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ROLES OF GLUTATHIONE-S-TRANSFERASE P1 (GSTP1) GENE, IN PROSTATE CANCER DETECTION

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Abstract

Prostate cancer is the leading cause of cancer related death in most developed countries, and is the most common malignancy in males.

Early detection of prostate cancer, multiple biopsies, and improved treatment currently represent the most critical strategies to decrease prostate cancer mortality.

The development of prostate cancer is a multi-step process through a series of morphologically distinct lesions initiated by genetic and epigenetic changes.

DNA methylation is a covalent chemical modification resulting in the addition of a methyl group (CH₃) group at the carbon 5 position of the cytosine ring. Even though most cytosine methylation occurs in the sequence context 5'CG3' (also called the CpG dinucleotide) some involves CpA and CpT dinucleotides [1]. DNA is made up of four bases, thus there are 16 possible dinucleotide combinations that occur. The human genome is not methylated uniformly and contains regions of unmethylated segments interspersed by methylated regions [2]. In contrast to the rest of the genome, smaller regions of DNA, called CpG islands ranging from 0.5 to 5 kb and occurring on average every 100 kb, have distinct properties.

DNA methylation is brought about by a group of enzymes known as the DNA methyltransferases (DNMT). The DNMTs known to date are DNMT1, DNMT1b, DNMT1₀, DNMT1p, DNMT2, DNMT3A, DNMT3b, with its isoforms, and DNMT3L [3].

In addition to the DNMTs, the other machinery of methylation includes demethylases, methylation centers triggering DNA methylation, and methylation protection centers [4, 5]

The best characterised gene found to be hypermethylated in prostate cancer is GSTP1, encoding the π-class glutathione S-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogenesis.

Methylation is highly tumour-specific but also prevalent in high-grade prostatic intraepithelial neoplasia lesions, which makes GSTP1 an attractive early detection biomarker.

Hypermethylation and inactivation of genes involved in DNA repair, such as GSTP1, may serve as initiating genome lesions for tumour development by increasing susceptibility to carcinogens, thus predisposing to further mutations and DNA damage.

GSTP1 functions in the conjugation and detoxification of potential carcinogens and has been demonstrated to have “caretaker activities”.

Promoter hypermethylation accompanied by loss of GSTP1 is one of the earliest and most common somatic genome alterations in prostate cancer.

Elevated GSTP1 expression (defending against oxidative genomic damage) is characteristic of proliferative inflammatory atrophy loss of GSTP1 activity in a subset of lesions may promote transformation to high-grade prostatic intraepithelial neoplasia and/or adenocarcinoma.

Key words: prostate cancer, glutathione-S-transferase P1 (GSTP1), prostatic intraepithelial neoplasia, adenocarcinoma.

Introduction

Prostate cancer has a set of problems, which are associated with its early detection, diagnosis and treatment. The diagnosis of early stage prostate cancer is very important for the management of the disease. Nowadays, the methods used in the diagnosis of prostate cancer are: prostate-specific antigen measurement, digital rectal examination, confirmed by histological examination of biopsy specimens. These methods are confounded by some limitations.

Genetic alterations such as: mutations, and epigenetic changes, can contribute to the malignant transformation and progression of prostate cancer. Many studies have demonstrated that, DNA hypermethylation may be useful as a biomarker in the early detection and diagnosis of prostate cancer.

An overview of genes and their expression profiles possibly involved in cancer is essential to gain a detailed understanding of molecular carcinogenesis.

Biomarkers are cellular, biochemical and molecular (proteomic, genomic and epigenetic) alterations by which normal, abnormal or simply a biologic process can be recognize or monitored. They are used to measure and evaluate normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention. In the field of cancer research and detection, a biomarker refers to a substance or process that is indicative of the presence of cancer in the body. It might be either a molecule secreted by a malignancy itself or a specific response of the body to the presence of cancer. Biomarkers are measurable in biological media such as: tissues, cells or fluids.

Glutathione S-transferase P1 as a biomarker in the detection of prostate cancer:

GSTP1 encoding the pi-class glutathione S-transferase, an enzyme capable of detoxifying electrophilic and oxidant carcinogens [4]. This genome change remains

the most common somatic genome abnormality of any kind (>90% of cases), in prostate cancer, appearing earlier and frequently than other gene defects, including the recently described fusions between TMPRSS2 and ETS family genes, that arise during prostate cancer development [6].

The associated loss of pi-class GST function likely sensitizes prostatic epithelial cells to cell and genome damage inflicted by dietary carcinogens and inflammatory oxidants, explaining the contribution of diet and lifestyle as factors to prostatic carcinogenesis [7].

GSTP1 CpG island hypermethylation, which is not present in normal prostatic epithelial cells (nor any other normal cells) seems to arise first in proliferative inflammatory atrophy lesions, the earliest prostate cancer precursors, which are characterized by simultaneous inflammatory epithelial damage and regeneration [8].

Hypermethylation of GSTP1:

The best characterised gene found to be hypermethylated in prostate cancer is GSTP1, encoding the π -class glutathione S-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogenesis. Methylation is highly tumour-specific but also prevalent in high-grade prostatic intraepithelial neoplasia lesions, which makes GSTP1 an attractive early detection biomarker.

Hypermethylation and inactivation of genes involved in DNA repair, such as GSTP1, may serve as initiating genome lesions for tumour development by increasing susceptibility to carcinogens, thus predisposing to further mutations and DNA damage.

GSTP1 functions in the conjugation and detoxification of potential carcinogens and has been demonstrated to have "caretaker activities".

Promoter hypermethylation accompanied by loss of GSTP1 is one of the earliest and most common somatic genome alterations in prostate cancer.

Elevated GSTP1 expression (defending against oxidative genomic damage) is characteristic of proliferative inflammatory atrophy loss of GSTP1 activity in a subset of lesions may promote transformation to high-grade prostatic intraepithelial neoplasia and/or adenocarcinoma.

Because GSTP1 is the most frequently methylated gene in prostate cancer, many studies have been made to detect prostate cancer in clinical samples, such as: plasma and serum [9, 10], prostate secretions.

Cancer cell DNA contains many somatic alterations, including mutations, deletions, amplifications, translocations, and hypermethylated CpG islands that affect the function of critical genes and contribute to the phenotype. Critical genes are represented by oncogenes and

tumour suppressor gene. Many studies suggested that when GSTP1 is inactivated, prostate cells appear to become more vulnerable to somatic alterations upon chronic exposure to genome-damaging stresses such as oxidants and electrophiles that are contributed by environment and lifestyle [11,12].

A number of studies have examined the ability of GSTP1 methylation in improving the sensitivity of standard histology for prostate cancer detection in needle biopsies.

Hypermethylation of GSTP1 in bodily fluids:

Many efforts are underway to develop non-invasive methods to quantifying methylation of genes in bodily fluids such as: urine sediments and in extracellular DNA present in peripheral blood plasma and serum [13]. Since alterations in DNA methylation are among the earliest and most common events in tumorigenesis, monitoring methylation patterns via bodily fluids in men at risk for harbouring prostate cancer (elevated prostate-specific antigen, detection of high grade prostatic intraepithelial neoplasm on serial biopsy) may detect disease that has been missed by needle biopsy. More than 75% of tumours originate in the peripheral zone of prostate gland, which surrounds the urethra. It is therefore conceivable that cellular debris and DNA shed into the urethra by the tumour is detectable in urine. Also, high levels of tumor DNA are reported in plasma and serum [14].

Cancers of the bladder and kidney also contribute cellular DNA to urine sediment. In this case, detection of prostate cancer specific DNA by methylation would require a panel of carefully selected methylation markers to both detect and discriminate among with a variety of urological malignancies.

Recent analysis of multiple loci (GSTP1, ARF, p16, MGMT) simultaneously, reported that methylation of at least one of the four genes in urine sediments was able to identify 87% of prostate cancer patients from controls (no evidence of cancer) with 100 % specificity [15,16]

Conclusions

Exploiting DNA methylation offers several exciting and promising opportunities for the management of prostate cancer. Promoter methylation is a frequent, early event and accumulates during multi-step prostatic carcinogenesis. Specific targets of hypermethylation in prostate cancers have been and continue to be defined. The development of these targets as methylation biomarkers for prostate cancer diagnosis and prognosis could contribute to the optimal identification and treatment of this disease.

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SURGICAL TREATMENT IN LEGG CALVE PERTHES DISEASE

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Abstract

Surgical treatment aimed at achieving or maintaining femoral head contention and offers the advantage of early mobilization by avoiding long orthopedic treatment. Content surgery can be achieved by addressing either femoral and acetabulum or both.

Key words: surgical treatment, advantage, procedures, results.

In a century since the first description Legg Calve Perthes disease is still mostly unknown and probably that is why the methods of treatment have not always the best results.

Surgical methods of treatment aimed at achieving or maintaining contention are supported by most authors because it offers the advantage of early mobilization and avoids long treatment with orthopedic inconvenience lengthy immobilisation. Content surgery can be achieved by addressing either femoral or acetabulum or both.

Lately surgery has gained ground against orthosis, especially in risk groups and children over 6 years.

There are several possibilities: Salter osteotomy (innominate), which is acetabulum inclination (slant and down) for a better coverage of femoral head, proximal femoral varus osteotomy when femoral neck is descending for 30° for better stability of the hip, the combination of the two, other^[4].

Varus osteotomy is preferred as an early treatment, because it allows accurate centering of the femoral head into acetabulum. Derotation must be achieved only if the child has a anteversion femoral head and is performed only a few

degrees^[1,2]. Innominate Salter osteotomy is an useful treatment in Perthes disease, but recovery of postoperative hip mobility may be difficult^[7].

Pathogenic surgery was abandoned row on row. Drilling, curettage and intraepiphyseal graftings give a rather middling result. Neck femoral and 1/3 of the upper femoral physis deperiostation described by Judet has been completely abandoned and even contraindicated. It was demonstrated that this intervention will only lead to a subperiostic hematoma that will make difficult blood circulation to the femoral head, with serious repercussions on the process of reconstruction^[4].

Surgery which are effect in LCP disease are recentering operations. This principle derives from surgical Salter's concept and make the surgery to have two objectives, to protect fragile areas of necrosis for mechanical aggression (pressure) and to introduce the femoral head into acetabulum so as to areas of hypertrophy be perfectly molded in acetabulum. These interventions make a reorientation of femoral head or a reorientation of acetabulum or both. In addition it appears that these interventions (osteotomies) have a trophic effect, accelerating the femoral head repair^[1,2,4].

A) Femoral osteotomies are the oldest procedures and consists in making a *simple osteotomy and sometimes associating varus and derotation*. Is performed sub or intertrochanteric (fig. 1) and offers the advantage of positioning the femoral head deep into acetabulum, protect it by the pressure exerted on the edge acetabulum forces and reducing pressure joints^[1,5].

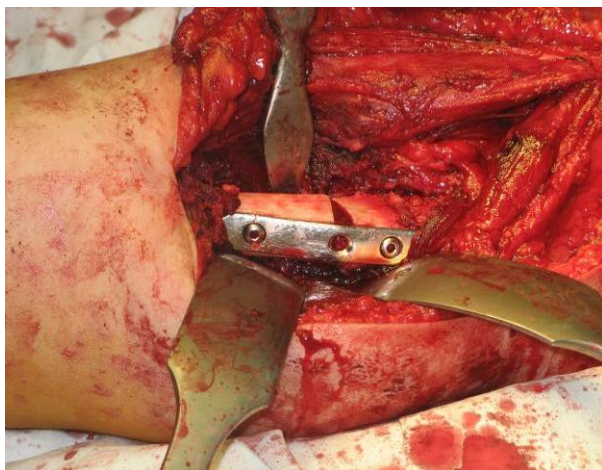


Fig. 1. Subtrochanteric osteotomy.

The objectives are restoring motility, matching between the femoral head and acetabulum and contention the head into acetabulum in abduction and internal rotation.



Osteotomy should be done preferably in the initial stage of fragmentation, trying prevention femoral head deformation by removing the pressures exerted on it (fig. 2).

Fig. 2. Fragmentation of the femoral head.

Of course, there are drawbacks of this procedure to be taken into consideration: varus osteotomy associated or not with derotation usually requires internal fixation and immobilization for 6 weeks, then at least one surgery with risks and costs for removing internal fixation and lower member is temporarily shortened by this procedure. Varus angle result should not be less than 110° because is known that it is decreasing with grow up. If an impaired cartilage growth is associate, the potential of remodeling can be decreased up to complete loss, and patient will remain with permanent shortening and limiting abduction, temporary or permanent [8]. Varus osteotomy supporters, with or without derotation reported satisfactory results in 70-90% of cases, and that argument also seems to shorten the treatment period to approximately 6 months from orthopedic treatment [6].

B) Acetabulum osteotomies

Innominate osteotomy (Salter) is performed to obtain contention by redirecting acetabulum and achieving a better coverage of anterolateral femoral head (fig. 3). It is placed in the flexion, abduction and internal rotation in trying to correct possible shortening during disease development. The objectives of this osteotomy consist in restoring complete hip motility, obtaining a round femoral head and its matching joint. Treatment should be performed early, and femoral head should be well positioned to flexion, abduction and internal rotation. Any residual adductors contracture is resolved by tenotomy. Osteotomy is stabilized by 2 or 3 wires and immobilization is maintained 6 weeks [7].

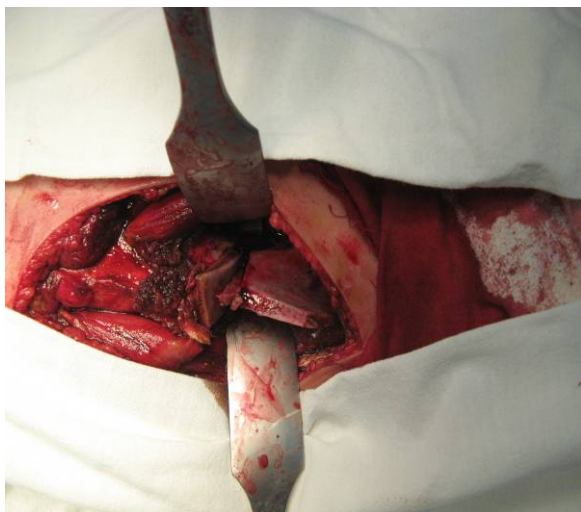


Fig. 3. Innominate osteotomy (Salter).

Disadvantages of this osteotomy consist in surgical risks, costs, need a second intervention for wires extraction. Also worthy consideration of that intervention is done the fact that operation is made on a bone previously unaffected and while this method can increase the forces acting on the femoral head by acetabulum lateralization and increased leverage abductors. Acetabulum may remain deformed, leading to loss of motility, particularly flex limitation. Anatomical satisfactory results however appears in 69-94% cases^[8].

Salter osteotomy produces an elongation effect by 0.5-1cm. which may compensate an consequent unequal leg length by disease. It appears that trophic effect achieved by this intervention is more versatile than femoral osteotomy, and authorization is walking early after Salter osteotomy, being allowed three months after intervention^[7].

Varus osteotomy associated with innominate osteotomy

Some short-term results of combination by the two procedures have been reported in patients with serious femoral head damage (Catterall 3 and 4). This combined procedure has the advantage of theoretically maximize contention femoral head and avoid complications arising in proceedings conducted separately. Femoral osteotomy directs femoral head into acetabulum while reducing the pressure on the femoral head which commonly appear after innominate osteotomy^[1,2]. Coverage achieved by the acetabulum osteotomy reduce the necessary degree for correction the femoral osteotomy and consequent minimizing complications such an excessive varus associate with abductors weakness and shortening^[7]. The sustainers of this process argue that the combined solution is surgical visa radical, definitive, allow early loading and shortens the duration of treatment. Disadvantages not exceed those of each process taken separately. At the same time, duration of the intervention is greater, potential for bleeding is increased and technically combined process is more difficult. They reported satisfactory results in 78% of patients and short term follow-up has been excellent^[8].

Shelf arthroplasty augmentation

This method was recently proposed as a first solution for children over 8 years in Catterall stages 2,3,4 with or without signs of head at risk, Herring type B or C, Salter Thompson B. Contraindications are other categories and hinge abduction. The authors suggested that this type of arthroplasty improves the anterolateral femoral head coverage and prevent subluxation and excessive epiphyseal lateral growth^[3]. Risk factors for adverse outcomes are age over 11 years, female sex, stage 4 Catterall^[8].

Triple osteotomy

The most recent time, there is no long-term studies, but in terms of this theory it can provide a good coverage of femoral head and architectural changes of the joint much more important. This intervention allows in each sector (varization, derotation and flexion) a maximum 30°

reorientation which provide the better femoral head containment in almost all forms of disease described.

Cheilectomy removed the anterolateral fragment of femoral head that acetabulum pressing during abduction. Process is addressed only to patients who have a big limitation of motility due to this phenomenon and must performed only after growth has ended to avoid further epiphyseal dislocation^[8].

In patients who prematurely closes growth cartilage, usually occur the great trochanter increase with painful gait and Trendelenburg limp and in these patients may come to question the *distal and lateral surgical great trochanter advancement*^[7].

Chiari osteotomy improve lateral femoral head coverage being useful in poor head coverage, even when starts early symptoms of degenerative arthritis^[7].

A major advantage of surgical treatment is that the final results are known, while in orthopedic treatment surgeon must decide when to interrupt treatment. Another advantage is the resumption of normal activity quickly after surgery^[8].

Disadvantages are the need to extract implants (pines or wires usually), the risk of infection and anesthesia. It is indicated to make arthrography, CT-3D or MRI before any surgery to assess acetabular cartilage contour and the femoral head, and its matching with acetabulum^[6,7,8].

If used correctly both treatment, orthopedic and surgery, have the same end results. Surgery is indicated particularly when the prognosis suggests a prolonged period of healing (big child with severe involvement of the femoral head). Because in the group I and II Catterall it is not a real deformation (only in rare cases of hip subluxation), surgical intervention is taken into account only for group III and IV Catterall. The geographical and social influence, largely for surgical indications. In some areas orthosis suitable for abduction may not be available, also may be that children and their families do not have a proper attitude to follow a treatment program with orthopedic methods. On the other hand, the surgical procedure should not be used if the patient can not be properly followed and reevaluated for potential complications^[4].

It is crucial to observe lower limbs inequality, especially if involving growth cartilage, or in they who were performed varus osteotomy. If the difference in length is significant, inequality is compensated with orthopedic shoes and the optimal age of the skeleton will be perform *contralateral distal femoral epiphysiodesis* to equalize the two legs, or if the length exceeds 4 cm femoral affected elongation by external fixation^[4,8].

The reconstruction procedures are used late in Perthes disease, to correct an preexisting femoral head deformity^[4].

In conclusion, although it tries to perform better correlations between risk factors, classifications, imaging, etc in attempt to achieve a good reflection in prognosis, yet is no unanimity in regarding Perthes disease treatment.

Surgical treatment attempt to shorten the disease evolution and improve prognosis, but significant studies on large lots

of patients and during long time are lack for the moment.

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METHODS TO DIAGNOSE CONGENITAL MALFORMATIONS IN NEWBORNS

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Abstract

Congenital malformations, congenital anomalies and innate defects (present at birth) are synonymous terms used to describe structural, functional or metabolic disorders present at birth. The science that studies the causes of these disorders is called teratology (Greek *teratos* = monster).

As genetic and malformative disorders are very diverse, appear at different ages and affect any system or organ, the patients who suffer of these diseases can be examined by a specialist doctor, all the medical practitioners facing genetic pathology should know some principles of genetic medicine. They also should know and apply the general methodology of genetic examination, should be able to indicate the necessary genetic explorations, as well as to correctly understand and interpret their results and should be able to advice genetically in a correct manner - within his/her area of competence – the patient and/or the family facing a genetic risk.

Major structural anomalies appear in 2-3% in live newborns and other 2-3% are discovered in children up to 5 years old, summarizing 4-6%. Birth defects are the first causes of infantile mortality, accounting for approximately 25% of all neonatal deaths.

Minor anomalies appear in approximately 15% out of the total of newborns. These anomalies do not alter the individual's health status, but they are associated with major defects in some cases, therefore they can serve as key elements for the diagnosis of more serious, hidden defects.

Key words: congenital malformations, genetic examination.

Classifying and defining congenital defects¹

1. Malformation – anomaly of morphogenesis produced through a primary, intrinsic and precocious process of abnormal development (morphogenesis). The organ does not develop normally (although the tissues are normal) or the differentiation is incomplete (the morphogenesis process doesn't end).

Examples: *spina bifida*, labial/palatine rupture, congenital heart defects, syndactylia.

2. Deformation – shape or position anomaly of a body part produced through the compression and deformation of a region otherwise normally developed both morphologically and structurally (fetopathic). Deformations affect the muscular-skeletal system and lead to the loss of symmetry, altering the alignment, distorting the configuration and the abnormal position of some structures; they are determined by multiplication factors that produce the limitation of the uterine space and/or the inability to

move (small /malformed uterus, big fetus, twin pregnancy, oligohydramnios). Unlike malformations and disruptions, deformations are reversible (if compression is seized) spontaneously or after orthopedic maneuvers.

Examples: some skull and face asymmetries, anomalies of the ear, crooked leg, deformations of the members.

3. Disruption – morphogenesis anomaly produced by altering or secondary destruction, extrinsic and late (fetopathy) of a fetal structure otherwise normally developed. The destructive processes produce shape and configuration modifications, unusual diffusions or fusions, loss of component parts. Disruptions are determined by extrinsic agents / ischemia, infections, embryonic forces (amniotic brides) / that destroy normal embryonic structures through compression and/or necrosis (consequence of thrombosis, embolies, compressions). By definition disruption is not genetic, although genetic factors may predispose to disruptive events (for example collagen defects that reduce the resistance of the amnion and make it more vulnerable to spontaneous ruptures). Disruptions usually appear in the form of multiple congenital anomalies.

