bone mineralization	AD	Variable	N -blue	Infrequent	Absent
VI	613982	*SERPINF1*		AR	Moderate/severe	N	Absent	Absent
VII	610682	*CRTAP*	Abnormal collagen post-translational modification	AR	Severe rhizomelia	N, grey	Absent	Absent
VIII	610915	*P3H1*		AR	Severe rhizomelia	N	Absent	Absent
IX	259440	*PPIB*		AR	Severe	Grey	Absent	Absent
X	613848	*SERPINH1*	Compromised collagen processing and crosslinking	AR	Severe	Blue	Absent	Present
XI	610968	*FKBP10*		AR	Mild to severe	N, grey	Absent	Absent
XII	613849	*SP7*		AR	Severe	N	Absent	Absent
XIII	614856	*BMP1*	Altered osteoblast differentiation and function	AR	Mild to severe	N	Absent	Absent
XIV	615066	*TMEM38B*		AR	Severe	N to blue	Absent	Absent
XV	615220	*WNT1*		AR/AD	Severe	White	Absent	Absent
XVI	616229	*CREB3L1*		AR	Severe	-	-	-
XVII	616507	*SPARC*		AR	Progressive bone fragility	White to slightly grey	Absent	Absent
XVIII	617952	*TENT5A*		AR	Moderate	Blue	-	-
XIX	259440	*MBTPS2*		XR	Moderate/severe	Blue	-	-

N – normal; DI - dentinogenesis imperfecta; AD – autosomal dominant; AR – autosomale recessive; XR – X linked recessive; *COL1A1* - Collagen, type I, alpha-1; *COL1A2* - Collagen, type I, alpha-2; *IFITM5* - Interferon induced transmembrane protein 5; *SERPINF1* - Serpin peptidase inhibitor, clade F, member 1; *CRTAP* - Cartilage associated protein; *P3H1*- prolyl 3-hydroxylase 1; *PPIB* - Peptidylprolyl isomerase B/cyclophilin B; *SERPINH1* - Serpin peptidase inhibitor, clade H,member 1/heat shock protein 47; *FKBP10* - FK506 binding protein 65; *SP7* - Transcription factor 7/osterix; *BMP1* - Bone morphogenic protein1/procollagen C proteinase; *TMEM38B* - Transmembrane protein 38 B; *WNT1* - Wingless-type MMTV integration site family, member 1; *CREB3L1* - cAMP responsive element binding protein 3 like 1; *SPARC* - SPARC/osteonectin; *TENT5A* - Terminal nucleotidyl transferase 5a; *MBTPS2*- Membrane-bound transcription factor protease, site 2

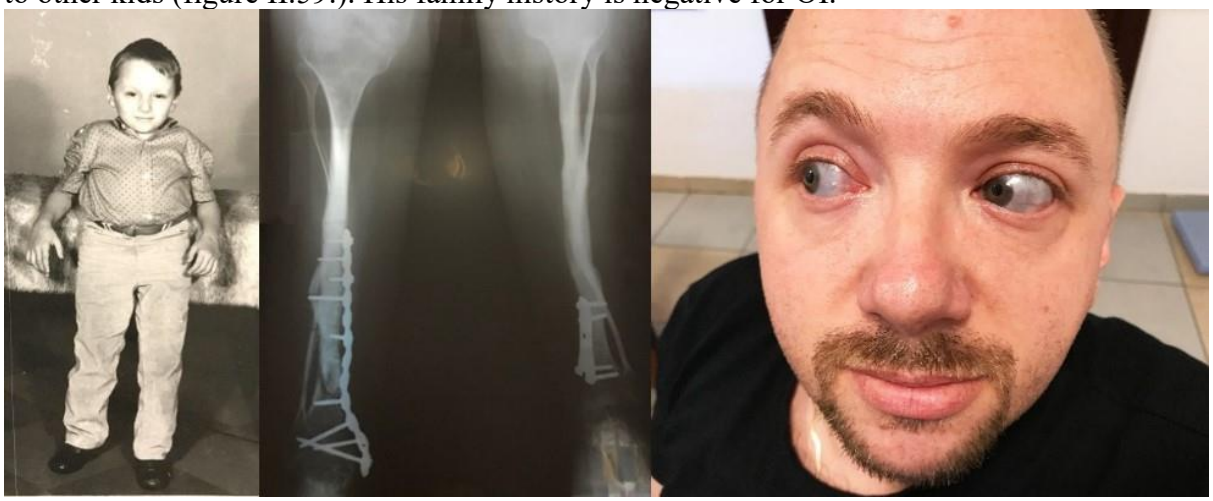
The penetrance for pathogenic variants in *COL1A1* and *COL1A2* genes is 100%. Expressivity is variable even within the same family [531].