Example: the absence of fingers or members.

4. Dysplasia – morphogenesis anomaly determined by the abnormal organization and functioning of the cells in a specific tissue. The effects of dysplasia are usually seen in all the body structures in which the tissue is placed. Therefore monogenic mutations appear and they are highly recurrent.

Examples: skeletal disorders, ectodermal dysplasia, connective tissue anomalies.

The diagnosis of congenital defects^{2,3}

For a correct and complete diagnosis of the different congenital anomalies, the following steps must be followed:

1. Family background (personal and family's antecedents).
2. Genetic examination and advice.
3. Clinical examination.
4. Paraclinical investigations.
5. Analysis and interpretation of data.

1. Family background

a) *Personal antecedents* refer to every stage in the individual's life and the history of the disease.

Schematically, we can distinguish:

1. Gestational anamnesis – that will refer to conception, the evolution of the pregnancy and birth.

Conception: reproductive antecedents and the age of the parents, their eventual chronic diseases, blood types, the date of the pregnancy's debut.

The evolution of the pregnancy: diseases and teratogenous exposures in the 1st trimester, the beginning and evolution of fetal movements, the quantity of amniotic liquid and echographical data.

In the anamnesis of the evolution of the pregnancy the emphasis will be on the mother's health or sickness, especially in the first 12 weeks of gestation, on the medication received and the eventual X-ray irradiation. The diseases (anomalies) induced in the next 6 months are usually the result of the action of mechanical factors (ambiguous), of infections or other factors that interfere with the growth of fully structured organs (hydrocephaly, microphthalmia, corioretinitis, malposition of the extremities). From the anamnesis of the anterior pregnancies, the eventual abortions or abortion risks will be considered.

Birth: gestational birth, the duration of labor, presentation, anomalies of the amniotic liquid, umbilical cord and placenta, APGAR score / resuscitation of the newborn, its morphological coordinates (weight, length, skull perimeter).

2. Neonatal anamnesis – it is equally important, as the difficulties in "the adaptation of the child to the extra uterine life" or a series of abnormal manifestations (apnea crises, sucking difficulties, eructation, hypotony, convulsions, cyanosis, and prolonged icter) can signal congenital malformations.

3. Postnatal anamnesis – refers to the evaluation of growth, social and psychomotoric development.

4. The history of the patient's disease: the moment of the debut, manifestations, clinical and paraclinical evaluations, medical care etc.

b) Family antecedents

The family's anamnesis offers a lot of information about the biological and social/legal relations, about the physical and mental state of the individuals in the family, about their reproductive function, which are all important information for establishing: the medical diagnosis, testing strategies, the way the malformation is transmitted, determining the recurrence risk, the identification of the persons with a high genetic risk etc. A history of the family is obtained in a "face to face" interview (with the patient and his/her parents) in a comfortable setting, without disturbing elements, in order to assure the confidentiality.

The family anamnesis will include the mother's and father's age, the eventual consanguinity, the genealogical tree (as detailed as possible) which records all the brothers and sisters, parents and all the ascending and collateral relatives, the sick and healthy ones, too.

2. Genetic consultation and advice

Most frequently, people ask for genetic advice in two situations:

a) if a person or its relatives have a congenital defect, in order to assess the risk of giving birth to sick children:

b) if in a family with healthy parents and one or more sick children there is a recurrence risk and the parents want to know the risk recurrence ratio.

The genetic examination and advice is performed by the genetician who is required to know and discover if the disease (defect) is hereditary or determined after conception (the transmission risk and even the prognosis differ a lot).

This is often difficult because the congenital element (present at birth) is not conclusive, since not all hereditary anomalies are congenital (present at birth) and not all congenital anomalies (present at birth) are hereditary. The familial character of the disease does not show beyond any doubt the genetic cause of the disease. The recurrence of the same disease in a family can be the consequence of the family's exposure to the same environmental factors. A genetic defect may also appear sporadically if it is a new mutation.

Differentiating a hereditary disease from a non-hereditary one can be complicated, as the clinical signs of a non-hereditary disease may be similar with those of a disease resulting from a genetic mutation.

If the disease is hereditary, knowing the dominant or recessive way of disease transmission is of great interest.

If a male individual who showed a pathological recessive feature linked to X marries a normal woman, none of his boys will carry and transmit the feature, but all the girls will carry and transmit it, although the disease will have no manifestations. The sons of such a carrier mother will have the risk of 1:1 to carry and express the abnormal feature (if they marry a normal woman, they will not transmit the disease). All the daughters have the risk of 1:1 of being carriers, like their mothers, but they are apparently normal.

If a congenital anomaly has been caused by environmental factors, the prognosis over the next pregnancy depends upon the possibility of the etiological agent (for example congenital rubeola, toxoplasmosis, X-rays administered therapeutically or teratogenous medicine) to interfere (reject, neutralize).

3. Clinical consultation

The physical examination is extremely important, because it represents, along with the medical history, the basis for a correct and complete clinical diagnosis. It is the first test of a clinician's qualification and it requires:

- optimal conditions (cooperative and relaxed patient, optimal light and temperature and, for a child, the presence of the mother);

- excellent technique, which includes a complete and methodical evaluation, attentive and comprehensive;

- a background of basic knowledge, represented by the knowledge of morphology and the understanding of the normal and abnormal morphogenesis.

a) General examination

The basic elements of the physical examination of a patient are:

- evaluation of vital signs (cardiac frequency, respiratory frequency, blood pressure);

- assessment of the growth parameters (weight, height, skull perimeter), the general habitus of the body and its proportions;

- evaluation of the general health and nutritional status;

- methodic evaluation of the systems and anatomical structures; the order of examination can vary, according to the patient's age and the main problems, but it is essential to perform a complete examination.

b) Dysmorphic examination

The objective is the recognition of the abnormal forms (dysmorphism), and so the identification of minor anomalies which can make a characteristic model.

Sometimes the diagnosis is instantaneous, because many syndromes have a facial stereotype appearance that allows for a rapid recognition, an immediate visual and mental diagnosis (for example Down syndrome, achondroplasia etc.).

The instantaneous recognition requires experience, which is acquired in time and may frequently lead to errors either because of the variable manifestations of the syndromes, or because of the temptation of making a quick diagnosis (before finalizing the evaluation of the patient or ignoring significant clinical data).

The diagnosis is most of the times analytical, based on a complete and detailed clinical evaluation. The shape, size, proportions, position, contour, folds, spacing, and symmetry of all the anatomical elements (whose normal morphology with the described variants must be well known) will be closely observed and described correctly (using the malformative semiology terminology). Special attention will be given to minor anomalies / subtle modifications of structures. The regional inspection of the surface structures will be completed by palpating them and through a series of active maneuvers.

Beside the initial examination (anamnesis, physical examination), psychological support and medical advice will be offered to the couple /the parents. At the end of the evaluation, the doctor has to present in a simple and accessible way a map of the identified problems (anomalies, signs, and symptoms) and a medical evaluation and health care plan (management).

4. Paraclinical investigations

These are focusing on the prenatal and postnatal period (in the last case some are specific to the investigation of certain apparatus and systems).

Prenatal diagnosis

Prenatal diagnosis is a complex, highly informative medical act, which allows for the diagnosis of numerous congenital anomalies and genetic diseases in the fetal life. This is correctly done only through a strong multidisciplinary collaboration, in which the genetician has an essential role in evaluating, diagnosing and eventually giving the genetic advice.

Establishing a prenatal diagnosis has been until recently done at the beginning of the second trimester of pregnancy. Prenatal diagnosis in the first trimester represents a great progress and not only the "last fashion",

allowing for and facilitating the diagnosis of chromosomal aberrations, hereditary disease in general, and especially of the hemoglobinopathies. Traditional or modified (modernized) techniques are being used. Direct visualization (fetoscopy), followed by chorion biopsy can be done, nowadays, between the 6th and the 13th gestational week. The chorionic villi suction biopsy can be guided through ultrasonography. The trophoblastic tissue obtained produces analyzable metaphases to determine the karyotype, culture tissue, good material for cytogenetic and biochemical analysis or for DNA extraction. Through specific Y-chromosomal probes the fetal sex can be established. But there are major risks for the fetus: injury or even death through bleeding, infection or tissue damage. The maternal risk appears to be insignificant. If the attempt to obtain tissue samples fails, the second option will be the amniocentesis, but this is more effective in order to assess the neural tube defects. The cytogenetic studies performed in the first trimester of pregnancy allow for the more precise and frequent detection of the chromosomal anomalies that the one made through the analyses of amniotic cells in culture, obtained in the second trimester of pregnancy (this is because many conception products are lost through spontaneous abortion in the first trimester). The higher frequency of miscarriages requires comprehensive studies to differentiate between spontaneous and induced miscarriages. There are many advantages of the prenatal diagnosis in the first trimester of pregnancy, including the optimization of therapeutic effects, the simpler and safer indication of therapeutic abortion, decrease of mother's anxiety etc⁴.

There is – in the future – the possibility that more pregnant women can be prenatally investigated through simpler and less harmful procedures.

The selection of pregnant women for establishing the prenatal diagnosis is based on the principle that the risk of a fetal anomaly can be at least equal to the risk of abortion induction by using the procedure of prenatal diagnosis. Over 200 genetic diseases can be diagnosed prenatally today.

Unlike non-invasive, without fetal risk screening methods, applied in a large number of pregnant women to identify pregnancies with high risk of abnormalities, prenatal diagnosing techniques are usually invasive (chorionic villi biopsy, amniocentesis, cordocentesis). This involves the harvesting and analysis of fetal cells to establish – for pregnant women selected on the basis of their high genetic and/or malformative risk – if the fetus is normal or not⁵.

Regardless of what procedure is used, prenatal diagnosis techniques must fulfill the following applicability conditions:

- security – depends on the experience of the person applying the procedure and is expressed through the ratio of immediate or late abortions following the procedure;

- accuracy – expressed through the quality of the results;

- quality control – which is done through the use of standardized procedures that refer to the qualification of the personnel, the functioning of the equipments and the accuracy of the results.

The tests have to be performed as soon as possible and the results must be obtained rapidly, so that if the pregnant woman carries an abnormal fetus, she can benefit of selective abortion, possible in the terms established by law⁶.

Prenatal diagnosis techniques are:

1. Fetal echography

The echography is the most frequently used method of visualizing the fetus (exceeding the radiography or RMN) as it has no risks for the mother and the fetus. It has been used as a prenatal diagnosis method since 1972, the initial purpose being that of detecting anencephaly. The informative capacity of the echographical investigations grew over the last years, as the apparatus have been improved and also fetal medicine experts have appeared.

The obstetrical information offered by the echography depends on the trimester in which the examination is done.

- in the 1st trimester (usually 10±2 weeks of amenorrhea) the echographical examination establishes the age of the pregnancy and determines the viability.

- in the 2nd trimester (usually 18±2 weeks) the echography allows for the diagnosis of twin pregnancy, assessment of the position of the placenta, gestational age – by measuring the biparietal diameter and the length of the femur, the screening of the anomalies of the fetal structures.

- in the 3rd trimester (usually at 32±34 weeks), the echographical investigations allow for the physician to establish the size and position of the fetus, the growth ratio, the intensity of the fetal movements, the gender, the anomalies of the amniotic liquid, the screening of the anomalies.

At present, in many countries the echography is a routine procedure for every woman in order to assess the fetus morphology and growth.

The sensitivity of the method in establishing the major congenital malformations is of 40-60%, and even higher for some types of anomalies (almost 100% for anencephaly, 85-90% for spina bifida).

The fetal defects that can be detected in the 2nd trimester of pregnancy are:

- anomalies of the nervous system: anencephaly, posterior fossa cyst, encephalocele, facial dysplasia, holoprosencephaly (anomalies of the cerebral ventricles and of the face), hydrocephaly, microcephaly, myelomeningocele, porencephaly (cystic lesions of the brain), rachischisis, *spina bifida*;

- cardiovascular defects: pericardial liquid collections, septal defects, *situs inversus*, valve defects, vascular anomalies, ventricular hyperplasia or hypoplasia;

- thoracic anomalies: esophagus atresia, diaphragmatic hernia, pleural effusions, intrathoracic cysts;

- gastrointestinal malformations: absence of abdominal muscles, ascites, cystic lymphangioma, intestinal atresia, laparoschisis (paraumbilical extrusion of abdominal viscera), mesenteric cysts, omphalocele (umbilical hernia of abdominal viscera);

- urogenital malformations: hydronephrosis, hydroureter, polycystic kidneys, renal atresia, teratomas, urethral valve;

- muscular-skeletal malformations: arthrogryposis, bone dysplasia, crooked leg, fractures, limbs paralysis, limbs reduction, mineralization defects;

- other anomalies: amniotic bride, Siamese twins, teratomas, tumors.

If multiple fetal anomalies are seen, amniocentesis and cytogenetic analysis should be performed. Between 15% and 30% of the fetuses that show echographically morphologic anomalies have chromosomal anomalies. A series of alarming echographic signs are associated with a high risk of chromosomal anomalies:

- the thickening of the nuchal fold identified in the 1st trimester of pregnancy is suggestive for Down syndrome;

- the excess of skin on the nape is suggestive for Turner or Down syndrome;

- the big placenta suggests the presence of triploids, fetal hydrops or thalassemia;

- precocious delay of growth appears in the case of trisomy and triploids;

- labial-palatine crests are visible in fetuses with trisomy or triploidy;

Echographical markers suggestive for the presence of fetal anomalies are:

- abdominal calcifications (meconium peritonitis)
- permanently flexed fingers (trisomia 18, arthrogryposis)

- defect of the common trunk, displayed as Fallot teratology or vascular defect (velo-cardio-facial syndrome)

- defects of ossification of the skull (anencephaly)

- thoracic deformations (skeletal dysplasia)

- urinary bladder hypertrophy (urethral valve)

- bone hypodensity (hypophosphatasia)

- cystic hygroma (Turner syndrome)

- facial hypoplasia and palatine rupture (trisomy 13 – holoprosencephaly)

- fractures (osteogenesis imperfecta)

- high number of choroidal cysts (trisomies)

- polydactylia (trisomy 13, Elis Van-Creveld syndrome)

- pterygium colli (Turner syndrome)

- lemon sign – skull in the form of a lemon (spina bifida)

- shortening of long bones (bone dysplasia)

- high volume of cerebral ventricles (hydrocephaly)

2. Amniocentesis

Amniocentesis is the procedure through which a sample of amniotic liquid (AL) is harvested through echographically guided transabdominal puncture. AL contains fetal cells that can be either DNA tested to detect the mutations, or cultivated for chromosomal analysis.

Over the last decade the health programmes in some countries confirmed the safety (lack of risks) and accuracy of prenatal diagnosis through amniocentesis. Abdominal

amniocentesis can be routinely practiced in the 16th week of gestation.

Amniocentesis is preceded by a thorough echographical examination to establish the number of fetuses, the conformation and viability of the fetus, the gestational age, the position of the placenta, the approximate volume.

Indications for amniocentesis:

- old age of the mother;
- the existence of a parental chromosomal translocation;
- older child that presented or still presents a chromosomal aberration;
- defects of neural tube to another descendant;
- congenital nephrosis in family antecedents;
- the presence in the family of a hereditary X-linked disease;
- the presence in the family of a metabolic hereditary disease.

The major complication of amniocentesis is the risk of 0.1-1% of inducing abortion, over the main risk of 2-3% for every pregnancy in the 2nd trimester.

Other rare complications are: small leaks of AL, infections, injury to the fetus. Further risks include the respiratory distress of the newborn and other benign orthopedic deformations.

3. Chorionic villi biopsy

It is the method of obtaining fragments of chorionic villi, which are embryonic structures that derive from the trophoblast, the external part of the blastocyst. The harvested samples contain both fetal cells and cells from the maternal decidua, which have to be carefully taken away before the analysis.

4. Cordocentesis

This is the procedure through which a sample of 2-3 ml of fetal blood is obtained, under echographical guidance, from the root of the umbilical cord. This is usually performed at 18-21 weeks of pregnancy in patients that come late for a prenatal diagnosis, when the cultures of amniotic cells give ambiguous results (mosaicism) or when the DNA diagnosis is not possible for a disease that can be identified through biochemical tests of the plasma or of the sanguine cells (some hematological diseases or hereditary immunological ones, bacterial infections, viral or parasite ones). The fetal blood needs only a few culture days for obtaining good quality chromosomal preparations. The abortion risk due to the procedure is of 2-3%.

5. Fetoscopy

The fetoscopy consists of introducing in the uterus, on a transabdominal way, a small and flexible instrument, called fetoscope that allows the visualization of the embryo and of the fetus and the collection of tissue from the conception product (skin, muscle, hepatic tissue). Fetoscopy detects defects and diseases that can not be detected through other invasive techniques (genodermatosis, muscular dystrophies, hepatic diseases).

It has to be practiced in aseptic conditions, with local anesthesia.

The optimal period of harvesting blood is from the 18th to the 20th week of gestation. The prenatal diagnosis of hemoglobinopathies, hemophilia A, Christmas disease, and granulomatous disease is now possible by the analysis of blood samples or fetal serum. Skin biopsy is successfully practiced for the prenatal diagnosis of the harlequin ichthyosis and bullous epidermolysis.

The complete visualization of the fetus allows the prenatal diagnosis of some severe genetic diseases. The optimal period is 16-17 weeks of gestation. The echographic examination is required before fetoscopy.

Among the complications of fetoscopy, evaluated globally at 1-1.7%, the rise of premature birth ratio with 7.2% must be included. However, no orthopedic anomalies or neonatal respiratory distress (at term newborns) determined by the fetoscopy have been reported.

6. The analysis of the fetal cells in the maternal blood

Three types of fetal cells circulate in the maternal blood: lymphocytes, erythroblasts and cells of syncytiotrophoblast. They can be identified and isolated in the maternal blood due to the antigenic differences between the mother and the fetus. Erythroblasts seem to be the most adequate cells for neonatal diagnosis out of the three cell types present in the maternal blood. According to recent data, the number of fetal cells in the maternal blood is higher in the case of pregnancies with fetuses having aneuploidy, especially trisomy 21, which would facilitate the detection of such anomalies⁷.

The lab analyses based on fetal cells are:

6.1. Chromosomes analysis

The diagnosis of chromosomal anomalies in the fetus represents the most frequent component of prenatal diagnosis, being indicated in the presence of a structural chromosomal anomaly in one of the parents or if in the family there is a child with a *de novo* chromosomal anomaly, such as pregnant women over 35 with a positive triple test or alarming echographical signs.

Sexual chromatin offers information regarding the number of sexual chromosomes and, through this, valuable data for establishing the genetic gender (XX or XY), and also the numeric gonosome anomalies.

- X chromatin results from the inactivation of an X chromosome at a XX woman who, through heterochromatization, will form the Barr corpuscle in the interphasic nucleus. The number of Barr corpuscles is equal to the sum of X chromosomes minus 1; therefore, the Barr test will be positive in a normal woman and negative for a normal man.

- Y chromatin represents the heterochromatin of the 2/3 distals of the long arm of the Y chromosome, visible in fluorescence under the microscope in the form of the F corpuscle; the number of F corpuscles is, of course, equal to the number of Y chromosomes.

The sexual chromatin test is simple, inexpensive and useful in practice, in well defined clinical situations. An

abnormal test represents a very serious option for a diagnosis, especially in a suggestive clinical context. However, we must add that the final diagnosis is made only through chromosomal analysis.

Determination of X chromatin is the most frequently used (Barr test) in practice, performed with a standardized method and a careful and correct interpretation. An abnormal result (negative Barr test in feminine patients, positive Barr test in masculine patients or the presence of two Barr corpuscles) will be confirmed by repeating the test and by an independent evaluation of another examiner. A delicate problem appears in the situation of certain positive results, but with a small percentage of positive X chromatin cells (under 10-12%); if a technical vice is excluded and the result is the same, a chromosomal analysis is required. Another delicate problem, rarely met, is the presence of a Barr corpuscle of abnormal dimensions, smaller or bigger than 1.5 microns, which can reflect a structural anomaly of

Y chromosome: a deletion of X or an isochromosome X. The chromatin test is rarely used, as it is laborious and required UV microscopy.

6.2. Biochemical analyses for metabolic diseases

Over 100 metabolic diseases can be diagnosed through biochemical studies performed on fetal cells or amniotic liquid. Most of them (being recessive autosomally) have a high risk of recurrence, a fact that justifies prenatal diagnosis.

6.3. DNA analysis

Numerous monogenic problems can be diagnosed through DNA analysis, using methods of direct detection of mutations or indirect diagnosis by the study of markers linked to the mutant gene.

We shall further show the main possible ways of paraclinical investigation in the postnatal period, in order to find malformations of the cardio-vascular, digestive, renal-urinary and central nervous systems.

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LEJEUNE SYNDROME – A MICRODELETION SYNDROME – CASE REPORT

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Abstract

Cri du chat syndrome (CdCS) was first described by Lejeune et al in 1963 as a genetic disorder caused by a variable deletion of the short arm of the chromosome 5. CdCS has an estimated incidence of between 1:15,000 and 1:50,000 live births. Among the mentally retarded population, the prevalence may be as high as 1:350. Approximately 90% of cases are the result of a de novo deletion, while the majority of the remaining cases are associated with translocations. We report a female newborn, which was thorough investigated. The fluorescence in situ hybridization (FISH) analysis, confirmed the deletion of the critical region for the Cri du chat syndrome (5p15.2).