The primary feature is liability for fractures (>90% of total cases) and osteoporosis. The associated features in some individuals and in some types of OI are: blue sclerae, young adult onset hearing loss, dentinogenesis imperfecta, joint hypermobility, short stature and progressive skeletal deformity [574].

There is a correlation between *de novo* variants and disease severity. While 60% of patients with mild OI have *de novo* variants, the majority (~100%) of individuals with progressive deforming disease / perinatal mortality have *de novo* variants [531].

II.4.7.2. METHODS

The couple asked genetic counseling because the woman is pregnant (first trimester) and the partner was diagnosed with OI. The consultant (37 year old patient) was diagnosed at birth with OI because he had a clavicle fracture and blue sclera. Since birth, he had many fractures (over 60), mostly on his legs, consequently now presents a shortened stature (1.36 m). In childhood, due to numerous fractures, he had a steady decrease in his condition compared to other kids (figure II.59.). His family history is negative for OI.



a b c

Figure II.59. Phenotype of proband

a. Proband at 5 years old; b. Bone splint technique for recurrent fractures; c. Blue sclerae

At the time of the first genetic consultation the consultant has short stature and blue sclera. He does not associate dentinogenesis imperfecta and deafness. The theoretical risk of the fetus to have the same disease is maximum 50%. The consultant was not molecularly tested.

For the consultant, a Clinical Whole Exome Sequencing test was performed focusing on 3583 OMIM disease genes and Exome of 20370 genes with target region capture followed by NGS.

By amniocentesis was obtained foetal material that cultured. After DNA extraction, a targeted sequencing was performed on both DNA strands of the relevant *COL1A1* region (the reference sequence is: COL1A1: NM_000088.3.).

II.4.7.3. RESULTS

Direct DNA sequencing analysis of *COL1A1* gene revealed a splicing mutation (c.1155+1G>C or IVS17+1G>C) in heterozygous state. This mutation is in the intron region in the first position following the 1155th base of the DNA strand, from G to C and significantly affects the binding of Exon 17 with intron 17 resulting in the transcriptional deletion of exon 17.

Mutation has been reported pathogenic being present in the LOVD database with 7 entries, all being substitutions in intron 17 (pathogenic splice-site variants). The frequency of this mutation in the normal population is very low (<1% in 1000 human genome project, dbSNP, ESP6500, BGI internal databases). The carrier of the mutation state was excluded for the foetus, so the pregnancy has been pursued further and a healthy baby was born at term.

To predict the consequences of mutation we used BDGP (Berkeley Drosophila Genome Project) algorithm that showed a significant impairment of exon 17 binding with intron 17 (boundaries) resulting in the transcriptional escape of exon 17. Prediction analysis of pathogenicity using Human Splicing Finder indicates alteration of the WT donor site, most probably affecting splicing. By using of S-CAP (Splicing Clinically Applicable Pathogenicity

prediction) 5 ′ core dominant we obtained a score of 0.834 corresponding to the heterozygous pathogenic variant status.

II.4.7.4. DISCUSSION

The prenatal diagnosis for OI should be considered in three main situations: 1) there is the risk of the birth of a child with OI in a family with autosomal dominant form of OI; 2) risk of OI type AD when the affected parent is the only one in the family; 3) there is risk of germinal mosaicism [425]. In our case we are in the second situation.

Because OI is a disease with important genetic heterogeneity and we needed an urgent prenatal diagnosis, the use of ordinary Sanger sequencing are time consuming and cost expensive. In this situation, the effective approach for screening mutation of osteogenesis imperfecta is whole exome sequencing [280, 346, 593].

The *COL1A1* gene is 17q21.33 and contains 52 exons. Gene expression of the *COL1A1* gene generates two different transcripts that are used for synthese of a protein of 140 kDa. Exons 6-49 produce alpha helical domain and each have a number of base pairs multiple of 9 [243].

Collagen type I consists of a triple helix consisting of two alpha1 chains (encoded by the *COL1A1* gene) and one alpha 2 chain (encoded by the *COL1A2* gene). Type I collagen is predominantly in the connective tissue of bones, corneas, dermis and tendons. Obviously, mutations in the *COL1A1* gene are more deleterious than those in the *COL1A2* gene.

The genetic defect in OI 1 is the reducing type 1 collagen (quantitative defect) or altering the structure (qualitative defect). In the first situation the phenotype is milder, while in the second situation the phenotype is more severe [175, 565].

The types of mutations in the *COL1A1* included in the Human Gene Mutation Database (HGMD®) are: missense / nonsense mutations -476; splicing mutation -234; regulatory -4; small deletion - 192; small insertions -61; small indels - 10; gross deletions - 24; gross insertions - 2; complex -2. In general, splicing mutations represent 8.72% of the total mutations inventoried in HGMD [532].

In LOVD the total number of variants was 2007. Most variants were reported in exon 37 (80 variants), exon 44 (69 variants) and exon 11 (68 variant). Most variants are substitutions (1541), and of these 302 are intronic as in the case of our proband. In intron 17 were described 16 variants [246]. The splicing mutations is the second most frequent and could generate exon skipping, intronic inclusion or activation of cryptic sites in introns and exons [18, 354].

In our case, WES focused on 3583 OMIM diseases and exome of 20,370 genes. Point mutations, micro-insertions, deletions, duplications (<20 bp) in the analyzed genes can be detected simultaneously. The global coverage is over 95%. According to the American College of Medical Genetics and Genomics (ACMG) there are five mutation types: “pathogenic”, “likely pathogenic”, “uncertain significance”, “likely benign” and “benign” [18].

Splice donor mutations are more common compared to splice acceptor mutations (1.5:1). The classic mutations are located in positions +1 and +2 at level 5 ′, respectively -1 and -2 at level 3 ′ [306].

As can be seen from the table II.41., the mutation identified in our patient was described in other seven cases: 5 mutations in OI type 1, two in OI II and one in OI IV. Only in two cases were the mutations identified through WES.

The pathogenicity of nucleotide substitutions was confirmed in 112 cases and in 906 cases pathogenicity prediction programs were used. In our case, the prediction algorithm using BDGP showed significant impairment of exon 17 binding with intron 17 resulting in the transcriptional escape of exon 17.

Prediction analysis of pathogenicity using Human Splicing Finder indicates alteration of the WT donor site, most probably affecting splicing. The use of the S-CAP showed a score

of 0.834 corresponding to the heterozygous pathogenic variant status. The program uses the classification of mutations into 6 regions: 5 ′ GT (5 ′ core) and 3 ′ AG (3 ′ core) dinucleotides, intronic variants upstream of a 3 ′ splice site (3 ′ intronic), variants lying in the canonical U1 snRNA– binding site, excluding the core 5 ′ SS (5 ′ extended) intronic variants downstream of a 5 ′ SS, and synonymous variants within the protein-coding gene (exonic). For each region the S-CAP score has a threshold that differentiates between benign and pathogenic mutations [262].

Table II.41. Characteristics of the mutation present in the proband [246]

Number	***Type***	***OI***	***Methods***	***Remarks***
1	Splice site	OI I	PCR, SEQ	ni
2	Splice site	OI I	unknown	Skips exon 17 (minor), activates exon 17 cryptic splice site
3	Splice site	OI III	PCR, SEQ	ni
4	Splice site	OI IV	unknown	Skips exon 17
5	Splice site	OI I	PCR, SEQ	ni
6	Splice site	OI I	HRM, SEQ	ni
7	Splice site	OI III	WES	ni
8	Splice site	OI I	WES	Skips exon 17

ni-no information; no 8 – our proband

Jagadeesh et al divides the variants into the regions mentioned above and using the score on each region classifies them into pathogenic and benign. It can thus be observed that the most common pathogenic variants are in the 3′core region and the 5 ′ core region, and most benign variants in the 3 ′ intronic, exonic, 5 ′ intronic region (table II.42.).