Key words: Cri du chat syndrome, microdeletion, fluorescence in situ hybridization.

Introduction

Cri du chat syndrome (CdCS) was first described by Lejeune in 1963 as a genetic disorder caused by a deletion of the short arm of the chromosome 5. CdCS has an estimated incidence of between 1:15,000 and 1:50,000 live births. (Higurashi M, 1990; Niebuhr E, 1978). Among the mentally retarded population, the prevalence may be as high as 1:350 (Niebuhr E, 1978). Approximately 90% of cases are the result of a de novo deletion, while the majority of the remaining cases are associated with translocations.

Various extensions of the deletion of 5p were described in the literature, but the region responsible for the hallmark feature of the syndrome - high-pitched cat like cry - was mapped on 5p15.3 (Gersh et al., 1995). Other significant features, based on which, the syndrome is usually suspected at birth, are: low weight (mean weight 2614 g), microcephaly (mean head circumference 31.8 cm), round face (83.5%), large nasal bridge (87.2%), hypertelorism (81.4%), epicanthal folds (90.2%), downward slanting palpebral fissures (56.9%), down-turned corners of the mouth (81.0%), low-set ears (69.8%), micrognathia (96.7%), abnormal dermatoglyphics (transverse flexion creases) (92%) and the typical cry (95.9%) (percentages from the Italian CdCS Registry (Cerruti, et al, 2006)).

Associated malformations, although not frequent, have been reported: cardiac, neurological and abdominal malformations, as well as hypospadias and chryptorchidism.

Extensive clinical and cytogenetic analysis has been performed on patients with Lejeune syndrome. The characteristic cat-like cry is probably due to anomalies of the larynx (small, narrow, diamond-shaped) and of the epiglottis (flabby, small, hypotonic), as well as to neurological, structural and functional alterations (Niebuhr E, 1978). The examination of clinical features confirms that the cat-like cry represents the most typical sign of the syndrome, not only at birth and in the first years of life, but also later. A longitudinal study carried out in 49 patients showed that the round face generally disappeared, the face becomes narrow, palpebral fissures frequently became horizontal, the nose will be long and coarse, and the patients display dental malocclusion. However, the phenotype remains recognizable in most patients.

Case report

The proband, (Fig. 1a, b; Fig.2) a newborn female is the first child of a healthy, unrelated young couple. Mother's age at birth was 23 years and father's was 24 years. The child was abandoned at the maternity. The newborn had intrauterine growth retardation, at birth the weight was 2530 g, head circumference was 31 cm, length: 48 cm and thoracic circumference: 31 cm. The proband was investigated at birth for high-pitched cry and facial dysmorphism. On examination the following anomalies were observed: microcephaly, round face, facial asymmetry, downwards slanting palpebral fissures, hypertelorism, strabismus, down turned corners of the mouth, low set ears, short neck, simian crease (Fig. 3), clinodactyly. No other associated malformations were noted.

Cytogenetics

Chromosome analysis from peripheral blood lymphocytes was performed. A number of 50 metaphases were analyzed and a deletion of the p arm of chromosome 5 was established (Fig. 4, Fig. 5). The karyotype of the patient is 46,XX,del(5)(p14→pter).

Cytogenetic analysis for the parents should have been performed, but it was not possible because they abandoned the child. This would have been helpful in order to establish whether there is a balanced translocation in one of them, or the deletion is de novo.



Fig. 1. Patient - facial dysmorphism. (a) front; (b) in profile.



Fig. 2. Patient - clinical features.



Fig. 3. Patient's hand. Note the simian crease.

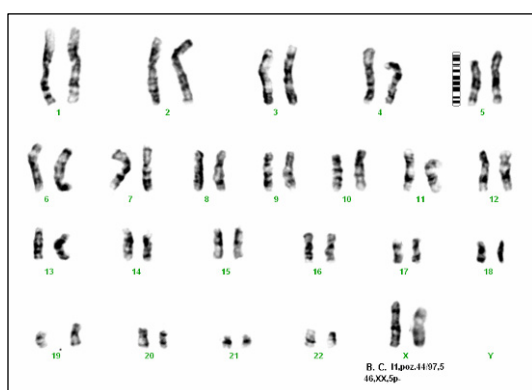


Fig. 4. Karyotype of the patient.
Note deletion of p arm of chromosome 5.

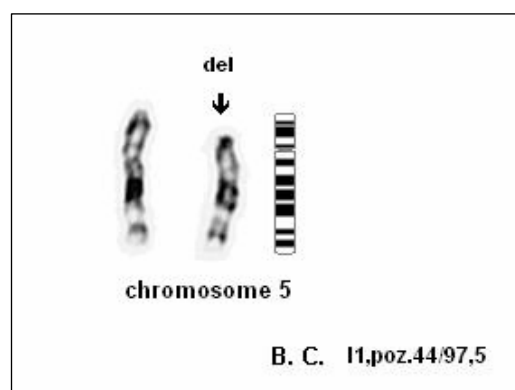


Fig. 5. Ideogram of chromosome 5, 5p deletion and normal chromosome of the pair.

To complete the investigation of the patient, FISH technique was carried out. The probe used was Vysis Cri-du-Chat Region Probe - LSI D5S23, D5S721 Spectrum Green. Slides were analyzed by fluorescence microscope. A total of 50 metaphases (Fig. 6) and 200 interphase nuclei

(Fig. 7) were analyzed. Only one signal for the specific probed used could be visualized in all cells analyses. The deletion of the critical region of the syndrome (5p15.2) was made certain.

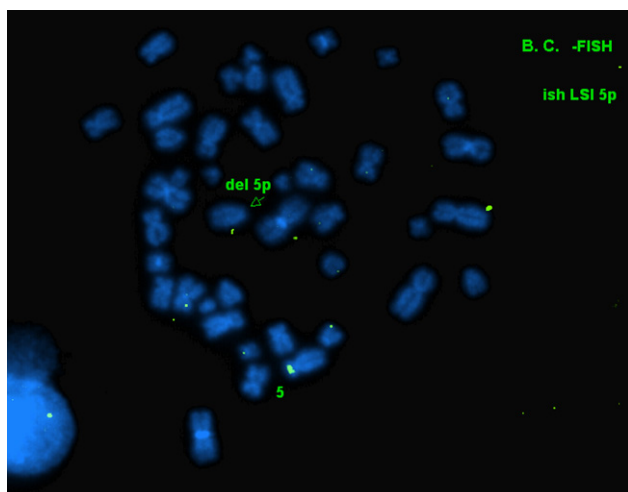


Fig. 6. FISH analysis. Metaphase spread using LSI 5p probe. Note only one green signal for the probe.

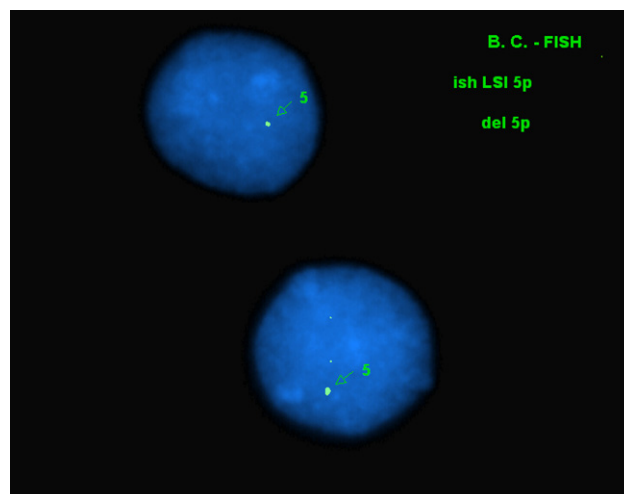


Fig. 7. FISH analysis. Interphase nuclei using LSI 5p probe. Note only one green signal for the probe.

Discussions

Cri du chat syndrome is usually diagnosed at birth due to the specific cat-like cry of the newborns. The diagnosis is first of all clinically if this hallmark is recognized, and also based on facial dysmorphism (facial gestalt).

The fact that the phenotype is well recognizable, in spite of the variability in deletion size, has led to the hypothesis that a critical region causes the characteristic clinical picture when present in a hemizygous situation. Niebuhr (1978) located this region in a narrow area around 5p15.2.

Cytogenetic analysis is important for assessing the diagnosis and molecular cytogenetic analysis (FISH) must be performed for confirming the cases.

Molecular-cytogenetic analysis by fluorescent in situ hybridization (FISH) has renewed the interest in this syndrome and allowed a molecular and phenotypic map of 5p to be defined. Our case could be diagnosed by this cytogenetic molecular technique, which assessed the fact that the critical region for the syndrome was deleted on one of the chromosome number 5. The importance of FISH for a precise diagnosis of 5p deletions was emphasized by the Italian study on 80 patients (Cerruti et al., 2006). Seven of the patients had not been correctly diagnosed by routine cytogenetics. FISH revealed that five of these patients had an interstitial deletion, one had a small terminal deletion and one had mosaicism. Subtelomeric FISH allows 5p cryptic chromosomal rearrangements to be found.

More recent studies identified two new genes, Semaphorine F (SEMAF) (Simmons et al., 1988) and δ -catenine (CTNND2) (Medina et al., 2000), mapped to the

“critical regions” involved in cerebral development, and thus their deletion was associated with mental retardation. Recently the telomerase reverse transcriptase (hTERT) gene has been localized in 5p15.33 and its deletion might contribute to the phenotypic changes in CdCS children (Zhang et al., 2003).

Genotype-phenotype studies showed a direct ratio between the clinical severity and the size of the deletion. A more severe phenotype and cognitive impairment were reported to be associated with a larger deletion. There was also identified a distinct region for speech retardation in 5p15.3, and assessed the fact that the condition of patients with a deletion in 5p13 appeared particularly severe.

Regarding the prognosis of the patients, studies have shown that after the first years of life, the survival expectation is high and morbidity is low. Mortality, already quite low in previous studies, has decreased in time from 9.67% in 1978 to 6.36% reported by Cerruti et al. in 2006. Also the percent of the children deceased in the first month and in the first year of life decreased meaningful in the later study. Mortality in patients with unbalanced translocations resulting in 5p deletions was higher than in those with isolated deletions, as reported by Wilkins also, because of the higher percent of associated malformations in these patients.

Studies have determined that in the first months of life the child confronts with sucking and feeding difficulties and with respiratory infections. There also have been reported intubations difficulties linked to larynx anomalies.

Psychomotor development is delayed in all patients but there is a variability related to deletion size and type as well as other genetic and environmental factors. Early

rehabilitation (physical therapy, speech therapy) is recommended for the neurological problems such as psychomotor and speech retardation.

Conclusions

Many cases of Lejeune syndrome have been reported in the literature, and a recent study performed by Cerruti et al. (2001) brought much to the phenotype-genotype correlation of the syndrome, as well as for the prognosis of the patients. But, to our knowledge, the case presented in this article is the first Romanian case of Cri du chat syndrome to be thoroughly investigated regarding the cytogenetic analysis, as FISH technique was available to assess the absence of the critical region for the syndrome.

For children with congenital abnormalities, an early clinical diagnosis confirmed through cytogenetic and molecular investigations, is important for providing personalized diagnostic and prognostic evaluation. The

thorough investigation is important for genetic counseling regarding the reproductive risk, particularly for patients with parental chromosome translocation involvement.

Studies have shown that several genetic and environmental factors can influence the psychomotor development. The results of Cerruti et al. (2006) showed an improvement in comparison with the past. In addition to the factors previously considered (home-rearing, early starting of physiotherapy), early education, the use of information technology and sport has certainly contributed to this result, also improving social insertion.

Recent studies results show a more optimistic aspect of the disorder than in the past, which may support caregivers and parents to work together in order to improve the quality of life of children and their families.

Our patient remains to be followed up and hopefully given the correct social insertion.

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PLURIMALFORMATIVE SYNDROME – CASE REPORT

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Abstract

Complete situs inversus is a genetic disorder with autosomal dominant, autosomal recessive or x-linked transmission, part of the group of ciliopathies and the subgroup of primary ciliary dyskinesias. We present the case of a 3 years and 8 month old girl, who associates complete situs inversus to chronic renal failure – left vesicoureteral reflux with secondary hydronephrosis, hypoplastic ectopic right kidney, hydrocephaly, bilateral varus equine foot and failure to thrive. She was first admitted in our hospital at 3 weeks of age and followed-up since then, necessitating complex medical and surgical therapy: treatment of recurrent urinary tract infections, surgical treatment of the VUR - terminal ureterostomy at 9 month, surgical treatment of the hydrocephaly – Medtronic – Delta valve – at 1 year and 6 month, surgical treatment of the varus equine foot at 3 years, nutritional and neurological recovery. The particularity of this case resents in the association of multiple malformations with a bad prognosis, because of progression of the renal failure and of the neurological impairment. She needs a complex follow-up with the collaboration of the paediatric nephrologist, paediatric surgeon, paediatric neurologist, brain surgeon and family doctor.

Key words: ciliopathies, situs inversus, hydronephrosis, hydrocephaly, child.

Introduction

Cilia are cellular organites with micro tubular structure, which are present on the surface of the majority of the human cells. Although they were described for the first time in 1853 (Purkinje and Valentin), the most discoveries related to their structure and function were made in the last 10 years. During almost 200 years cilia became from simple vestigial organites, cellular "antenna" that mediate a multitude of signalization ways.

Studies regarding cilia led to the definition of ciliopathies, a pathogenic group whose etiology is based on defects in the genesis, structure and/or function of the cilia, characterized by important genotypic and phenotypic heterogeneity.

Case report

Anamaria I., 3 years and 8 month old, first admitted in January 2007 and followed up since then in the Paediatric Clinic I, Children's Hospital "Louis Turcanu" Timisoara, is the first child of a mother who worked in a toxic

environment in the first 3 month of pregnancy. She was born premature (31 weeks gestational age), Apgar score = 7, birth weight = 2200 g.

History reveals a first admission to the hospital at 3 weeks of age, transferred from the County Hospital Arad to the Premature Department of our hospital with the following diagnoses: Acute renal failure. Urinary tract infection with *Candida albicans*. Left knee osteoarthritis with *Candida albicans*. Complete situs inversus. Bilateral hydronephrosis. Bilateral varus equine. Intraventricular hemorrhage grd. II. Neonatal seizures, prolonged admission until 3 month of age with a slow favorable course. She presented 2 more episodes of urinary tract infection with *E coli* at 4 and 6 month of age, and imaging was performed: cystourethrography showed massive left vesicoureteral reflux (VUR) with grd. IV hydronephrosis, urography confirmed the left hydronephrosis and showed an ectopic pelvic right kidney with delayed, poor secretion after 3 hours; renal scan using 99mTc-labeled dimercaptosuccinic acid (DMSA) revealed a normal sized left kidney with poor excretion and stasis and a pelvic right kidney with reduced capture and excretion, relative function 27%-right kidney and 73%-left kidney. At 9 month of age she was admitted to the Paediatric Surgery Clinic, Children's Hospital "Louis Turcanu" Timisoara for the surgical treatment of the vesicoureteral reflux – terminal ureterostomy. From May 2006 until January 2007, the patient was non-compliant to follow-up and therapy.

The patient was first admitted to our clinic with fever, unrest, disuria, failure to thrive. Physical examination revealed weight = 5600 g, height = 64 cm, cranial circumference = 47 cm (all parameters under 3rd percentile), temperature = 38,2°C, pallor, normal respiratory findings, HR = 98/min, BP = 90/60 mm Hg, permeable ureterostomy in the left iliac fossa, normotensive anterior fontanella, spastic hypertonia of the legs, motor retardation, bilateral varus equine. Biological findings showed positive inflammatory markers, GFR = 38,26ml/min/1.73m2, leucocyturia and negative urine culture (urine collection after initiation of antibiotherapy).

Abdominal ultrasound: situs inversus, liver (left) with normal structure, left hepatic lobe = 76,7mm, portal vein = 7,16 mm, spleen (right) with normal structure, long axis = 64 mm, left kidney with irregular shape and contour, 58,3/36,3 mm, pelvic dilation of 22,6/9,67 mm, grd. II hydronephrosis, right kidney undetectable, bladder with normal walls. (Fig. 1)

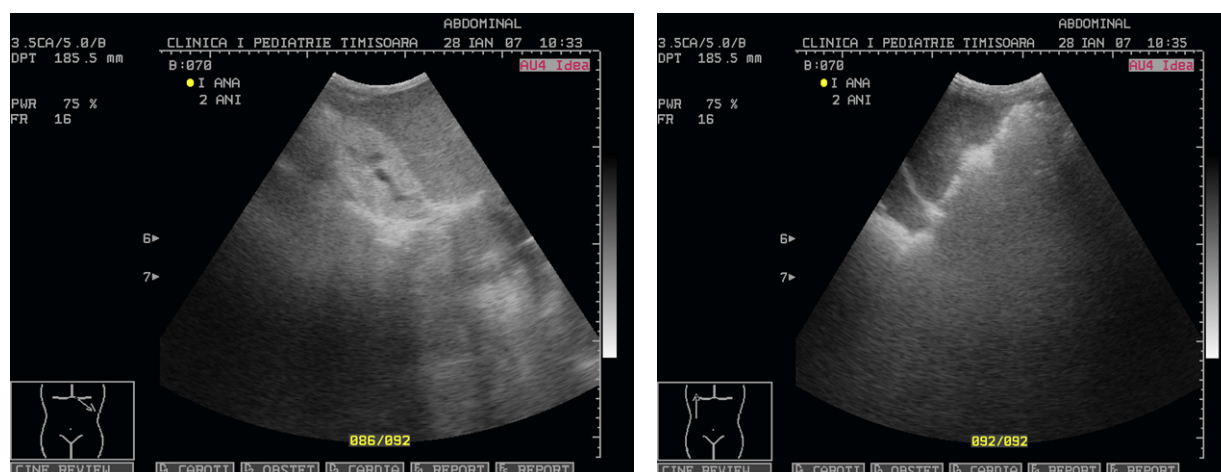


Fig. 1: Abdominal ultrasound.

Cardiac ultrasound: atrioventricular and ventriculoarterial concordance, mitrale and tricuspidian grd. I regurgitation, normal origin of the coronary arteries, right aortic arch, descendent aorta on the right side of the spine, situs inversus totalis.

EKG: normal sinusal rhythm, 130/min, QRS axes 130°, indirect signs of right ventricular hypertrophy, aspect of dextrocardia.

Recurrent urinary tract infections were the reason for completing imaging in this case and an abdominal MRI was performed (February 2007), which revealed: complete situs inversus, liver on the left and spleen on the right side, right position of the heart, left kidney with normal position, 5,8/3 cm, with preserved function and pyelocalyceal hypotonia, right kidney ectopic, anterior of the sacral vertebrae, 4/2,1 cm. Conclusion: complete situs inversus, ectopic right kidney. (Fig. 2)

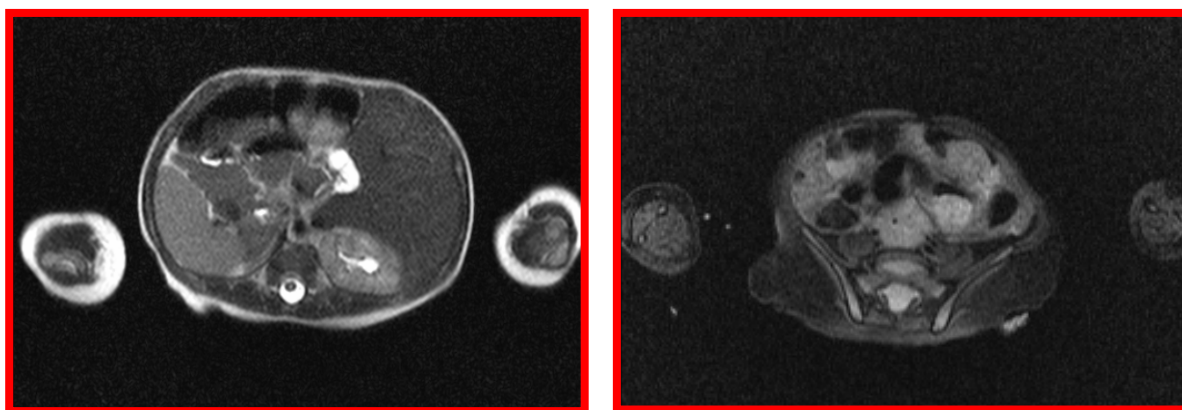


Fig. 2: Abdominal MRI.