Table II.42. Comparison between pathogenic and benign variants in different regions of the gene [244]

Region / **Variant**	***3′ intronic***	***3′ core***	***exonic***	***5′ core***	***5′ extended***	***5′ intronic***
Pathogenic	1419	5835	317	7044	2279	165
Benign	2357091	42880	1899048	58942	210618	2191871

II.4.7.5. CONCLUSIONS

Osteogenesis imperfecta is a rare condition characterized by genetic heterogeneity (allelic, nonallelic, clinical). WES is the technique to be applied as the first intention to screen for mutations of genes involved in OI pathogenesis. In silico analysis of the pathogenic variant using dedicated programs is absolutely necessary to establish the classification of the variant in the 5 categories of mutations, especially because this is extremely important in the prenatal genetic counselling.

III. FUTURE DIRECTIONS OF CAREER'S DEVELOPMENT

Developing one's teaching career is closely related to maintaining the motivation for professional development. The perception of a teaching position and the position taken by a specialist depend on the compatibility between what the individual considers to be suitable for himself (skills, needs, preferences) and what the position actually involves (constraints, opportunities, responsibilities). Career guidance is therefore a relatively stable pattern of talents, values, attitudes and occupational activities.

The specific roles of the university teaching staff are multiple: teaching and educational abilities, research and expert roles, institutional and community activities, professional and personal development.

Given my experience in teaching and research, my next actions will be focused on several directions, the main aim being to continue my professional development and increase the quality of research.

The main objectives are: completing professional standards, developing basic skills, increasing professional performance based on continuous self-assessment, in accordance with performance criteria.

Scientific research requires a work style and a pattern of conduct involving a serious approach when working on the chosen theme, moral and professional responsibility, teamwork and communication of the research results, which must be correctly interpreted and present scientific value.

According to the trends in Genetics research my main areas of research will be:

- developing research projects at global level, such as:
- A. Nutrigenomics (identifying correlations between the genetic status of the patient and his/her nutritional profile or the use of certain nutrients as foods/drugs)

  Considering the alarming increase in human pathology associated with dietary imbalances I believe that one future research direction could be the genetic characterization of Romanian patients with metabolic syndrome - diabetes, obesity and cardiovascular disease - by identifying potentially pathogenic gene polymorphisms. However, the potential benefits of nutrigenomcs, along with other genetic or genomic clinical applications, require balancing with potential socio-ethical risks, promotion of personalized nutrition interventions and individual responsibility. Key risk factors raised by the socio-ethical context in which NGS applications will be implemented need to be considered.

  Modern genetics has advanced beyond single-gene disorders to gene families, exomes, transcriptomes, and ultimately, to the complete genome. Severe challenges in data processing and interpretation remain, but the enormity and potential closure of the undertaking is inspiring. The completion of the Human Genome Project was the starting point for new projects towards complete understanding of the function of genes and of the regulatory regions in the genome. Sequencing data are revealing unexpected functions for non coding regions of the genome and also the effect of the variability among individuals. Most traits, diseases and responses to environmental stimuli are highly complex, with environmental factors and genetic networks of interactions. The factors and genetic network underlying a trait may be elucidated by a combination of bioinformatics approaches, and the emergent properties of such approaches may be more revealing than the search for individual candidates for a trait or process. The identification of genomic variation in large numbers of individuals helps to distinguish neutral variants ('non-pathogenic' variants) from variants disrupting gene function ('pathogenic variants'). Identification of genotype-phenotype correlations in various genetic diseases and especially those related to the prenatal and neonatal period and the

development of diagnostic methods adapted to certain genetic diseases should be the key to improving the diagnosis of monogenic and multifactorial diseases in the next decades.

- **B. Malformative pathology** (improving diagnostic methods in order to identify the genetic causes of congenital anomalies would allow better preventive measures).