Neurological impairment was the reason for a complete neurological assessment: Electroencephalogram, ultrasonography and cerebral MRI (02.2007), which showed an active tetra ventricular hydrocephaly. (Fig. 3)

Correlating the clinical, biological and imagistic data, the following diagnosis was established:

- Left vesicoureteral reflux with secondary grade II hydronephrosis
- Hypoplastic ectopic right kidney

- Recurrent urinary tract infection
- Chronic renal failure stage II
- Complete situs inversus
- Active hydrocephaly
- Recurrent seizures
- Failure to thrive
- Bilateral varus equine

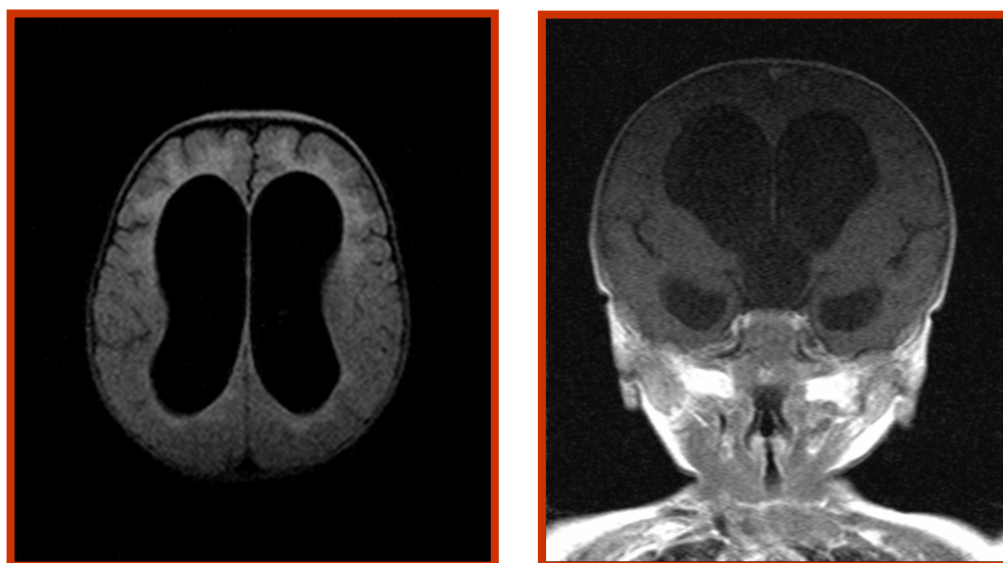


Fig. 3: Cerebral MRI.

Medical therapy consisted in antibiotics: Cephtriaxone 100 mg/kg/day, iv, 7 days, followed by prophylaxis with Nitrofurantoin 2 mg/kg/day once daily, and symptomatic treatment: antiemetic, antipyretic, anticonvulsive medication and vitamins (D, group B). Surgical therapy of hydrocephaly, consisting in ventricular-peritoneal drainage with Medtronic – Delta valve, was performed (February 2007). Diet recommendations were: caloric intake of 120 kcal/kg, aprox. 100% RDA for age, protein intake 0,6 – 0,8 g/kg/day – 100 – 120% RDA, supplementation with essential amino acids and ketoacids, phosphate restriction and no fluid restriction.

Clinical course was initially favorable, she still presented until now 2 -3 episodes of urinary tract infection/year, explained by particular factors such as – complex urinary tract malformation, presence of ureterostomy, secondary immune deficiency – chronic renal failure is characterized by lymphopenia, inappropriate response of polymorphonuclear cells to bacterial infection and insufficient Fc receptors on the macrophages and malnutrition by atrophy of lymphatic organs, depletion of LT, L NK, reduction of bactericidal activity of polymorphonucleares, low levels of secretor IgA, alteration of complement system. Surgical treatment of varus equine was performed July 2008.

Possible complications are those of VUR: renal scars, hypertension (aprox. 10% of the children with renal scars), chronic renal failure (in USA 8,4% of the cases with chronic renal failure are due to reflux nephropathy) and urolithiasis (19,1 – 29,8 %, after Millnier et al., hypercalciuria present in over 50% of the children with VUR being a risk factor) and those due to long time administration of antibiotics: rash, hepatic toxicity, medullar toxicity, antibiotic resistance.

Situs inversus per se has no complications but can be associated in 5 – 10% with cardiac anomalies (transposition of great vessels), especially when situs inversus is incomplete (up to 95%).

Short time prognosis of the urinary tract infections is good, long time prognosis depends on the frequency of the recurrent infections, progressive evolution of renal failure and neurological impairment.

The case needs a complex follow-up with the collaboration of the paediatric nephrologist, paediatric surgeon, paediatric neurologist, brain surgeon and family doctor.

Discussions

The case is particular due to the complex plurimalformative syndrome: the literature mentions the association between situs inversus and hydrocephaly, the association between situs inversus and renal anomalies such as renal dysplasia/hypoplasia/tumors but not the association between all three elements.

VUR is considered the most frequent malformative uropathy of the child. The prevalence in the healthy population is unknown, the incidence is 1,3% after Ransley¹ and between 29 – 50% in the population with urinary tract infections, with a higher rate in girls.

Renal hypoplasia is frequent unilateral, with a higher frequency in boys, with an incidence of 1/300 newborns, is often associated to VUR.² Clinical forms: renal dysplasia, aplasic type (loss of cortical and medullar structure, hystopathologic diagnosis), ischemic small kidney (hypoplasia of the renal artery, history of high blood pressure) and small pyelonephritic kidney (history of recurrent pyelonephritis).³ Ectopic kidneys are associated in 25 – 70% of the cases with VUR.

Complete situs inversus was first described in 1793 by Baillie. It is a ciliopathy, with autosomal dominant, autosomal recessive or x-linked transmission.

In the embryonic stage of gastrula the clockwise rotation movement of the nodal cilia (mobile, structure 9+0) determines an extra cellular fluid flux towards the left side and implicit

the left – right asymmetry.⁴ The anomalies of these cilia are implicated in the generation of complete situs inversus and situs ambiguus (polisplenia, agenesis of the spleen, annular pancreas, horseshoe kidney etc.).

Primary ciliary dyskinesias are a heterogeneous group of disease, 90% show structural changes of the outer-arm or inner-arm of dynein. They are the result of defects of the mobile cilia (structure 9+2). The mobile cilia contain over 250 proteins with structural and/or functional role. Any defect at any of these proteins generates motility anomalies (hyper motility, hypo motility, asynchronism) and consequently the reduction of the mucociliary clearance. Clinical implications can be the appearance of recurrent or chronic respiratory infections, hydrocephaly, and fertility problems. Primary ciliary dyskinesia is associated in 50% of the cases with situs inversus, association known as

Kartagener syndrome. In this case the mutation is on the gene DNAH 11 (7p21) – motor subunit of dynein, "left-right dynein", responsible for the rotation movements of the cilia of ciliar cells of Hensen nodule. (Fig. 4)⁵

Other ciliopathies are generated by defects of the primary cilia (structure 9+0, immobile). These are receptors for a multitude of signalization ways, which maintain the cellular homeostasis. Impossibility of maintaining this homeostasis determines the appearance of polycystic kidney disease, as well as of some syndromes that can associate retinitis pigmentosa, impaired sense of smell, obesity, diabetes, hypertension, mental retardation: Senior-Loken syndrome, Joubert syndrome, Biedl-Bardet syndrome.⁶

It seems that the neural tube defects appear too as a consequence of primary cilia anomalies, there also are several syndromes unidentified yet.⁷

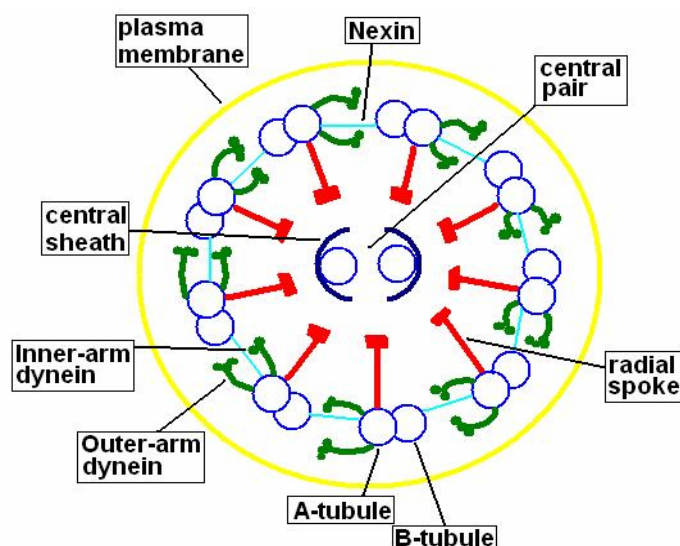


Fig. 4: Structure 9+2.

The association situs inversus – hydrocephaly was first mentioned in the literature by Greenstone in 1984. In 2002 Tallon et al demonstrated in mice that the mutation of the gene Mdnah 5 (chromosome 15) generates the absence of the outer-arm of dynein and clinic situs inversus and hydrocephaly in all cases.⁸ Hydrocephaly is the consequence of ependymary cilia alterations as part of the primary cilia dysfunction. There also is demonstrated (2007, a 5 children study group) that the mutation of the gene 1p 31.3, which encodes the nuclear transcription factor IA (NFTIA) is

associated to central nervous system anomalies such as agenesis of corpus callous + hydrocephaly + ventriculomegaly + Chiari type 1 malformation + "tethered spinal cord" + VUR.⁹

Early development of chronic renal failure (reflux nephropathy) in this case is due to the coexistence of several risk factors: recurrent urinary tract infections and renal scars, association with other renal anomalies, low birth weight, neurological impairment.

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COWS MILK ALLERGY - CASE PRESENTATION

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Abstract

Paper aim is to present the case of six months old baby, admitted in Clinic II Pediatrics for agitation, growth impairment.

Key words: milk protein allergy, children, colic.

Background

Cows milk allergy is one of the most common food allergies prevalent during infancy and it can manifest as skin rash, diarrhea, regurgitation or even constipation. Childhood cow's milk allergy is a diagnosis encompassing various syndromes. Antigen-immunoglobulin E (IgE) antibody interaction is classically involved in mast cell degranulation in IgE-mediated food allergy, while non-IgE mediated cow's milk allergy is mostly mediated by cellular mechanisms. The diagnosis of cow's milk allergy largely relies on a good knowledge of the clinical expression of the disease. Considering the multiples possibilities of reaction to cow milk this condition has to be considered as a potential etiology in colic, failure to thrive.

Case presentation

A 6 months old boy was admitted in our clinic for: colic, sleep disorders, poor weight increase (weight below 2 SD). His father had also sleep disorders as a child and frequent migraines as adult. The child was born at 36 gestational age, his birth being precipitated by an accidental trauma; with 2650 g weight and 9 Apgar score.

He received natural alimentation for 1 month and at 2 months after mixed fad with mother milk and a normal milk formula. He had a good neonatal evolution, with growth in weight ~ 1.5 kg in the first month; at 6-8 weeks of age sleep disorders begun with periods of prolonged wakefulness crisis marked by agitation and crying without apparent reason. Also erythematous lesion appeared on the abdomen and face and poor growth weight. He was consulted by a neurology pediatrician who diagnosed spasms in extension, myoclonic seizures and recommended clonazepam. Excepting inconstant amelioration of colic attack no improvement in weight gain. Associating pallor and poor appetite he was directed for pediatric evaluation.

Clinical examination revealed an infant with 6.1 kg weight, 72 cm pale, with a discrete muscular hypotonia, subcutaneous cellular tissue in the lower chest and abdomen; thorax with rickets sign. On heart and lung auscultation examination was normal. Also exam of the gastrointestinal and renal apparatus was normal. Intestinal transit disorders consisted in constipation and occasional regurgitation. Infant was reagent, with normal reflexes

present, frequently crossing legs. Left Babinsky sign was positive.

Laboratory investigations revealed anemia associated with hypoproteemia and sideropenia. Also hypoglobulinemia was documented $\gamma=6,7\%$ with IgA deficiency. Normal liver and renal parameters were registered; biochemical fecal examination was normal, with acceptable digestion assessment.

In evolution, during his admission in the hospital a neurological crisis was observed, with spasms in extension + opisthotonus, lasting about 5 minutes, marked uneasiness, accompanied on the set, followed by deep sleep. In evolution, a generalized erythema rash on the trunk, abdomen, with rapid remission (~ 10 h) ad integrum was observed. Subsequently anamnesis revealed that the rash appeared after cheese (casein) ingestion.

Cow milk allergy was suspected and we found occult blood in the stool. Total serum IgE level was increase (but specific Ig E for beta lactoglobulin or casein was negative).

We considered that the child has an allergy to cow milk proteins (rash associated, colic, anemia), complicated with anemia secondary to hypoproteinemia and hyposideremia associated with malnutrition (IP = 0.8). Milk was replaced with a dietetic formula with intense hydrolyzed proteins and avoidance of any milk products. The diagnosis was sustained by the good evolution of the baby after exclusion of milk, with improvement of clinical status, weight gain (weighing ~ 700 g / 3 weeks).

For differential diagnosis many conditions were considered as follow:

1. Allergic enteropathy (immune mediated type IV) from other allergen-soybean, rice, manifested by malabsorption, weight stagnation, rectal bleeding or occult bleeding and / or diarrhea stools was excluded by the absence of mentioned food allergen.

2. Eosinophilic gastroenteropathy (allergy nonIg E digestive determination) associate malabsorption with weight deficit, vomiting-location at a gastric level, or diarrhea in colitis. Exclusion: no clinical specific sign, biological: without eosinophilia.

3. Autoimmune enteropathy lesions secondary reactions of a type IV-lymphocyte Th1h/LfTs the imbalance, expressed clinically by failure to thrive, malabsorption, accompanied by elements of autoimmunity was excluded, clinical-absence of other autoimmune diseases (thyroid, hepatitis) and normal biological parameters.

4. Coeliac disease (atypical) associate, anemia, occult bleeding and frequently selective IgA deficiency. He was

excluded because the disease onset was before gluten administration, and he gained weight on gluten present diet.

5. Other weight causes of poor growth like digestive malformations, cardiac, renal, chronic infections were excluded clinically and biological.

Dietary therapy of the complications, besides exclusion of milk and milk products, a vitamin supplements (vit.B6, vit D) and minerals (Fe) was necessary.

Monitoring of allergy and complications need assessment of nutritional status, anthropometric parameters every two months and biological control at 6 months.

Particularity of the case was that allergy to cow milk manifested by weight deficit, colic, rash without the classic manifestations of diarrhea or rectal bleeding

Conclusion: Literature shows that most organs can be affected by allergy PLV with various expressions:

1. Gastrointestinal disease: gastroesophageal reflux, eosinophilic gastroenteropathy, colitis, constipation.

2. Respiratory diseases: asthma and allergic rhinitis (6% of asthma-crises triggered by food allergens), hemosiderosis of the lung

3. Skin: atopic dermatitis, multiform erythema, angioedema

4. Sistemic diseases:

-anaphylactic shock

-weight stagnation, anemia

We should always keep in mind cow's milk allergy as a possible etiology in many cases.

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PRENATAL GENETIC DIAGNOSIS IN MUCOVISCIDOSIS (CYSTIC FIBROSIS) BY CLASSIC AND EARLY AMNIOCENTESIS

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Abstract

Mucoviscidosis is the most common genetic autosomal recessive disease in Caucasian populations, a potentially lethal disease and therefore prenatal genetic diagnosis is essential for couples with increased risk of having children with mucoviscidosis.

Our goal was to detect CFTR mutations in fetal genomic DNA isolated from amniotic fluid collected by classic amniocentesis and early amniocentesis in order to establish if the fetus is healthy, just a carrier for one CFTR mutation or both alleles are affected and the fetus has mucoviscidosis.

Key words: mucoviscidosis, CFTR mutations, amniocentesis.

Introduction

Mucoviscidosis or cystic fibrosis is caused by mutations of the CFTR gene (cystic fibrosis transductance conductance regulator), located on chromosome 7 in locus q31.2. Over 1600 mutations and sequence variations (polymorphisms) have been described until now, however only one mutation was found in over 70 % of the investigated chromosomes - $\Delta F508$. Mucoviscidosis is the most common genetic autosomal recessive disease in Caucasian populations, with an incidence of 1/2200 – 2500 live birth and a carrier frequency of 1 in 22 persons^{1,2}.

Prenatal genetic diagnosis involves sampling of the amniotic fluid by amniocentesis. Amniocentesis is an invasive procedure which can be carried out between the 15 and 16th week of pregnancy or prior to the 15th week of pregnancy - early amniocentesis (most commonly between 12–14 gestational weeks). Early amniocentesis results are available 4–6 weeks before standard amniocentesis and 1–3 weeks after chorionic villus sampling (CVS), presenting an attractive method for prenatal diagnosis in the early second trimester, despite a somewhat higher rate of immediate post procedure complications^{11,12,13}.

Amniocentesis should be preceded by genetic counseling, in which the family pedigree and genetic risk are evaluated and the advantages and risks of the procedure are explained.

Material and methods

Ten couples were selected for performing prenatal diagnosis. Eight couples were carriers for CFTR mutations. The couples had children with mucoviscidosis (registered in the database of the National Centre for Mucoviscidosis, Timisoara), that were genetically tested and had both mutations identified, or had deceased children clinically diagnosed with mucoviscidosis, with or without a molecular diagnosis. Two couples had fetal hyperechogenic bowel diagnosed at routine ultrasonography. Genetic testing was carried out for each parent, regardless of their family history or previous genetic tests.

Molecular diagnostic was performed on Genomic DNA isolated from venous blood samples collected on EDTA from both parents and on amniotic fluid samples collected by classic amniocentesis (16th week of pregnancy) in 9 cases and by early amniocentesis (13th week of pregnancy) in one case.

By classic amniocentesis we collected 15 – 16 ml of amniotic fluid (1 ml for each week of pregnancy); the first 5 – 6 ml were not used, only the remaining 10 ml, in order to avoid contamination of the amniotic fluid with blood or maternal cells.

The amniotic fluid was also visually inspected after sampling to detect any possible traces of blood. Brown or green tinged amniotic fluid is aspirated in 1–6% of midtrimester amniocenteses and may be associated with an increased risk (5–9%) of perinatal mortality and pregnancy loss. Analysis of discolored fluid samples indicates that in most cases the discoloring pigment is hemoglobin. Vaginal bleeding prior to amniocentesis seems to predispose for presence of discolored amniotic fluid¹³.

By early amniocentesis only 600 μ l of amniotic fluid was collected. It has been suggested that removal of amniotic fluid at amniocentesis, especially when performed early in gestation, may affect fetal lung development. Lung function tests performed after birth to babies subjected to amniocentesis apparently demonstrated lower dynamic compliance and higher resistance compared to controls. Other studies could not document an effect on neonatal lung

function tests but noted a significantly higher incidence of respiratory distress and admissions to special care units for neonates subjected to chorionic villus sampling (CVS) in the first trimester. Apparently, both amniocentesis and CVS performed in the first trimester may impair antenatal lung growth. Therefore only a reduced volume of amniotic fluid was collected¹³.

DNA was isolated immediately after sampling in order to obtain the best results. Due to the low cellularity of the amniotic fluid, before DNA extraction, the samples of amniotic fluid were subjected to a mild centrifugation (2,000 rpm), the resulting supernatant (approximately 9 ml) was removed, the remaining sedimented fetal cells being resuspended in about 1 ml remaining fluid. These procedures aimed at concentrating fetal cells in the amniotic fluid and increasing the DNA quantity obtained following extraction. In the case of amniotic fluid collected by early amniocentesis we used the whole quantity without centrifugation of the sample. The blood samples taken from the parents were either immediately processed or preserved at -20°C for later analysis^{14,15}.

For detection of CFTR mutations we used the Elucigene CF29 kit (Tepnel Diagnostics, UK). The kit can identify 29 mutations considered to be the most common among Caucasian populations. At the same time, Elucigene CF29 can identify the normal allelic variant for the locus characteristic for the $\Delta F508$ mutation, which is the most common CFTR mutation (approximately 70% for Western and Central Europe populations and the USA), so that one can differentiate between $\Delta F508$ heterozygots (carriers of the mutation) and $\Delta F508$ homozygots (patients have mucoviscidosis and both alleles are affected). For the rest of the mutations, differentiation between heterozygots and homozygots is not possible, but their low incidence seldom induces the occurrence of homozygots. The method used by Elucigene CF29 is based on ARMS-PCR (amplification refractory mutation system)^{15,16}.

For genomic DNA isolation from blood collected on EDTA and from amniocytes found in the amniotic fluid we used commercial kit - Qiagen QIAmp DNA Blood Mini. For the extraction of genomic DNA from the amniotic fluid, the extraction protocol (Qiagen) used for blood samples was slightly modified in order to enhance the concentration of isolated DNA. Thus, we used a greater volume of amniotic fluid (600 - 1200 μ l), the final elution time was extended to up to 5 minutes and the volume of the buffer solution was reduced to 150 μ l or even 100 μ l. DNA concentration was measured with NanoDrop 1000. For the amniotic fluid samples the results were situated in the 1 – 2 ng/ μ l interval and for the sample collected by early amniocentesis the DNA concentration was 0,5 - 1 ng/ μ l. The recommended DNA concentration for an optimal PCR amplification is situated in the 1 – 10 ng/ μ l interval. The samples of fetal DNA isolated from amniotic fluid were used without dilution^{14,15,16,17}.

Isolated genomic DNA was amplified following the amplification program from Elucigene CF29 work protocol:

AmpliTaq Gold polymerase activation at 94°C for 20 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, primer attachment stage at 58°C for 2 minutes and extension at 72°C for 1 minute. At the end of the amplification program, the extension stage of the last cycle, at 72°C, was programmed to last 20 minutes. For one sample we used a PCR master mix which contained 1.5 μ l DNA polymerase (Ampli Taq Gold), 8.5 μ l sterile deionized water, 2.5 μ l buffer solution for dilution and 12.5 μ l staining solution. The resulting mix was divided into four equal parts of 5.5 μ l in four sterile 0.5 ml Eppendorf tubes, each tube containing 16.5 μ l primer mixt (TA, TB, TC, TD). Subsequently, 20 μ l were taken from each tube which were then introduced into a thin 0.2 ml PCR tube followed by 5 μ l of the extracted genomic DNA^{14,15}. Electrophoresis of PCR products was carried out on agarose gel 3% (NuSieve 3:1; Cambrex BioScience), with ethidium bromide (20 μ l ethidium bromide to 20 ml gel). The migration buffer solution used was TBE (Tris-Borat-EDTA), and migration took place in a Sub-Cell GT DNA Electrophoresis system (Bio-Rad) apparatus. We used the 50 Base-Pair Ladder (Amersham-Pharmacia Biotech) as marker of the size of the fragments obtained by PCR reaction (dilution: 80 μ l sterile deionized water, 10 μ l staining solution and 10 μ l Ladder 50 bp). In each well of agarose gel 20 μ l of the PCR products were introduced, and adjacent to these we loaded 20 μ l 50 Base-Pair Ladder dilution^{14,15}. The migration took place at 4 – 5 V/cm calculated to the distance between the electrodes (for a gel of 6 x 7 cm and a distance of 20 cm between the electrodes, we used an electric potential of up to 80 V), until the dye front had migrated 5 cm from the loading wells towards the anode (1 to 1.5 hours)^{16,17}. The gels were visualized with an UV transilluminator at 260 nm and photographed with a Canon Powershot A710 digital camera with filters adapted to the corresponding wavelength of ethidium bromide light emission. The fluorescent signals for the sample collected by early amniocentesis were weak, but by increasing the exposure time when the gel was photographed we managed to obtain proper results which allowed us to establish the molecular diagnosis^{15,16,17,18}.

Results and discussion

The photos of the gels were analyzed and the results were interpreted according to the diagram shown in Figure 1.

Genetic testing of the parents in one investigated couple showed that the mother was a carrier for $\Delta F508$ mutation (genotype $\Delta F508/N$), and the father was a carrier of the G542X mutation (genotype G542X/N). The couple had already had a child with mucoviscidosis^{19,20}, complete form, with pulmonary and pancreatic involvement, who was genetically tested and presented the G542X/ $\Delta F508$ genotype (compound heterozygote). Both the $\Delta F508$ mutation and the G542X mutation are severe mutations which induce the occurrence of complete forms of the disease.



Figure 1. Interpretation of the results obtained by electrophoresis of PCR products.

The electrophoregram for this case showed the presence of a 279 pb fragment which, according to the interpretation diagram, corresponds with the G542X mutation. For the locus of the $\Delta F508$ mutation, the normal sequence was found (160 bp), the mutant sequence characteristic for the $\Delta F508$ mutation being absent (Figure

2). The fetus was only a carrier of G542X mutation (heterozygote). As for the disease to become manifest requires the existence of at least two mutant alleles, the fetus was going to be clinically healthy. The couple was informed the fetus health condition, and the recommendation made during genetic counseling was to continue the pregnancy¹⁵.

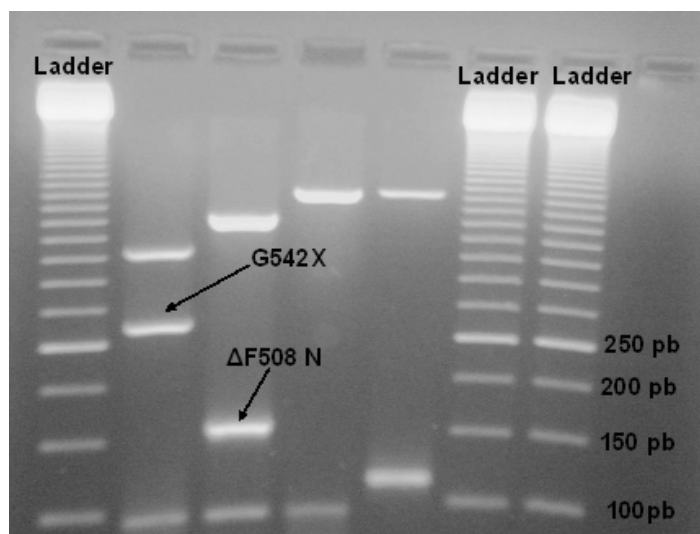


Figure 2. Electrophoresis of PCR products – G542X heterozygote, G542X/N genotype.

Three couples were carriers for $\Delta F508$ mutation (severe mutation) and had in their family history children with mucoviscidosis or deceased children of mucoviscidosis^{21,22}. In all these cases we established by prenatal diagnosis that the fetuses were only carriers for $\Delta F508$ mutation (genotype $\Delta F508/N$, figure 3) and the genetic counseling recommended the continuation of pregnancy. In one of these cases, due to the anatomical particularities of the mother it was not possible to collect an uncontaminated (with blood) sample of amniotic fluid. Therefore we isolated the fetal amniocytes by culture and the fetal genomic DNA was isolated from cultured amniocytes.

In one investigated couple, family history showed a deceased child, who had died in its first month, clinically diagnosed with mucoviscidosis but without molecular diagnosis. The parents were genetically tested and the results showed that the father was a carrier of the $\Delta F508$ mutation, and the mother was a carrier of the 621+1 G>T mutation. Prenatal diagnosis showed that the fetus was a compound heterozygote for the two mutations (genotype $\Delta F508/621+1G>T$), a condition that confirms the diagnosis of mucoviscidosis, both alleles being affected (Figure 4). The parents were informed of the result, receiving full information on the child's chances of survival with early proper treatment. The couple decided to terminate the pregnancy¹⁵.

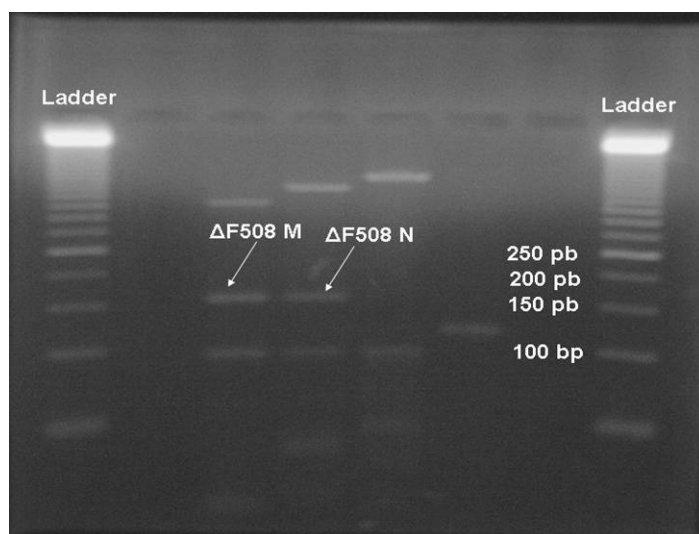


Figure 3. Electrophoresis of PCR products – heterozygous genotype, $\Delta F508/N$.

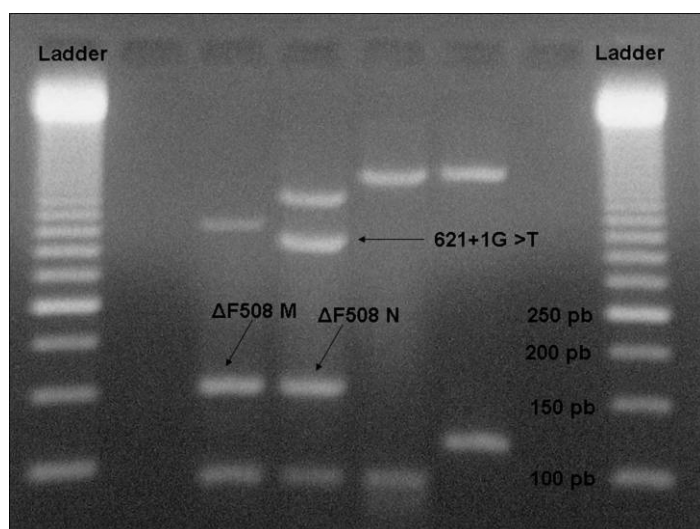


Figure 4. Electrophoresis of PCR products – compound heterozygote, genotype $\Delta F508/621+1 G>T$.

A normal genotype was found in the prenatal genetic diagnosis for 5 couples. The $\Delta F508$ mutation or other mutations were absent. In figure 2 and figure 3 we can observe the electrophoresis band characteristic for the normal sequence for the $\Delta F508$ locus ($\Delta F508 N$, 160 pb). The parents were previously tested. In one couple the mother was a carrier for G542X, and the father being a carrier for $\Delta F508$. The couple had in their history a deceased child with the clinical diagnosis of mucoviscidosis, who was not genetically tested. Following the result of prenatal diagnosis, the recommendation was to continue the pregnancy, the fetus having none of the mutations of the parents.

In other couple the mother was a carrier for N1303K mutation and the couple had a child with mucoviscidosis who was genetically tested but only one mutation (N1303K) was found. Since it was possible that the father was a carrier for a CFTR mutation which could not be detected by Elucigene CF 29, the only variant which was acceptable for the fetus was a healthy genotype, situation which was found

in this case and the recommendation was to continue the pregnancy.

Two couples had echographic findings (hyperechogenic bowel) which can be a hallmark for mucoviscidosis and recommended the prenatal diagnosis. However in these cases the results were negative for mucoviscidosis and a normal fetal genotype was found.

In only one case we used amniotic fluid collected by early amniocentesis. Both parents were carriers of $\Delta F508$ mutation and had a deceased child with mucoviscidosis. The volume of collected amniotic fluid was small (0,6 ml) due to the risks of the procedure to the fetus and the interpretation of electrophoregram was difficult due to the reduced quantity of isolated DNA. Weak fluorescent signals were photographed on the gel and we had to increase the exposure time in order to have suitable results (Figure 5).

Improved ultrasound technology, increasing experience with ultrasound-guided needle manipulation and patient preference for more private, earlier genetic diagnosis have motivated a shift from second trimester amniocentesis toward earlier procedures - CVS and "early" amniocentesis.

Early amniocentesis refers to procedures performed before 15 weeks' gestation (most commonly between 12–14 gestational weeks). The approach technique is somewhat different from that used at midtrimester, for 2 reasons. First, ultrasound guidance is essential as the size of the fluid pocket is much smaller and requires greater experience to access safely. Second, if one pushes the needle slowly into the pocket, there is a much higher likelihood of tenting the fetal membranes, which did not adhere yet to the uterine

wall. A 22-gauge needle was used, which is inserted to the myometrium. After fetal position is verified, the needle is advanced in a single, swift thrust into the pocket of fluid. Sometimes, rotating the needle may also help to overcome tenting of the membranes. One ml of amniotic fluid per every week of gestation is aspirated into a syringe, transferred into sterile tubes and sent to the laboratory for processing¹³.

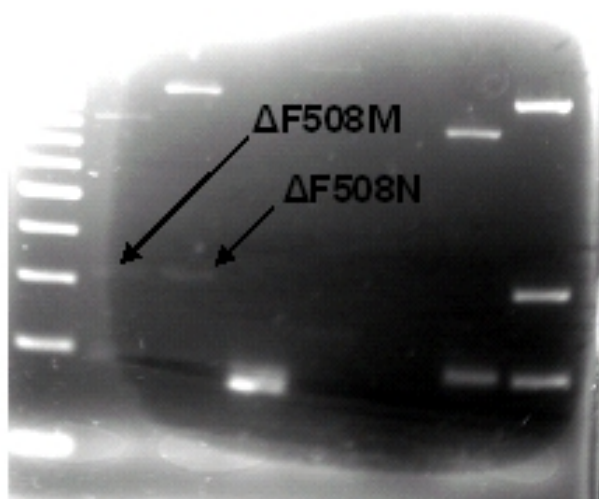


Figure 5. Electrophoregram of PCR products obtained by amplification of isolated DNA from amniotic fluid collected by early amniocentesis. Increased exposure time of photo.

One important study which systematically looked at early amniocentesis was done in Canada (Canadian Early and Mid-Trimester Amniocentesis Trial-CEMAT Group) published in 1998²³. In this trial, 4,374 women were randomized to either early amniocentesis (between 11 and 12 6/7 weeks) or midtrimester amniocentesis (between 15 and 16 6/7 weeks). This and subsequent reports from the trial demonstrated that compared to midtrimester amniocentesis, early amniocentesis was associated with a 4-fold risk of a technically difficult (twice the risk of requiring multiple needle insertions) or unsuccessful procedure (1.6% vs. 0.4%), a 10-fold risk of chromosome culture failure (2.4% vs. 0.25%), a higher rate of fluid leakage following the procedure (3.5% vs. 1.7%), a greater risk for pregnancy losses (7.6% vs. 5.9%), and a significantly higher risk (1.3% vs. 0.1%) of having a baby with talipes equinovarus (club foot)²². Early amniocentesis (EA) was considered as an early prenatal diagnosis technique option due to the concerns related to CVS and the wide spread use of amniocentesis at 15–16 weeks gave false reassurance that amniocentesis could be used safely at an earlier gestational age. The ultrasound-guided amniocentesis technique was moved down in gestational age with procedures being undertaken in the 11–14 gestation weeks. Early observational studies were not able to identify the risks of the procedure and it was only after 3 randomized trials were completed (Sundberg, 1997; CEMAT, 1998 and EATA, 2004)^{23,24,25} that the true risks of the procedure were identified. These early amniocentesis

risks included higher total pregnancy loss, a significant increase incidence of musculoskeletal foot deformity, a significant increased culture failure rate, and an increased post amniocentesis rate of leakage compared with the gold standard mid-trimester amniocentesis. The early amniocentesis window considered in these randomized trials was 11 weeks and 0 days to 13 weeks and 6 days. The specific risk of EA in a gestational window of 14 weeks 0 days to 14 weeks 6 days has not been clearly defined¹³.

Conclusions

Prenatal diagnosis can be performed on samples of amniotic fluid collected by normal amniocentesis in the 16th week of pregnancy or by early amniocentesis prior to the 14th week of pregnancy, however, due to the greater risks for pregnancy and fetus of this early procedure and because the volume of collected amniotic fluid is reduced (0,5 – 1 ml) it is recommended to perform classic amniocentesis.

Early amniocentesis is attractive because of a shorter learning curve, the availability, the low rate of maternal-cell contamination and the early gestational timing.

Also, for an accurate prenatal diagnosis in mucoviscidosis it is required to have a good sampling technique for the amniotic fluid in order to prevent its contamination with blood or maternal cells, and a good technique for DNA isolation.

Mutation detection by ARMS-PCR with Elucigene CF29 is applicable only to those couples in which at least

one of the parents is a carrier of the $\Delta F508$ mutation, or when both parents carry different CFTR mutations, other than $\Delta F508$, because the kit can differentiate between the

condition of heterozygote (carrier of a mutation) and homozygote (diseased) only in the case of $\Delta F508$ mutation.

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IMPLICATIONS OF PREMATURE RUPTURED MEMBRANES LABOR IN NEWBORNS' INFECTIOUS PATHOLOGY

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Abstract

Objectives: Evaluation of the premature ruptured membranes incidence in a neonatology service, identification of microbial agents involved in producing infections with intrapartum onset and analysis of clinical and evolutive parameters involved in these cases.

Material and method: Anamnestic, clinical and paraclinical data research, on all newborns resulted from premature ruptured membranes labors registered in year 2007 in the "Bega" Hospital in Timisoara.

Results: In the studied sample, 7.19% of the total cases have presented premature rupture of membranes. It can be noticed an increased incidence of this kind of cases in newborns with a low birth weight (three times bigger than in newborns with normal birth weight). The distribution of cases according to the ovular rupture duration indicates $\frac{1}{2}$ for 12-24 hours, $\frac{1}{4}$ between 24 and 48 hours and $\frac{1}{4}$ over 48 hours. Most frequently involved infectious agents were staphylococcus aureus and gram-negative bacillus. A manifest infection was identified in 40.83% of cases; a generalized infection was found to be present especially in newborns with low birth weight.

Conclusions: The risks for newborns developed infections increases with the prolonging of premature ruptured membranes labor's duration and the newborns' low birth weight. Prolonged labor in cases with premature rupture of membranes indicates the need for starting early antibiotic therapy in resulting newborns.

Key words: premature rupture of membrane, newborn, infection

Introduction

The premature rupture of the amniotic membranes (PROM) represents a clinical and biological evolving entity which is rather frequent and with a significant high-risk degree at newborns with low birth weight (1). Neonatal infections continue to be major causes of morbidity and

mortality in the newborn. This is despite improvements in antimicrobial therapy, advances in neonatal life support measures, and the prompt recognition of perinatal risk factors for infection (2, 3). Perinatal mortality and morbidity associated with preterm delivery remain a major health problem, although improved methods of obstetrics care, delivery, and neonatal care for the preterm (4).

The objectives of the study are:

- Appreciating the PROM incidence in a neonatology department on a limited period of time;
- Organization of PROM on stages according to the latency period (the time interval from the appearance of PROM till birth) and the newborn category to which it appeared;
- Identifying the microbial agents involved in producing the infections with intrapartum debut;
- Analyses of the clinical-evolving parameters of the cases included in the studied sample;
- Identification of the means and measures capable to reduce the incidence of PROM and of the consecutive complications through methods of rapid diagnosis and efficient therapy.

Material and methods

Anamnestic, clinical and paraclinical data research, on all newborns resulted from premature ruptured membranes labors registered between January 1st 2007 and December 31st 2007 in the Neonatology Department Bega, Timisoara.

Results and discussions

The studied sample was of 1668 newborns, of which a number of 120 cases (7.19%) had PROM. This incident had a greater frequency at newborns with a low birth weight, 3 times higher than the category of newborns with a weight over 2500 g (Tab. 1).

Table 1. Distribution of PRM in relation to their birth weight.

Weight at birth	Total alive newborns	PRM (Nr.)	PRM (%)
< 1000 g	6	1	16,66
1001-1500 g	29	6	20,68
1501-2000 g	41	10	24,39
2001-2500 g	78	11	14,10
> 2500 g	1514	92	6,03
TOTAL	1668	120	7,19

The repartition of the cases in relation to the duration of ovular rupture reveals a balance of almost 1/2 during the period 12-24 hours, 1/4 between 24-48 hours and approximately 1/4 in the period over 48 hours.

In this distribution the high frequency of the interval of over 24 hours seems suggestive, a period associated with a high risk of infection. Analyzing the incidence of the infection in the studied sample, it may be noticed that the infection appeared at 49 newborns (40.83%), the **generalized infection (GI)** appeared especially at newborns

with low birth weight and the **local forms (LF)** to those with weight over 2000 g (Tab. 2). The infection and the risk of generalized infection rise proportionally to the immaturity degree and the weight handicap of the newborn.

The risk of infection was high when PRM was prolonged and when it was associated with a maternal chorioamnionitis and with fetal or neonatal suffering. The cases which had PROM the aspect of the amniotic liquid was modified in proportion of 20.83%.

Table 2. Distribution of cases in relation to the incidence of the infection

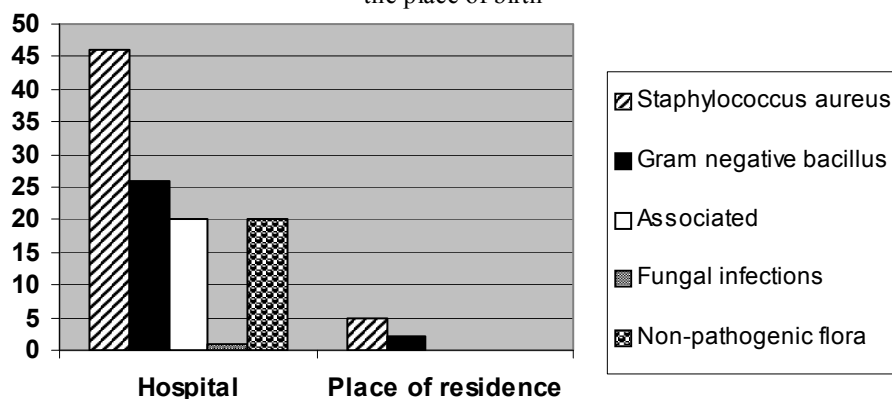
Weight at birth	GI (Nr.)	GI (%)	LF (Nr.)	LF (%)
< 1000 g	1	100	0	0
1001-1500 g	2	33,33	2	28,57
1501-2000 g	6	60	2	20
2001-2500 g	2	18,18	6	54,54
> 2500 g	2	3,26	26	28,26
TOTAL	13	10,83	36	30

Most of the studied PROM cases (95) were graded with an APGAR score between 8-10, 12 cases with APGAR 6-7 and 6 cases with a score under 5.

113 (94.16%) of the newborns with PROM were born in the maternity, the rest being born at the place of

residence. Of the etiological agents, the staphylococcus aureus (42.50%) and the gram-negative bacillus (23.33%) dominate, the place where the labor with PROM happened being of no importance (Fig. 1).

Fig.1. Distribution of cases in relation to the etiological agent and the place of birth



Further on we studied the precocious neonatal mortality at those who had PROM for a period longer than 24 hours. In relation to the total number of cases included in the lot, precocious neonatal mortality represents 4.16%

(Tab. 3). The risk of precocious neonatal mortality rises proportionally and significantly in relation to low birth weight in PROM cases.

Table 3. Predominance of precocious neonatal mortality in PRM cases.

Weight at birth	Total alive NB	PRM	Deceased
< 1000 g	6	1	0
1001-1500 g	29	6	2
1501-2000 g	41	10	1
2001-2500 g	78	11	1
> 2500 g	1514	92	1
TOTAL	1668	120	5

The causes that contributed to the lethal evolution:

- cerebral hemorrhage (5),
- generalized infection,
- respiratory distress syndrome,
- congenital malformations (6, 7).

All PROM cases were under anti infectious treatment after prevailing the bacteriological probes, the therapy being changed when necessary, according to the antibiotic sensitivities.

Conclusions

1. Incidence of prolonged PROM (over 24 hours) is significantly high to the newborns with low birth weight (under 2500 g).

2. The risk of generalized neonatal infection rises according to the duration of the ovular rupture and low birth weight.

3. The precocious neonatal mortality of the newborns with a weight under 2500 g is significantly higher if the baby is born after prolonged PROM.