  According to the World Health Organization (WHO), 6.9 million children under the age of five died in 2011 – nearly 19 000 children each day. Efforts to improve child survival can be effective only if they are based on accurate identification of the causes of diseases, to determine the effectiveness of disease-specific interventions, and to assess trends in disease burden in relation to national and international goals. Starting from data gathered through research project PN-II-PT-PCCA-133/2014 on the theme of "Implementing a diagnostic algorithm based on complex analysis of the genomic profile for patients with congenital and developmental abnormalities", I hope to continue these researches in direction of elucidation the molecular mechanisms causing congenital and developmental abnormalities. The final targets are: designing better prevention measures and clinical management and eventually, more specific therapies. Phenotypic and microarray data collection will improve clinical interpretation of genomic results from individuals with rare genetic disorders. Array CGH, Next Generation Sequencing, PCR, MLPA, MS-MLPA are the most advanced methods for assessing genomic anomalies and have demonstrated the potential to clarify unexplained phenotypes at the molecular level and to identify candidate gene with alterations at genomic level. Transfer of these technologies from research to clinical practice is a necessary step for the national implementation of new prevention and intervention methods in accordance with European standards. The genome profiling diagnostic algorithm that we hope to develop will provide a uniform tool for the investigation of complex disorders and will allow prevention, prediction and personalization of medical care.

- C. Prenatal Diagnosis (Validation of methods and some minimally invasive diagnostic protocols for both the pregnant patient and the fetus).

  Karyotyping is the predominant technique for prenatal diagnosis of chromosomal abnormalities, but most chromosome banding techniques are time-consuming and limited to resolutions of 5 to 10 Mb. Conventional karyotyping only identifies chromosomal anomalies in about 35% of pregnancies with fetal ultrasound abnormalities, depending on the types of these anomalies.

  Molecular cytogenetic techniques such as fluorescent in situ hybridisation (FISH), quantitative fluorescent PCR (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) overcome some of those limitations and are used as adjuncts to conventional methods for detecting common chromosome numerical anomalies, but none of them provides a genome wide screening for unexpected imbalances. Array-based comparative genomic hybridization (array-based CGH) technology can simultaneously evaluate regions across the entire genome and allow for detection of unbalanced structural and numerical chromosome abnormalities of less than 100 kb. The array-based CGH platform used for clinical prenatal diagnosis is able to enhance the detection rate by 10 to 16% of pregnancies with foetal ultrasound anomalies but not detected by conventional cytogenetic or other molecular cytogenetic techniques. Array-based comparative genomic hybridization possesses a number of significant advantages over conventional cytogenetic and other molecular cytogenetic techniques, providing a sensitive and comprehensive detection platform for unexpected imbalances in the genome wide.

Low-coverage whole-genome sequencing of maternal plasma DNA was highly accurate in detecting common trisomies. It also enabled the detection of other aneuploidies and structural chromosomal abnormalities with high positive predictive value. The discovery of cell-free fetal DNA in maternal plasma and the invention of massively parallel sequencing (MPS) have made non-invasive prenatal testing (NIPT) for some aneuploidies a clinical reality.

In the field of prenatal diagnosis, I have two targets: to continue the research concerning a non-invasive method of prenatal diagnosis of trisomy 21 and to apply a molecular method like array-CGH or NGS to confirm the possible pathologic cases. The first target is based on the results of the research carried out using an internal grant from the University of Medicine and Pharmacy "Grigore T. Popa" (Internal Grant UMF Iaşi 28210 / 16.12.2011 on the theme of "Quantification of methylated fetal DNA - a new approach to non-invasive prenatal diagnosis of trisomy 21"). Thus, I plan to develop a method of investigation based on molecular medicine, a topical method in the planned project, validating diagnostic protocols with major impact in the area of prenatal diagnosis.

The major objective is to adapt and validate a new method for non-invasive prenatal diagnosis of the unbalanced chromosomal abnormalities. With the subsequent implementation in clinical practice of this new test, it will be possible to eliminate the risks of classical invasive prenatal diagnosis. In addition, I hope to validate a quick method with sensitivity and specificity close to 100%. This study involves the following scientific objectives: identifying a target group of pregnant patients with high risk of aneuploidy, molecular testing by combining two methods of molecular biology MeDiP Q-PCR and Real Time, statistical and mathematical interpretation of results, data validation through a conventional prenatal diagnosis, linking and integration of information in order to achieve an innovative protocol for prenatal diagnosis.

The ideal setting to advance prenatal diagnosis and increase its resolution would be to apply a molecular method in high risk pregnancies in conjunction with chromosomal analysis. This will increase the detection rate for likely pathogenic CNVs up to 5%. To avoid interpretation problems these arrays should cover all known pathogenic CNVs and have a low-resolution backbone for the detection of relatively large CNVs thus keeping the detection of CNVs of unclear significance to the minimum. The implementation of this method, corroborated with the improvement of the prenatal screening method, will ameliorate the quality of prenatal diagnosis and reduce the number of children with chromosomal abnormalities.