4. Prolonged PROM imposes a precocious antibiotherapy for the newborn, in relation to the etiology and the antibiotic sensitivities.

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POSTAXIAL HYPOPLASIA OF THE LOWER EXTREMITY IN CHILDREN – CASE REPORT

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Abstract

Background: Postaxial hypoplasia of the lower extremity is a rare, congenital disorder characterized by partial or total absence of the fibula. It occurs in about 7 to 20 per million living birth. Males are twice affected as females. It has variable expression, ranging from mild to severe deformity and associated anomalies of the foot. **Material and methods:** We present a 14 months old male toddler, product of a non-consanguineous marriage, born at term, after an uncomplicated pregnancy. The patient presents mild shortening of the left lower extremity and foot deformity: syndactyly of the first and second toe and absence of the fifth toe. Evaluation was made by history data, clinical and genetic examination, laboratory and imaging studies. **Results:** No familial incidence of malformations, congenital infections or teratogenic factors were noticed. Completely absent left fibula and associated anomalies: tibiotalar valgus, tarsal coalition with 3-ray foot aspect; limb-length discrepancy of 2,5 cm were diagnosed. Observation and nonoperative management are appropriate in this case. Special shoes are prescribed to enable the child to gain maximal function. **Conclusions:** The diagnosis was made by the absence of the left fibula with associated skeletal anomalies. The functional, social and psychological state of the child will be considered. The patient must be monitored throughout his growth.

Key words: postaxial hypoplasia, lower extremity, rare disorder, child

Introduction

Postaxial hypoplasia of the lower extremity (fibular hemimelia) is rare and has variable expression, ranging from mild to severe deformity. It is a congenital disorder characterized by partial or total absence of the fibula. Agenesis of the fibula is the most common manifestation of a spectrum of dysplasias of the limbs, a reduction malformation affecting the long bones. Congenital absence of the fibula was first described by Goller in 1698. O’Rahilly and Frantz’s classification would be used to describe true congenital absence of the fibula and corresponding portion of the foot terminal as complete paraxial fibular hemimelia.¹ A constellation of lower-extremity features accompanies fibular hemimelia. Shortening of the limb is the most common sign and the

anomaly is mainly unilateral.¹ Generally, reported cases involve normal pregnancy with no familial incidence.¹ Theories of origin correspond to the sixth and seventh week of embryological formation with genetic versus teratogenic factors.¹ In type II deformity fibula is completely or almost completely absent. An affected extremity typically displays a valgus foot and ankle, shortening of the leg, anterior bowing of the tibia and knee, tarsal coalition and radiographic absence of one to several lateral rays.² Males are twice affected as females. It is estimated to occur in about 7 to 20 million living births.

Case report

We present an 14 months old male toddler admitted in the First Pediatric Clinic of “Louis Turcanu” Children’s Emergency Hospital for recurrent wheezing. He is second in birth order, product of a non-consanguineous marriage. The child was born at term, delivered vaginally, after an uncomplicated, normal pregnancy. No familial incidence of fibular hemimelia, no congenital infections were present. Mother wasn’t exposed to teratogenic factors. At birth he was noticed to have a left foot deformity (syndactyly of the first and second toe, an absent fifth toe) associated with a discreet shortening of the left limb.

Clinical findings: A leg-length discrepancy of 2,5 cm was associated with calcaneovalgus deformity in the left foot and skeletal anomalies: syndactyly of the first and second toe, the absence of the fifth toe; genu valgus with lateral axis displacement; a skin dimple in the left midtibial area. (Fig.1)

X-ray series (long-leg standing; pelvis/hip; tibia/fibula; ankle/foot) were done. The long-leg series noticed left fibula agenesis. (Fig.2) The ankle/foot series noticed: tibiotalar valgus and tarsal coalition; a 3-ray foot; metatarsals associated with three phalanges, two of which are fused to form only three toes; limb-length discrepancy. (Fig.3)

Ultrasonography examinations (transfontanelar/cardiac/abdominal) noticed normal aspects. Laboratory studies pointed out normal data. Orthopedic, genetic, ophthalmologic, cardiology and neurologic evaluation was done and no other abnormalities were noticed.



Figure 1. Clinical aspect of the left lower limb.



Figure 2. Long-leg standing X-ray aspect.



Figure 3. Ankle-foot X-ray series.

By the Achterman and Kalamchi classification system, is a type **II** of fibular hemimelia with completely absent fibula in this case.³ Fibula hemimelia is associated with deficiencies of the lateral aspect of the foot, calcaneovalgus deformity and associated skeletal anomalies: syndactyly of the first and second toe, the absence of the fifth toe, tarsal coalition.

By Stanitski D.F. and Stanitski C.L. system, the patient is classified as having a type **IIISc3** fibular hemimelia, where **III** is the complete absence of the fibula;

S is the spherical, ball-and-socket ankle; **c** is the presence of the tarsal coalition and **3** is the 3-ray foot.³

We considered to be a sporadic incidence of postaxial hypoplasia of the lower extremity in this case.

Therapy: Because the deformities are mild, observation and non-operative management is appropriate in this case. Special shoes are prescribed to enable the child to gain maximal function. Because the discrepancy between lower extremities may progress with growth, in the future procedures in the left foot may include resection of talar coalitions and fusions.

Follow-up: This patient must be monitored throughout his growth. The clinician must inform the family of what they might confront with. The limb-length discrepancy is one of the most difficult to address.³

Outcome and prognosis: Genu valgum associated with postaxial hypoplasia of the left lower extremity is progressive and it can adversely affect alignment of the lower limb in this case. Special shoes will facilitate an increase in weight bearing activity with minimal discomfort.⁴ The patient and his parents are at risk of emotional problems due to limb's cosmetic troubles.³

Discussions

Presentations of postaxial hypoplasia of the lower extremity (fibular hemimelia) vary widely, ranging from

what appears to be an absent fifth toe in a newborn or a minimal difference in limb lengths, to severe fibular deformities that are immediately apparent. The ipsilateral tibia may be hypoplastic, bowed or normal.^{5,6} Postaxial hypoplasia of the lower extremity can be frequently associated with femoral deficiency, deformities of the lateral aspect of the foot, or is part of a malformation syndrome.^{3,6} The most associated anomalies are skeletal and includes: syndactyly, brachydactyly, clinodactyly of fingers and toes and facial dysmorphism. The pediatrician must look for associated abnormalities, including problems with alignment and stability, because the clinical appearance may evolve with growth and development.³

Table I. Clinical findings of postaxial hypoplasia of the lower extremity.³

<ul style="list-style-type: none"> ▪ Fibular anomaly (shortening/complete absence) ▪ Femoral hypoplasia ▪ Lateral patellar subluxation ▪ Genu valgus with lateral mechanical axis displacement 	<ul style="list-style-type: none"> ▪ Coxa vara ▪ Tibial deformities (shortening/bowing) ▪ Ankle anomalies (valgus/valgus-ball-and-socket aspect) ▪ Foot deformities (absent tarsal bones/tarsal coalition/absent foot rays)
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Postaxial hypoplasia of the lower extremity is usually sporadic, with a negligible recurrence risk for the patient's siblings.^{7,8} In a small percentage of cases, a familial incidence (autosomal recessive) has been reported.^{7,8} In type II deformity patients have unilateral absence of the fibula, anterior bowing of the tibia, foot deformity with absent rays and marked shortening of the leg.^{9,10,11} These aspects are observed in about 35% of the cases. Prognosis and rehabilitation are mainly dependent of the limb malformation's severity and the possibility of orthopedic correction.¹²

Conclusions

Postaxial hypoplasia of the left lower extremity was diagnosed by the absence of the fibula with associated skeletal anomalies in the foot. The functional, social and psychological status of the patient will be considered. The ultimate goal of the treatment is to enable the child to gain maximal function by achieving adequate lower-extremity alignment, length and stability. The patient must be monitored throughout his growth.

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CORRELATION OF VILLOUS ALTERATIONS IN CELIAC DISEASE PEDIATRIC PATIENTS WITH RISK FACTORS ANALYZE

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Abstract

Objectives: There are many studies concerning the risk factors involved in celiac disease etiopathogeny. The aim of this research was to establish a correlation between presence of several risk factors and villous alteration severity by introducing a risk score. **Material and methods:** The present study was performed on a group of 25 pediatric patients with celiac disease diagnosed between September 2005 and November 2008; celiac disease severity was classified using Marsh criteria. Five celiac risk factors have been analyzed: gluten administration before age of 5 month in artificially nourished infants, presence of first and/ or second degree relatives diagnosed with celiac disease, presence of several autoimmune conditions (type I mellitus diabetes, autoimmune thyroiditis, rheumatoid arthritis, polyendocrine autoimmune conditions), Down syndrome and viral infections in patient’s medical history (adeno, herpes or rubella virus). Odds ratio (OR) and relative risk (RR) have been calculated for each of them using an original formula, and the risk score was computed for each patient. The calculated score was compared with the intestinal morphological result. **Results:** We found a strong correlation between the computed score and the villous alteration’s degree ($r=0,94$). Finally, we estimated the score parameters: sensitivity, specificity and positive predictive value, which validated our score. **Conclusions:** We consider very useful an assessment of risk differentiation in celiac patients with positive serology, knowing that the majority of gluten enteropathy subjects present the latent form of illness, without typical symptoms, according to celiac ice-berg described in 1991.

Key words: celiac disease, villous atrophy, antiendomysium antibodies, antitransglutaminase antibodies

Introduction

Celiac disease is an immune-mediated enteropathy caused by a permanent sensitivity to gluten in genetically susceptible individuals (DQ2 and/or DQ8 HLA haplotype). It occurs in symptomatic subjects with gastrointestinal and non-gastrointestinal symptoms, and in some asymptomatic individuals, including subjects affected by: type I diabetes, Turner syndrome, Williams syndrome, IgA deficiency and first degree relatives of individuals with celiac disease (1). Recent studies incriminate as potential factors involved in

celiac disease pathogeny: viral infection with adenovirus (based on structural similarity of 206 – 217 amino-acids gliadine sequence and E1b protein elaborated during infection), rubella, herpes virus infection and/or Plasmodium Yoelli parasite infection (2).

Objectives

In this study we proposed to establish several risk factors contribution in celiac disease appearance and disease’s manifestation form. It is well-known that classic risk factors (early gluten exposure – under 5 month of life in formulas fed infants, first degree relatives with celiac disease, several infectious factors, autoimmune or genetic diseases association), play an important role in celiac disease appearance. The aim of this research was to establish a relation between presence of five risk factors and the severity of intestinal morphological alteration by introducing a risk score. We also assessed this score by establishing its specificity, sensibility and accuracy.

Material and Methods

The study developed between September 2005 and November 2008. The lot of study consisted in 25 patients, aged 7 month – 18 years, sex ratio G/B 16/9. Before including each patient in the lot of study we obtained written informed consent from their parents. IgA selective deficiency was considered exclusion criteria. Celiac disease diagnosis was based on clinical and biological assessment of malabsorption syndrome, positive serological tests and small intestinal biopsy followed by histological exam of biopsy sample. Serological tests consisted in assessment of IgA anti endomysial antibodies (EMA) using indirect immunofluorescence technique on smooth muscle of monkey esophagus. ImmuGlo™ Anti-Endomysial Antibody test kits were provided by Immco Diagnostics. Assessment of IgA human tissue transglutaminase antibodies (hu-tTG) was made using ELISA technique. ImmuLisa™ anti-hu tTG antibody IgA ELISA kits were provided also by Immco Diagnostics. For intestinal biopsy we used Watson Capsule for children aged less than 6 years old and superior digestive endoscopy followed by controlled biopsy taken in patients aged more than 6 years old.

The 5 risk factors that have been present before celiac disease in the lot of study are represented by: 1.gluten

administration before age of 5 month in artificially nourished infants; 2. presence of first and/or second degree relatives diagnosed with celiac disease; 3. several autoimmune conditions (type I mellitus diabetes, autoimmune thyroiditis, rheumatoid arthritis, polyendocrine autoimmune conditions); 4. Down syndrome and 5. adeno/herpes or rubella virus infection in patient's medical history.

Odds Ratio (OR) and relative risk (RR) have been calculated for each of these risk factors, according to statistical formulas for transversal studies (3). Using an original formula, the risk score was computed for each patient:

$S = \sum OR_i \times FR_i$ (OR_i = Odds Ratio for each of 5 risk factors, FR_i symbolic represented by values of 0/1, indicates risk factor presence or absence and \sum = sum).

The calculated score was compared with intestinal morphological result. Finally, we estimated the score parameters: sensitivity, specificity, positive predictive value and accuracy.

Statistic data processing was made by using SPSS12 application.

The lot of study was divided based on intestinal villous alteration degree, using Marsh classification (1992), modified by Oberhuber (1997) as following: type II hyperplastic (infiltrative lympho-plasmocytic lesions in villous corion, associated by glandular crypt enlargement) and type III destructive (including partial and subtotal villous atrophy – type IIIa, IIIb respectively and total villous atrophy – type IIIc). (4)

Results and discussions

Distribution of villous alteration type in studied lot indicated 36% of patients with hyperplastic villous lesions (Marsh II type) and 64% of patients presented different degree of villous atrophy (Marsh III type).

Lot characteristics are shown in table I.

Table I - Lot characteristics.

No of patients	Risk factors					Type II Marsh	Type III Marsh
	Early gluten exposure <5 month	Ist and/or IInd degree relatives	Autoimmune conditions	Down syndrome	Adeno/herpes/rubella virus		
25	17	16	15	4	13	9 36%	16 64%

Risk factors weight can be assessed by calculating OR and RR.

OR represents a quotes ratio - probability of having an exposure to certain risk factors in subjects that are now presenting un effect (celiac disease in our case) reported to probability of having the same exposure in subjects that are not presenting un effect now. (3)

RR represents a risk ratio – risk of certain effect appearance (celiac disease in our case) in subjects who have been exposed to a risk factor, reported to the risk of the same effect appearance in subject not exposed to the same risk factor. (3)

OR and RR hierarchy for analyzed risk factors are showed in table II.

Table II – OR and RR for celiac disease's risk factors.

Risk factor	Early gluten exposure <5 month	Down syndrome	Autoimmune conditions	Adeno/Herpes/rubella virus	Ist and/or IInd degree relatives
OR	2.70	2.43	2.05	1.39	1.29
RR	1.46	1.29	1.30	1.12	1.09

These results indicate values greater than 1. That confirms the hypothesis that all analyzed factors represent risk factors for celiac disease. OR value for early gluten administration before 5 month old in formula fed infants, Down syndrome and autoimmune conditions association are greater than 2. The results indicate that these 3 factors have a greater weight in influencing the progression of celiac disease to more severe forms of villous injury.

We calculated the risk score values for each patient using the formula mentioned above ($S = \sum OR_i \times F_{ri}$). Then,

we statistically analyzed the medium score value for the lot of patients with Marsh II – hyperplastic villous injury, for the lot of patients with Marsh IIIa and Marsh IIIb together – partial and subtotal villous atrophy – and finally, for the lot of patients with total villous atrophy – Marsh IIIc type.

Processing data with Spearman correlation index for each three values of score averages classified based on villous alteration type, we obtained the value $R=0,943$. The result indicated a strong correlation and validated the proposed score.

Table III - Risk score values.

Marsh classification of villous alterations		Hyperplastic (type II) N = 9 patients	Partial/subtotal villous atrophy (type IIIa, IIIb) N = 7 patients	Total villous atrophy (type IIIc) N = 9 patients
Score	Average	4.39	5.44	5.70
	SD	1.94	2.39	2.22

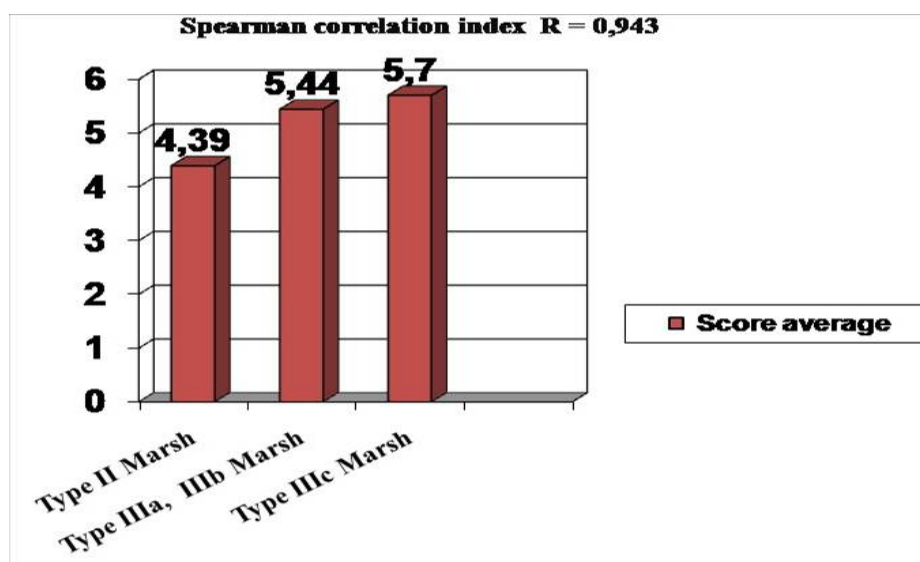


Figure 1 - Score average for different Marsh degree villous alteration.

In order to statistically compare the score averages of type II Marsh lot (N=9 patients) and type III Marsh lot (N=16 patients), we used Wilcoxon test and we obtained the value $p=0,05$, indicating the presence of a statistically significant difference.

Classifiers quality assessment is realized by sensibility and specificity analyze. So, we assessed sensibility and specificity variation for discrimination limit values between classes in accordance with quartiles

between average score of type II Marsh lesions group (4,387) and type III Marsh lot (5,579). Quantitative index values varying with limit values between classes (quartiles) are showed in table IV.

Although the specificity value is not high enough, the sensitivity of the proposed score has a sufficiently high value in order to recommend this score as a useful tool for assessment of celiac disease risk level in certain subjects.

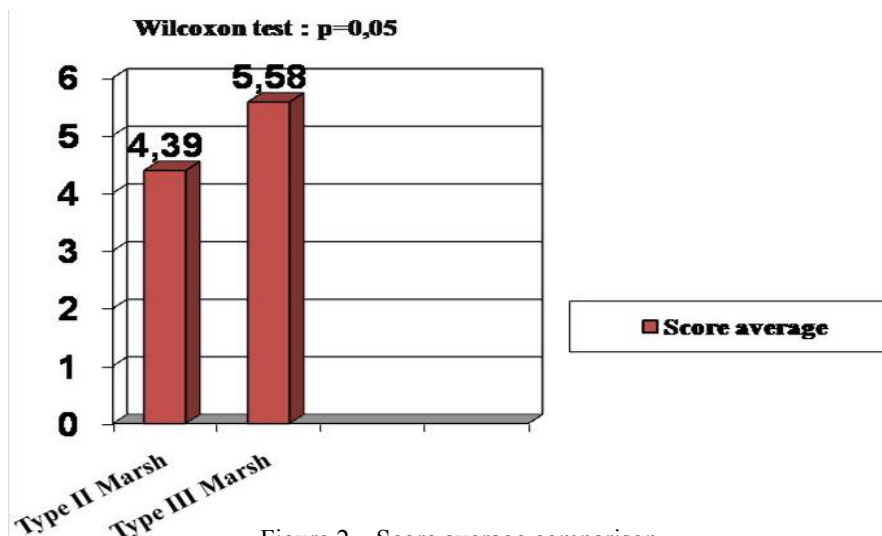


Figure 2 – Score average comparison.

Table IV - Quantitative index of risk score.

Limit	4.387	4.685	4.983	5.281	5.579
Sensibility	0.694	0.690	0.611	0.611	0.528
Specificity	0.474	0.474	0.632	0.684	0.737
Positive predictive value	0.714	0.714	0.759	0.786	0.792
Negative predictive value	0.450	0.451	0.462	0.481	0.452
Accuracy	0.618	0.618	0.618	0.636	0.600

Conclusions

A scoring scale awards the advantage of having a synthetic perspective upon patients possible evolution. Medical literature offers few attempts of computing celiac disease appearance score in accordance with risk factors exposure. Comparing our results with other statistic studies based on celiac disease risk factors identification in genetically susceptible individuals (DQ2 or DQ8 HLA haplotype), we found a strong concordance (5), (6). Our results emphasize the necessity of serological screening (EMA, hu-tTG antibodies) in certain patients groups

(autoimmune conditions, genetical diseases, viral infection) as well as in subjects with first/second degree relatives diagnosed with celiac disease (7). After serological screening, histological confirmation of gluten enteropathy in early stage is indicated, followed by gluten exclusion in order to stop the illness progression to its most feared complication – intestinal lymphoma. We consider very useful a risk factors hierarchy in patients with positive serologic tests, based on the fact that most celiac patients present silent or atypical form of diseases, in accordance with celiac ice-berg described by Richard Logan in 1991. (8)

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REPRODUCTIVE TOXICITY INDUCED BY URANYL ACETATE DEHYDRATE

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Abstract

Our study looked at the potential adverse effects of uranyl acetate dehydrate (UAD) exposure on skeletal abnormalities in fetuses tried to determine a dose - effect relationship for UAD exposure via three different routes and tried to obtain an overall understanding about the toxic effects during the period of organogenesis. Pregnant female Sprague Dawley rats received uranyl acetate dehydrate in doses of up to 1 mg/kg/day delivered via orogastric gavage, subcutaneous injection or subcutaneous implantable osmotic pumps during gestational days 5-16. Gross inspection and histology examination of fetal organ samples was performed following C-section delivery of pups on E20. Maternal toxicity was indicated by significant reduction of body weight gain during pregnancy, behavior changes and death in some cases. Increased rate of abortion, fetal developmental arrest and decreased fetal viability was noted in females exposed to 1mg/kg/day UAD. Significant reduction in the average size of the litters was noted and size/weight of the newborns in females receiving 0.830 mg/kg/day UAD. No gross fetal malformations were noted however nonspecific target organ changes were recorded.