- D. Reproductive disorders (developing protocols and algorithms for diagnosis and investigation in cases of sterility or infertility in order to discern the etiology and choose the optimal method of reproduction)

  Current clinical evaluation of infertile couples is relatively simple. After a history and physical examination, circulating hormone levels are assessed, semen analyses are performed and a genetic evaluation is restricted to the current limits of clinical testing: a karyotype and perhaps a Y chromosome microdeletion test.The diagnoses are usually descriptive of the observed problem, with no mechanistic insight into aetiology. Research advances could be used to cure infertility, and to ensure the birth of a healthy baby for all couples desiring parenthood. Future genetic marker studies in large samples with detailed phenotypic and clinical information will yield valuable insights into disease risks, disease classification and co-morbidity for many diseases associated with reproduction. Novel genes and pathways will provide new targets for biomarker discovery and new drug targets for drug development or repositioning of drugs currently on the market or in clinical trials. The real benefits will the convergence of

genetics, genomics and biological research in well-phenotype data sets to develop methods of diagnosis and treatment for the many common diseases associated with reproduction. Future directions will include analysis of rare coding variants and functional annotation of variants in regulatory regions through continuing advances in genetics and genomics. In future, I will try to perform a research study in this direction by applying array-CGH or other molecular methods that could improve the diagnosis of reproductive troubles and will give hope to childless couples.

- E. Oncogenetics (evaluation of the genetic causes of cancer and validation of protocols for the investigation of hereditary or familial cancers that would allow detection of individuals at risk and their early treatment)

  Cancer is a genetic disease caused by an accumulation of mutations leading to uncontrolled cell growth and proliferation. Genetic changes in cancer can be either inherited through the germ line, resulting in cancer susceptibility, or acquired as somatic mutations. Aberrations implicated in tumorigenesis include point mutations and extended genome rearrangements, such as translocations, inversions, small insertions/deletions, and copy number variants (CNVs). Cancer is a gradual process of accumulating genetic alterations that drive the transformation of normal cells into highly proliferative cancer cell types. Genome rearrangements have long been recognized as hallmarks of human tumours and have been used to diagnose cancer. Techniques used to detect genome rearrangements have evolved from microscopic examinations of chromosomes to the molecular based approaches. The availability of next-generation sequencing technologies may provide a means for scrutinizing entire cancer genomes and transcriptomes at unparalleled resolution. Of particular interest are the methods that have been used to detect genome rearrangements and the potential that next-generation sequencing technologies may offer to the field. Cytogenetic methods have had a significant impact on our understanding of cancer and ultimately led to the development of an effective treatment for chronic myelogenous leukaemia. The development of aCGH methods and other microarray based approaches has revolutionized research into structural genomic variation in cancer cells because such methods have enabled high-throughput interrogations of cancer genomes at higher resolutions. These technologies are suitable for the detection of specific types of variation, such as CNVs. Advancements in next-generation sequencing technologies speak of the promise of cost-effective resequencing of cancer genomes and transcriptomes at an unprecedented depth, allowing the simultaneous detection and quantification of multiple molecular events on a genome-wide scale. It has become evident that tumour genomes contain a vast spectrum of aberrations that include a small number of characterized mutational hot spots and a large number of rare changes. Since both of these classes likely harbour cancer-driving mutations, the need for more resequencing studies is unquestionable if we are to approach a comprehensive view of cancer genomes. Another emerging feature of cancers is their heterogeneity, whereby the genomes of individual tumours, as well as cell subpopulations within the same tumour, show sequence variation. Studies of such variation will be enabled by single-molecule sequencing technologies. However, computationally efficient methods for the analysis of short read data and the effective handling of repeats must be developed and implemented.

  Developing research in this direction is provided as I am part of the multidisciplinary team within the Oncogenetics Department at the University of Medicine and Pharmacy "Grigore T. Popa" Iasi. The immediate objectives of this project are to identify families at risk of hereditary cancers, such as breast or colon cancer, followed by the

identification of the type of mutation, which will improve the prevention of these maladies.