Key words: uranium toxicity, fetal toxicity, congenital malformations, uranyl acetate dehydrate.

Introduction

Uranium makes up approximately 2–4mg/kg of the earth's crust (ATSDR, 1999). It is more plentiful than silver or tin, with abundance equal to that of molybdenum or arsenic. Although in nature there are more than 100 different uranium ores, uranium typically occurs as the mixed oxide U₃O₈, in amorphous (pitchblende) or crystalline forms (uraninite).

Depleted uranium (DU) is a man-made, radioactive, heavy metal derived from uranium ore. It is chemically identical to natural and enriched uranium, although it is approximately 40% less radioactive than the naturally occurring metal (ATSDR, 1999).

It reacts with most non-metallic elements; it has pyrophoric properties and may spontaneously ignite at room temperature in air, oxygen and water. These unique properties make it appealing for use in many civilian and military applications.

DU is used as: radiation shielding, for missile projectiles, as target elements in plutonium production reactors, for gyroscope components, and as counterweights or stabilizers in aircrafts. Additional applications for DU also include X-ray radiation shielding in hospitals, as counter weights for rudders and flaps in commercial aircrafts, in keels of sailing yachts and as ballast in both military and non-military airplanes. (Hindin et al., Environmental Health, 2005) Military applications for DU consist of production of distinctly powerful projectiles (e.g., bullets/ penetrators, missile nose cones) and also as a protective armor for tanks. As a projectile, a DU penetrator ignites on impact under high temperature; it has a low melting point.

In the early days of the Manhattan Project, a very extensive toxicology program on uranium was carried out (Tannenbaum, 1951; Voetglin and Hodge, 1953).

Nowadays, the biokinetics, metabolism, and chemical toxicity of uranium, including the adverse effects on main target tissues, are established (Taylor et al., 1997; Craft et al., 2004; Brugge et al., 2005).

Until recent years little attention was paid to the potential toxic effects of uranium on reproduction and development. Moreover, most experimental studies on uranium-induced developmental toxicity have been performed in a sole species of mammals, mice (Albina et al., 2003)

Potential mechanisms of toxic action of DU alloy include mutagenicity and genotoxicity, disturbances in cell division, changes or inhibition of protein or steroid synthesis, disturbance or inhibition of enzyme systems, and disruption of behavioral patterns involved in normal reproduction. The end product of these mechanisms may be: 1) increased or decreased cell death; 2) disturbed cell-to-cell contact; 3) reduced biosynthesis; 4) increased morphogenetic pattern formation; or 5) disruption of tissue structure that may lead to abnormal pathogenesis in the reproductive system or developing fetus. If the repair processes inherent to fetal tissue become overwhelmed, dysmorphogenesis of the developing fetus may occur resulting in too few cells or cell products being formed to affect structure and functional maturation of the developing individual.

It has been shown that uranium is a developmental toxicant when given orally or subcutaneously to mice. Decreased fertility, embryo/fetal toxicity including teratogenicity, and reduced growth of the offspring have been observed following uranium exposure at different gestation periods (Domingo, 2001).

An increased incidence of cleft palate and dorsal and facial hematomas was found among litters from pregnant Swiss-Webster mice dosed with uranyl acetate dehydrate (UAD) at 1–50 mg/kg per day by gavage on gestational days 6–15.

A dose-related increase in liver weight was found among pups with increasing maternal dose levels of UAD. Brain, heart, lung, kidney, and spleen weights of pups with exposure to uranium during gestation and lactation were not significantly different from the weights of these organs from control animals. Fetotoxicity, characterized by significant decreases in fetal weight and incomplete bone ossification at several sites was observed in offspring born to dams exposed to 1 or 2 mg/kg per day.

Domingo et al., found that if the uranyl acetate dehydrate was given by means of single subcutaneous injections of (4 mg/kg) to the pregnant females, the number of dead and reabsorbed fetuses and percentage of postimplantation loss was greatest on day 10 of gestation. Also, fetal weight was significantly reduced and a higher incidence of skeletal variations occurred among surviving offspring as compared with negative controls.

There are only a few human studies so far that looked at the relationship between depleted uranium and congenital malformations in humans. The present studies were a result of the observations made in military combat area in the postwar period.

The Nuclear Policy Research Institute, USA reports that as early as 1995-96, Iraqi doctors suspected a rise in leukemia and birth defects among children born or treated at the Women and Children's Hospital in central Basrah, Iraq's second largest city.

The Iraqi studies, the only population-based studies available, have their limitations including a lack of independent measures of exposure such as tissue and urine samples, no control city for comparison, mobile population so that some exposed individuals moved from the area while unexposed people moved into the area and, as a retrospective study, a question of assessment bias.

Additional information comes from, Imad Al-Sadoon et al., 1999, who performed an analysis of registered congenital malformation among births in Basrah, Iraq for the period from 1990 to 2000. In general there was an apparent increase in the incidence rate from 1995 upwards. In 2000 such incidence was almost six folds higher than in 1991. To improve statistical efficiency of the data collected and overcome small numbers of cases recorded, the pattern and incidence of congenital malformations are grouped into three periods, 1991 to 1994, 1995 to 1998 and 1999 to 2000.

The incidence rate for the first period was 2.5 congenital malformations per 1000 births while the respective figure for the second period is 4.57 and for the third period was 13.49. Congenital heart diseases and

chromosomal aberrations were reported at a higher frequency during the latter years. Such unusual malformations as phocomelia and ichthyosis, which were not reported in 1990 have been recorded later though in small numbers. The frequency of cleft lip and palate followed a similar trend. No apparent trends were observed in the remaining malformations.

Our study looked at the potential adverse effects of UAD exposure on skeletal abnormalities in fetuses, tried to determine a dose - effect relationship for UAD exposure via three different routes and tried to obtain an overall understanding about the toxic effects during the period of organogenesis.

Material and methods

Sexually mature male and female Sprague- Dawley rats were obtained from the University of Medicine and Pharmacy "Victor Babes", Timisoara in joint collaboration with "Pius Branzu" Experimental Surgery Research Center in Timisoara, Romania location where all the animals experiments were performed.

Female rats were mated with males (2:1) until copulation was detected. Finding of sperm (plugs) indicated copulation and the day of detection was considered as day 0.5 of gestation. Experiments involving the above mentioned animal species was approved by the Commission of Ethics - University of Medicine and Pharmacy "Victor Babes", Timisoara, Romania.

Uranyl acetate dehydrate (UAD) was purchased from SPI-ChemTM (West Chester, PA).

Physical, chemical properties as well as handling instructions were obtained and followed as described in the Material Safety Data Sheet (MSDS – UAD).

The chemical was administered by oral-gastric gavage or subcutaneous injections where 0.9% NaCl solution was used as a vehicle. For the oro-gastric administration animals received anesthesia prior the procedure with Isoflurane (Hospira Worldwide Inc.) Animals received 0.830 mg/kg/day or 1 mg/kg/day of UAD by either gastric gavage (10 animals) or subcutaneous injections (20 animals) received the treatment between days 6 through 15 of gestation.

An equal number of animals for all groups, labeled as control animals received the same regime but instead of UAD they received 0.9% NaCl solution throughout the same gestational days as the experimental groups.

An additional group of animals (20 animals) received UAD through subcutaneous implantable osmotic pumps (Alzet – 200 microliters, Cupertino, CA) that contained a UAD&0.9% NaCl (0.830 or 1 mg/kg/day of UAD) mixture that was implanted at day 6 of gestation. The use of osmotic pumps offer the benefit of saving critical time by eliminating the need for frequent animal handling and repetitive injection schedules reducing the stress on the animals as well as since it is a dependable drug delivery systems have proven invaluable in predictably sustaining compounds at therapeutic levels, avoiding potentially toxic or misleading side effects and ensuring accurate research results. The pumps were surgically implanted through a

minimal incision on the back of the animal in the cervical area and the wound closed with two interrupted 4.0 vicryl sutures (Ethicon, Inc.). Sutures were removed 4 days later and the implanted pumps were removed at the time of the caesarian section of the pregnant females. Animals that displayed signs of end point criteria or severe signs of distress were euthanized and excluded from the study.

Animals included in the study were monitored on daily basis and parameters such as food consumption, body weight gain and clinical signs of toxicity were regularly followed.

Cesarean sections were performed on all females, after previous euthanasia with carbon monoxide (CO), on gestation day 20. After median laparotomy and exposure of the gestational uterus the neonate rat pups were extracted, counted, weighed and examined for external variations, visceral malformations and skeletal abnormalities as showed below.

After delivery, rat pup fetuses were carefully dissected and numerous organ samples were harvested. Presumably targeted organs by the UAD toxicity were: heart, lungs, liver, kidney, intestine, muscles and bones. Additional samples of skin, placenta, uterus, umbilical cord were harvested as well.

The collected samples were placed in histology recipients, stored overnight in Formalin 10% and then sent to the Histology Core at the University of Medicine and Pharmacy “Victor Babes”, Timisoara where analysis of the samples was performed. Slides were prepared from embedded samples in blocks that were cut at 3µm thickness and stained with haematoxylin and eosin (HE). Resulting slides were read in a double blinded fashion for accuracy by two independent investigators.

The Student t test was used for comparing differences between the groups of animals.

Measurements within each experiment were averaged, and mean values and SD were derived from the averaged values. Results are presented as mean with statistical significance set at $p < 0.05$. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 14.0 for Windows.

Results

The UAD administration either by oro-gastric gavage, subcutaneous injection or osmotic pumps resulted in maternal toxicity in all treated animals.

At 0.830 mg/kg/day administered through oro-gastric gavage on gestational days 6-15, maternal toxicity was indicated by a significant reduction in body weight gain during gestation and a decrease in body weight at termination. On the average, the weight gain in the UAD group was about 8 grams compared to the control group where an average of 24 grams was recorded. (Figure 1)

When given by means of subcutaneous injection or osmotic pump the same amount of UAD (0.830 mg/kg/day) produced significant maternal and fetal toxicity. Almost all rat pups at the time of the C-section were either dead or extremely small for their gestational age. In one case we noticed the uterus filled with pus like collection with lack of

pups. We concluded that all pups experienced developmental arrest that occurred between or around gestational age 6-15. At this dose 2 pregnant females died on gestation day 7.

Animals exposed to UAD at 0.830 mg/kg/day by all 3 routes experienced a weight loss during the pregnancy in 100% of cases in some cases severe.

Administered by subcutaneous injection or osmotic pump of 1 mg/kg/day UAD on gestational days 6-15 produced maternal toxicity, death in many cases and fetal death in all cases. Two pregnant females died on day 7 of gestation and we only recorded fetuses in 2 cases out of 10 females. In the event of fetus presence the pups were in low numbers (2 in the mentioned cases) and they were extremely small for their gestational age when compared to the age matched control. At gross inspection the pups appeared to have developed and then experienced developmental arrest at an early gestational date.

In one case that received 1 mg/kg/day UAD through subcutaneous osmotic pump we noticed a local infection that we concluded stopped the normal functioning of the pump thus not allowing the UAD to produce a systemic effect. In this particular case the female at the time of the C-section produced 13 normal looking pups with a 4.5 gram average weight.

With 0.830 mg/kg/day administered through oro-gastric gavage, we did not notice any maternal death at any stage of the gestation. Females in the UAD exhibited a decrease in appetite, food consumption and a decreased level of activity noted by the animal caregiver staff after attentive monitoring of animals 3 times a day.

A decrease in the number of pups was noted among the UAD group animals compared with the control group. A normal litter can carry anywhere from 7 to 16 pups fact confirmed by the number of neonates in the control group. Females in the UAD group had significantly smaller litters. The number of fetal deaths and the sex ratio for live fetuses were unaffected by treatment.

When given by means of subcutaneous injection or osmotic pump the same amount of UAD (0.830 mg/kg/day) proved to produce more effective maternal and fetal toxicity than the gavage route and we recorded a decreased number of fetuses on average 12 in the females that did present with pups at the time of the C-section.

When looking at the birth weight of the pups we noted that there was a statistically significant difference between animals in the UAD groups and control group originated pups. (Figure 1)

Examination of the live fetuses revealed no dose-dependent or statistically significant increases in the incidence of fetal gross external alterations.

Additional experiments with lower amounts of UAD helped us conclude that the no-observable-adverse-effect level (NOAEL) for maternal toxicity was < 0.5 mg/kg/day and the NOAEL for embryofetotoxicity was also < 0.5 mg/kg/day as no significant increases in the incidence or type of malformations were observed at this dose.

Samples of intestine harvested from pups originated from mothers treated with UAD by means of gavage, 0.830

mg/kg/day, had signs of minor to severe damage consisting of epithelial cell lifting and/or separation and necrosis to mid villus level. The crypts had a normal architecture.

However, the results indicated that UAD was not toxic for the intestine, as measured by histological appearance at 0.830 mg/kg/day. (Figure 1)

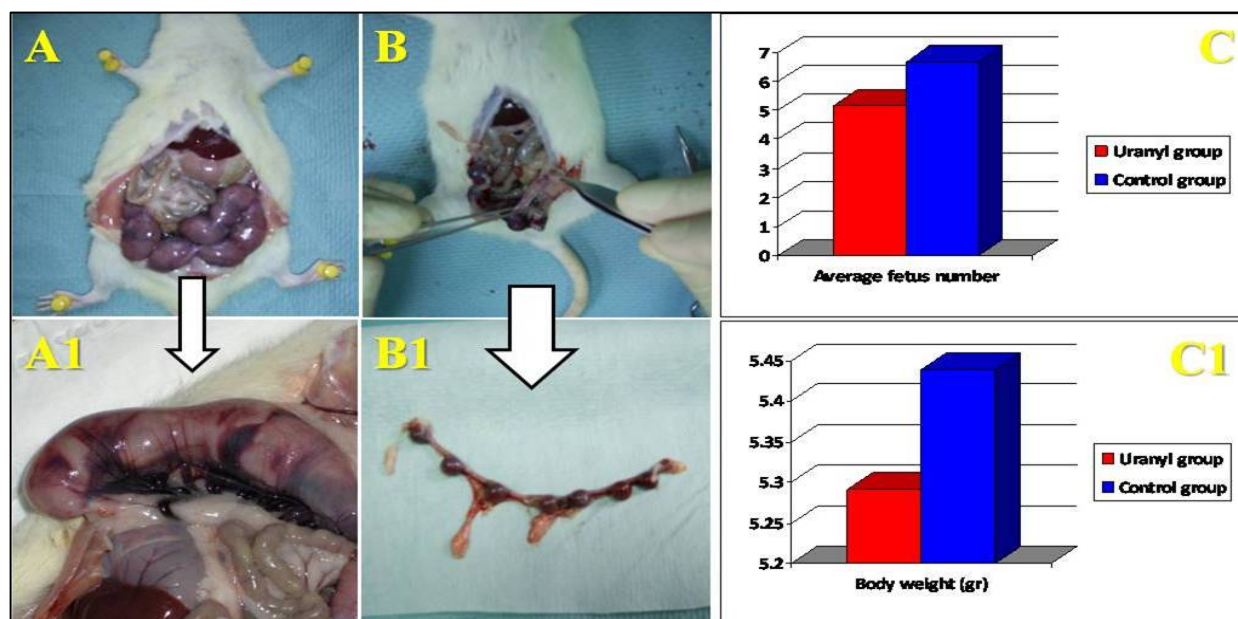


Figure 1 – A, A1 – Normal pregnancy vs. pregnancy in rats that received UAD ; lack of fetuses and small size uterus (B,B1). Decreased number of pups with a significantly lower body weight in females that received 0.830 mg/kg/day UAD.

The alveolar structure of the lungs of UAD rat pups originated from females that received, 0.830 mg/kg/day, was altered, the thin histological weft disappeared and large

spaces were formed by the damage to the interalveolar septa. In the other regions of the lung, the amount of fibrous connective tissue was slightly increased. (Figure 2)

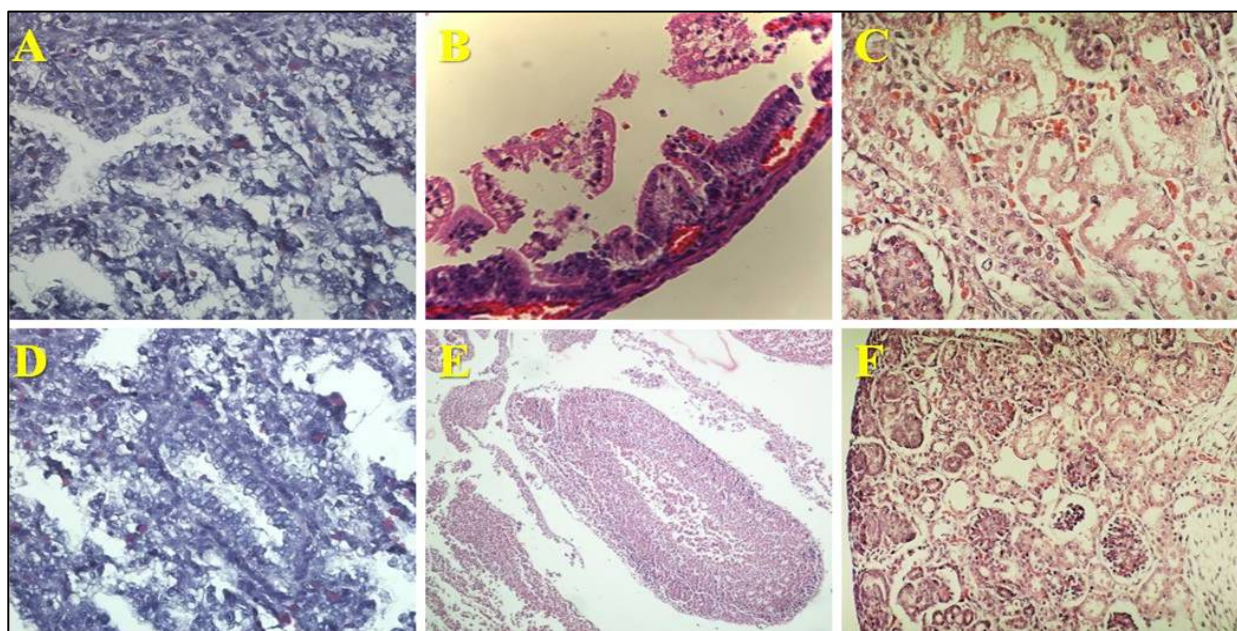


Figure 2 – A;D – Lung H&E staining 40X – organ aspect altered, the thin histological weft disappeared and large spaces were formed by the damage to the interalveolar septa with slightly increased amount of fibrous connective tissue. B;E – Intestine H&E staining 40X - minor to more severe damage consisting of epithelial cell lifting or separation and sometimes necrosis to mid villus level. C;F – Kidney H&E staining 40X - signs of immaturity and marked hyperemia changes.

We found similar changes in the few surviving rat pups that originated from females that received 1 mg/kg/day by subcutaneous injection or osmotic pump delivery system.

There was no gross external alteration to the structure of the lung noted in either of the two groups examined. Since the caesarian section produced some degree of prematurity in the rat pups examined in both the control and the experimental group we conclude that the above mentioned changes were not significantly different between the groups.

Several samples of bone and muscle were taken from each fetus including femur, upper and extremity, ribs and in cases whole body sections were examined.

There was no gross external difference between femurs originated from fetuses of females that received,

0.830 mg/kg/day. The histological aspect of femur bone sections from UAD rat did not reveal any pathological changes.

Bone samples from animals belonging to both the control and the UAD groups showed presence of cartilage cells and immature reticular cells without any significant changes.

In isolated cases we noticed areas of necrosis when looking at sections of ribs but since this was a sporadic observation we concluded that this can't be generalized. We concluded however that the UAD fetuses bone sample slides depicted decreased ossification when compared to their control counterparts. (Figure 3)

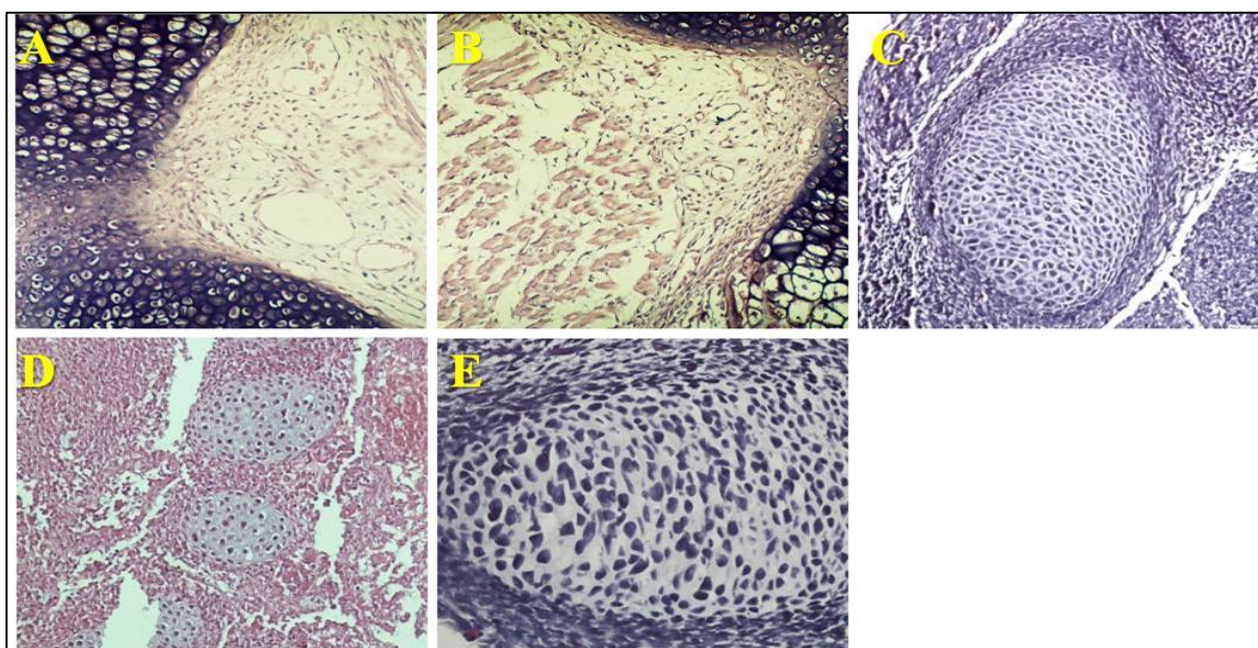


Figure 3 - Bone sections – A;B – femur – without pathological changes , normal appearance cartilage cells and immature reticular cells – C;E – humerus – normal appearance – D- ribs and intercostals muscle section – decreased ossification when compared to the control group animals.