- F. The Pathology of Rare Diseases Project (multinational teams participating in research aimed at this vast area, would enable the development of research protocols to improve the care of these patients) that I am part of and use to sign up for the grants competitions organized at national or international level;

  Since the field of rare diseases in Europe is a priority area of medical research for the years 2014-2021, I will try to establish a multinational partnership that would attract European funding in order to enable us to investigate genetic disorders at molecular level with the best tools available.

- Provision of feasible solutions to improve the material resources of the subject and careful use of the equipment with a view to improving scientific research;
- Obtaining the certificate in the shortest time possible would give me the ability to manage the PhD students in the medical genetics field, and would increase the prestige of Iasi medical school, allowing young researchers to choose the most current research topics;
- Preparation of monographs covering different areas of medical genetics that could be a first for the Romanian medicine (from Mendelian Genetics to Genomics, Epigenomics, Chemogenomics, Nutrigenomics) or would mean solving some problems that have not been addressed in the recent literature (Clinical Cytogenetics);
- Validate research results through the development of articles to be published in international journals or indexed in international databases – a minimum of 1 ISI paper/ year and 2-3 papers BDI/year (as main author);
- Attending national and international conferences with a high level of scientific communications;

It is clear that future research efforts cannot be achieved only at individual level, so an important goal will be to authorise and collaborate with other colleagues from the department so we could integrate in national or international interdisciplinary teams. In addition, I believe that these teams should also include young people (PhD students, interns, graduate and Masters students) whose enthusiasm would increase the energy of the team.

In academic medicine, diagnosis and teaching activities are intricate and form a whole. Academic career development involves continuous study and improvement of knowledge, which is useful in the development of the medical genetics profession – represented by the genetic counselling consultation and the application of advanced cytogenetic techniques or molecular genetics to certify the clinical diagnosis.

The main directions of development for the medical activity will be: prenatal genetic diagnosis, reproductive disorders, diagnosis of malformation, and validation of new diagnostics laboratory collaboration with specialists from other fields. All these fields require a process of continuous accumulation of medical information, which I propose to capitalize by participation at congresses as guest lecturer.

In this respect, the main actions proposed are:

- The organization of national and international scientific seminars in the field of genetics with the participation of key speakers;
- Active participation in the specialized committees of the Ministry of Health (Medical Genetics and Rare Diseases Commission);
- Permanent improvement of medical care through continuous information, conducted by consulting databases, specialist websites or magazines;
- Learning new techniques for diagnosis of genetic diseases and their introduction into clinical practice;

- Development of assessment and investigation protocols for patients / couples with reproductive disorders in women with amenorrhea (primary and secondary) or men with azoospermia;
- Collaboration with local or foreign specialists for the diagnosis of patients with complex clinical symptoms.

In addition, it is important to communicate information gathered during the process of research and medical diagnostics. To this end, I propose several actions summarized below:

- Develop educational materials with sufficient imagery, representative of the researched topics, presented in good IT formats (video and PowerPoint) and periodically revised and completed;
- Develop themed modules (sets of lectures, learning packages based on the course structure);
- Prepare and publish revised editions of the university courses tailored to each direction (at a maximum 5 year interval);
- Work with colleagues from other universities in an effort to develop a common course of Medical Genetics;
- Improve topics and courses offered to interns from various medical fields who study medical genetics;
- Develop and expand the postgraduate programs, both through courses for various categories of doctors and for Masters and PhD programs;
- Select students expressing an interest in genetics through a themed workshop, where they can expand their initial level of theory and practice, on the basis of which they will be able to develop scientific papers that will be presented in communication sessions; then, based on this focal point I will select the best ones, channelling them to research topics that will be continued in their PhD programs. In this context, I believe that PhD students should be encouraged to gain mandatory skills to conduct research in genetics topics like:
  - PhD students will acquire the basics of fundamental genetics;
  - develop interest and motivation for the study and application of genetics in medical practice*;*
  - understanding medical genetics development prospects, given its transdisciplinary character;
  - understand how microscopic and molecular genetics techniques are applied;
  - engage in scientific documentation in the field and exploit the knowledge gained through the development of scientific papers / participation in conferences and congresses;
  - support PhD students in research projects development
  - encourage PhD students to participate in and obtain mobility grants abroad;
  - develop theses of high scientific quality and finalise their studies

I consider that all this actions will bring me the elements necessary for a better development in career and also the professional prestige that will confer to Medical Genetic School of Jassy a leader position in Romania.

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