The analyzed muscle samples in the case of UAD pups showed signs ranging from normal aspect to atrophy and to inflammatory infiltrate.

With regards to the kidney, on gross inspection there was no significant difference between the experimental and control animals however in one case the most remarkable abnormality detected was kidney hypoplasia. Histology did not reveal any major abnormalities in the kidneys of UAD fetuses except signs of immaturity and marked hyperemia, changes that were also seen in the control animals. (Figure 2)

Macroscopic examination of the livers did not point out any significant changes, we didn't notice any structural changes at 0.830 mg/kg/day of UAD. The average weight of livers from animals in both groups did not differ. There weren't any signs of fatty liver or lobular necrosis.

Histology revealed that there wasn't any degree of injury to the liver cells. Cell had a normal aspect but an inflammatory cell infiltration in portal areas and sinusoids was noted in the rat pups originated from females that received, 0.830 mg/kg/day. Extensive congestion and inflammatory cells aggregating in hepatic sinusoid lumen was noted as well in the UAD fetuses. (Figure 4)

Where there were no fetuses the uterus was harvested and histological analysis showed only signs of pregnancy, like (increased mucosa thickness) and from gross observations "resorbing sites" - locations where presumably fetuses were located.

There were also area of degeneration and micro calcifications noted in the placenta of females exposed 0.830 or 1 mg/kg/day of UAD. (Figure 5)

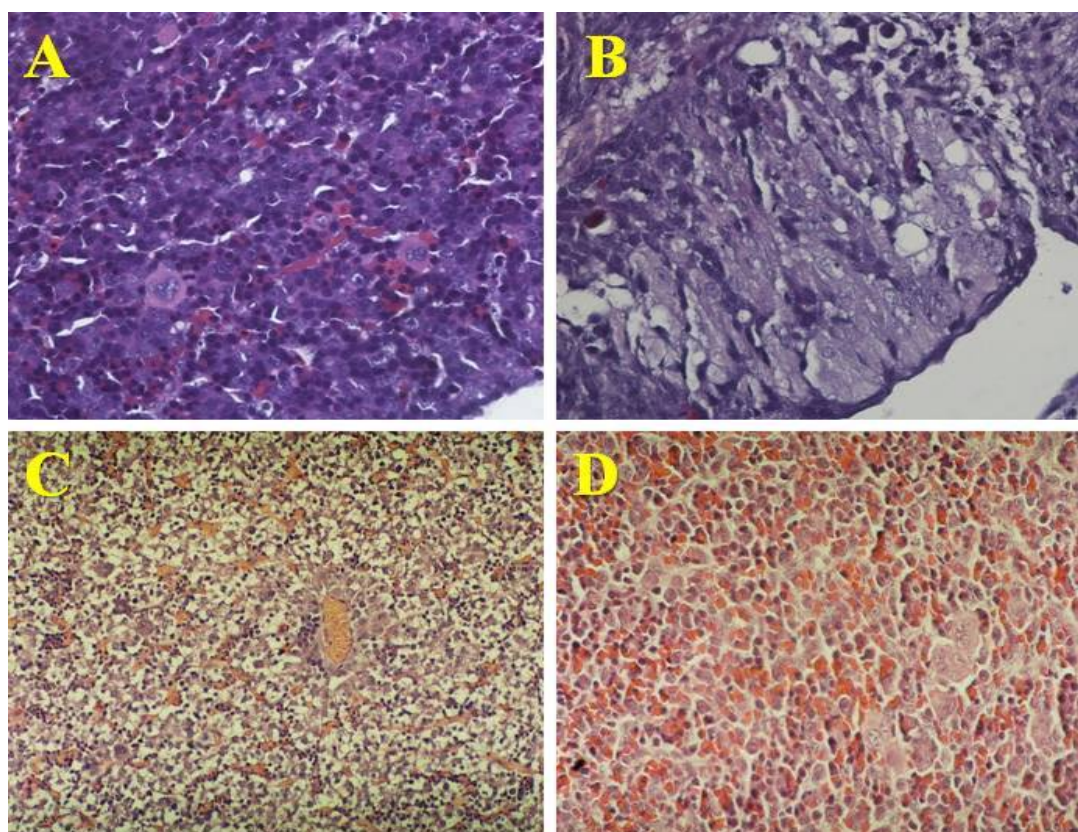


Figure 4 – Liver sections H&E staining - congestion and inflammatory cell infiltration in the liver.

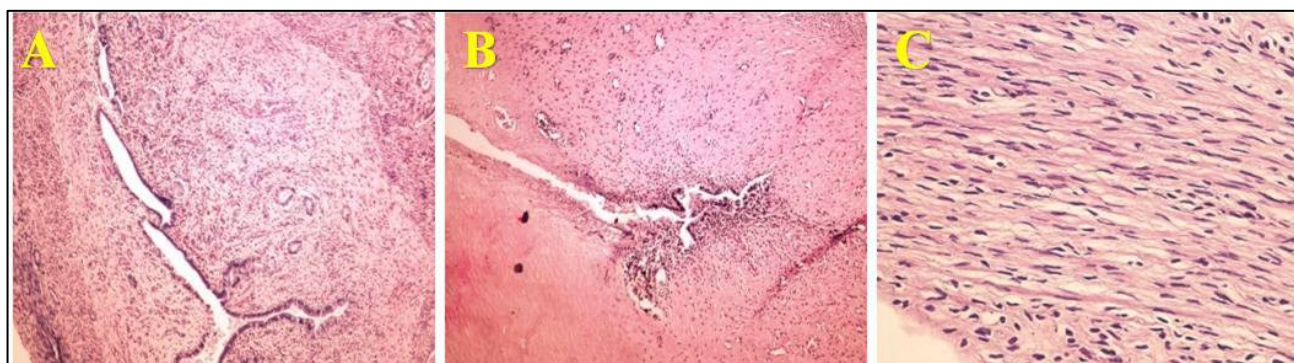


Figure 5 – Uterus H&E 40X sections – myometrium (C) and endometrium (A&B) – specific pregnancy changes with increased width of mucosa layer. Depth and number of glands are increased appreciably. Glands are straighter and are lined by columnar epithelium. Myometrium of normal appearance.

Discussions

In the last decade, many parts of the world suffered substantial demographic, social, cultural, economic, and ecological turbulences as direct or indirect consequences of the war scenarios. Public attention worldwide has been drawn to an apparently increased incidence of malignant diseases in the areas of military activities.

This increase was associated with alleged radioactive and/or chemical contamination by military equipment and weapons.

Our study looked at the embryotoxicity and teratogenicity in rats following different routes of administration of UAD.

In general terms, there is a good agreement between the dose and the effect obtained though as our previous hypothesis; it appears that the route of exposure (oral vs. subcutaneous administration vs. gradual delivery via osmotic pump) can determine the degree of toxicity of UAD. The results of our study are similar to other studies where uranium was administered orally or subcutaneously to mice. Effects such as, decreased fertility demonstrated by

the reduced number of fetuses, reduced size of the pups, embryo/fetal toxicity following uranium exposure at different gestation periods (Domingo et al., 2001), were also encountered in our study.

Arfsten et al., found irregular estrous cycles identified in uranium-exposed females as compared with satellite control females over a three-month interval.

Females in the uranium-exposed group that did not have litters over the first seven months of the experiment did not have any litters over the last five months of the experiment. The authors concluded that the decrease in reproductive success in uranium-exposed animals may have been an indirect effect resulting from decreased food intake as evidenced by depressed body weights and irregular estrous cycles. However, it is possible that there was a direct chemical interaction on the reproductivity of uranium-exposed breeders given the fact that reproductivity continued to be poor once uranium was reduced to background levels in their diets.

In a study by Maynard and Hodge (1949), rats (50/sex) were exposed to dietary levels of uranyl nitrate of 2% (about 460 mg/kg) for one day. Males and females were then paired, over a period of 7 months. Declines in total number of pups born (1959 vs. 1725; 12% decrease) and litter size (8.6 vs. 7.6; 7% decrease) were observed with treatment, but the actual number of litter bearing females increased from 43/50 to 44/50 with treatment.

Other investigators found that maternal toxicity was apparent at a dose level of

5 mg/kg/day indicated by decreased body weights as compared with controls, suggesting that the observed developmental variations may have resulted from a maternal toxic response. However, it was concluded that some of the fetal effects were independent of maternal toxicity. A significant reduction in body weight gain during pregnancy, treatment-related signs in behavior, abortion, and death have been reported to be general criteria for the existence of maternal toxicity in rodents. We found that females in the UAD exhibited a decrease in appetite, food consumption and a decreased level of activity.

Several studies looking into the changes of behavior patterns in animals exposed to uranium have showed that there is an accumulation of the chemical in the brain of animals exposed by various routes to the element.

According to Houpert et al. following chronic ingestion of DU, uranium accumulated mainly in the hippocampus. The hippocampus is known to be involved in the spatial working memory processes and previous experiments have shown that this kind of memory was altered in rats after repeated DU inhalation or after chronic ingestion.

Some other behavioral changes were observed after uranium exposure, such as sleep-wake cycle modification, increased anxiety-like behavior, or changes in exploratory activities. We found very similar changes in behavior in exposed females throughout pregnancy. However, the mechanism by which uranium induces such effects still remains to be elucidated. The most probable hypothesis is a direct chemical or radiological effect on one or more

cerebral areas, although an indirect effect can't be entirely excluded.

Since our results did not provide any concrete evidence of lung pathology as a result of exposure of pregnant females to UAD during gestation we hypothesize that the lung can only be affected when directly exposed to particles of uranium via air. One study found that "uranium dust" caused lipid peroxidation and micronucleiformation; however, chemical analysis of the dust revealed that there was no uranium component in the dust, and thus, these results are likely due to the other chemical components of the dust or to the particles themselves. (Ohshima S. et al., 1998) The other study found that insoluble DU induced neoplastic transformation of human bronchial cells consistent with the possibility that exposure to particulate DU may cause lung cancer, although that study did not consider specific genotoxic events that may have led to the transformation. (Yang, Z. H et al., 2002). Investigators have provided evidence that miners exposed to uranium particles have an increased incidence of lung cancer compared to other exposure routes.

Since this route of exposure, inhalation is nonexistent in case of the fetuses this could explain in part the normal appearance of the lungs in these neonates.

Bosque et al., (1992), found that fetotoxicity was evidenced by a significant decrease in fetal body weight and significant increases in the incidence of several skeletal districts unossified or with decreased ossification in the 1 and 2 mg/kg/day UAD groups.

Our study found that skeletal defects were not present at 0.5 mg/kg/day, whereas internal or skeletal malformations were only evident in the 1 mg/kg/day UAD when fetuses were found to be present in the uterus at the time of the c-section.

Tasat et al., (2007), notice ultra structural alterations in the nucleus and the cytoplasm of osteoblasts after in vivo exposure to uranium of adult rats (2 mg/kg; 1x) demonstrating a clear toxic effect.

Uranium exposed flat cells covering bone exhibited fragmented and swollen rough endoplasmic reticulum cisternae, scattered free ribosomes, and few coated vesicles with a fuzzy content. Uranium-treated osteoblasts showed signs of severe alterations, exhibiting absence of cell membrane and Golgi complex, swollen and fragmented RER cisternae presenting floccular content, and puffy nuclei with fine granular content when compared to non treated animals. Cell, (osteoblasts) viability was determined after 24 h in culture. None of the assayed doses of uranyl nitrate (0.1–100 μ M) affected cell viability, which always remained close to control values. After 24 and 48 h in culture, cells exposed to 0.1–100 μ M uranyl nitrate failed to show the typical morphologic features resembling those of apoptotic cells, such as pyknosis and nuclear fragmentation.

In our study gavage administration of UAD, 0.830 mg/kg/day to pregnant females failed to produce any significant alterations of osteoblasts structure in the fetuses in all segments of bones analyzed.

It is well documented that metals induce imbalance in oxidative metabolism, mainly through the increase in

reactive oxygen species (ROS), triggering apoptosis in many cell types including bone cells. It is well known that radiation, certain toxic drugs, chemical agents, and metal traces, can increase the physiological production of ROS. The balance between the production and detoxification rates of ROS determines their intracellular steady-state concentrations, which under pregnancy conditions might be kept in balance.

Up to a certain unknown concentration of uranium we think that there is a compensative mechanism for the increased ROS that protects the newborns from increased cell apoptosis and indirectly skeletal malformations.

Uranium is a classic nephrotoxin, and its use at high doses for experimental induction of nephrotoxicity is well established with renal failure being reported. (Bosque et al., 1993)

Several studies have looked at the chronic exposure of adult animals to uranium but little is known about the effect on fetus kidneys in females exposed to the element.

In our study at the gavage dose of UAD 0.830 mg/kg/day that we used there was no damage to the kidney detected and in the case of 1 mg/kg/day we weren't able to obtain evidence of kidney damage because of the low number of specimens available.

Donnadieu-Claraz et al. investigated the effect of chronic exposure to uranium in rat kidneys that received uranium in the drinking water (40 mg uranium liter⁻¹).

Microscopic analysis showed that proximal tubule cells from contaminated rats had increased numbers of vesicles containing dense granular inclusions. These inclusions were composed of clusters of small granules and increased in number with the exposure duration. The authors identified these characteristic granules as iron oxides. Uranium was found to be present as a trace element but was never associated with the iron granules. These results suggest that the mechanisms of iron homeostasis in kidney could be affected by chronic uranium exposure.

Other animal studies, like the one described by Novikov and Yudina (1970) where they administered female rabbits (6 to 8/group) oral doses of uranyl nitrate of 0, 0.02,

0.2, and 1 mg U/kg/day for 12 months noted no differences when compared with controls with respect to serum urea, creatinine or chlorides. (U.S. Environmental Protective agency 1989)

The lack of signs of liver toxicity after oral gavage of the substance is similar to results reported by other authors such as Domingo et al. (2003) that investigated the Influence of maternal stress on uranium induced developmental toxicity in rats. They found that there were no changes in the liver associated with UAD exposure with or without stress up to 0.830mg/kg/day administered either by oro-gastric gavage or subcutaneous route.

The investigators however noted that while uranium was not detected in the control and the restraint only groups and it not produced any major changes to the liver in the experimental animals it significantly accumulated in kidney, spleen, and liver of UAD treated dams.

There are no studies to our knowledge that looked at the effects of uranium on the fetus intestine, however Dublineau et al. addressed the biological consequences of a contamination with depleted uranium on intestinal properties such as the barrier function and/or the immune status of this tissue. Their study concluded that depleted uranium is not toxic for the intestine after acute exposure (204 mg/kg). Nevertheless, depleted uranium seems to modulate the expression and/or production of cytokines (IFN gamma) and chemokines (MCP-1) in the intestine. The amount that these rats received, 204 mg/kg, far exceeds the amount that the fetuses were exposed to in our study so we consider our findings regarding the intestine as collaborative with the dose.

The current investigation might be the basis for further studies on the potential role of uranium exposure on maternal and fetal toxicity and its role in the genesis of congenital malformations following other ways of exposure such as inhalation or through wounds.

Acknowledgements

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COLONIC TUBE ESOPHAGOPLASTY WITH NECROSIS OF THE GRAFT A CASE REPORT

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Abstract

Colonic tube esophagoplasty is a well known technique used for esophageal replacement. In a hand of a skilled surgeon this alternative for esophageal substitution has proven effective and a good substitute but not without problems. This case report illustrates a possible serious complication of colonic tube esophagoplasty and the solution find to resolve it.

Key words: colonic tube esophagoplasty, graft necrosis.

Introduction

The need for esophagoplasty continues to decrease over the last years. Each replacement organ used for the operation for esophageal replacement has proved effective and a good substitute but not without problems. The most common used techniques for esophageal substitution are gastric tube interposition in an isoperistaltic or antiperistaltic fashion, colonic interposition, gastric transposition, or jejunal interposition graft. Though nothing can truly replace the original esophagus these substitutes for the esophagus have stood the test of time as a viable alternative and many patients have had their substitute for almost 50 years and some for more than 50 years. Gastric tube and colonic tube esophagoplasty are delicate techniques, with precise steps; any mistake in performing these steps could cause massive damage to the patient(3).

Case report

A 1 year 7 month boy was hospitalized in Intensive care unit of Pediatric surgery Department of Hospital for Children "Sf Maria" Iasi, after massive caustic ingestion (home made soap). After specific treatment the child developed progressive severe dysphagia, which required a Stamm gastrostomy for alimentation and repeated sessions of endoscopic dilatation. The barium swallow showed a full-length ischemic stricture of the entire 2/3 distal esophagus, so 5 month after the caustic ingestion, in February 1999, we decided to perform a colic tube esophagoplasty.

In the operating room, the patient was placed in the supine position with a small sand bag under the shoulder with the neck extended and turned to the right side. A tube is placed through the nose into the esophagus to allow easy dissection. A careful dissection and then isolation of the cervical esophagus was done. The abdomen was then opened through a midline incision, mobilization of the colon

was done, and the graft was chosen on the territory supplied by the upper left colic artery with the length measured from the site of the antrum to the esophagostomy site. After choosing the colonic graft, inspection of the upper left colic artery pulsations was done. Then the middle colic and marginal vessels are clamped by bulldogs, and the colon is left inside the abdomen (to verify adequate circulation).

Esophagectomy was done by cutting the left triangular ligament of the liver followed by dissection of the esophagus encircling it with a tape (fig. 1). After freeing the esophagus from all its attachments, the esophageal hiatus is explored by dissection with the help of retractors inside the hiatus. With blunt and sharp dissection, the esophagus was freed as high as possible as, higher than the pulmonary ligaments. Care is taken to avoid entering the pleura. The cervical and abdominal teams simultaneously do blunt finger dissection of the esophagus; until the dissection was complete (fingers of both surgeons touch each other). The esophagectomy then is done by cutting the esophagus at the cardia with closure of the gastric end. The esophagus then is passed upward by traction from the cervical team with a long silk sutured to the esophageal end. The silk is sutured to the proximal end of the colon and pulled through the posterior mediastinum and out of the cervical incision.

The colon was re-evaluated and resected after verification of its vascularity and length. The graft was washed with diluted iodine solution and passed behind the stomach in an isoperistaltic manner. To facilitate passage through the chest, a silk suture is applied to the proximal end of the colon and pulled through the cervical incision until the colon is in place, in the posterior mediastinum. Redundant parts are resected. A single layer, end-to-side or end-to-end, esophago-colic anastomosis is done. The incision is closed after a drain is placed in the mediastinum and another near the cervical anastomosis.

The initial post operatory course was favorable, but at 5 days the child developed fever, dysphagia, pus and necrotic tissue leakage through mediastinal drainage and signs of severe sepsis. The thoracic X-ray showed image of a large mediastinum, suggestive for a mediastinal collection. With massive large spectrum antibiotherapy, good drainage the evolution was favorable but with progressive total dysphagia. One month later the barium meal revealed a full length ischemic stricture of the colic graft owing to inadequate blood supply.

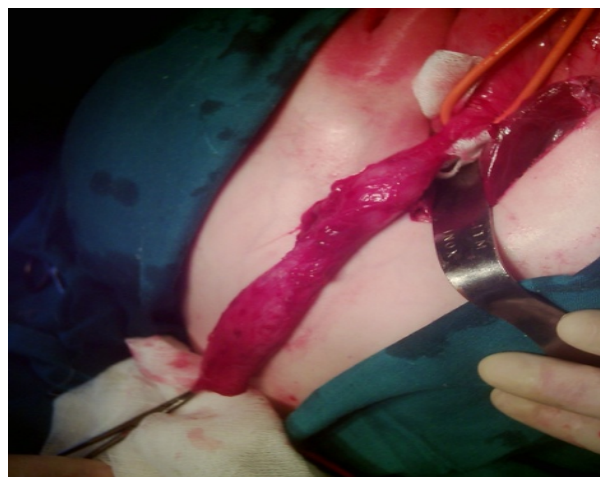


Fig.1. Esophagectomy.

The decision to perform another esophagoplasty was made 1 year later. The abdomen was entered through the previous midline incision. The previous colonic graft was closed distally with a stapler. One attempt was made to take off the former graft, but the adhesions due to necrosis were too hard, with great risk of lesions of pleura or great vessels, so the colic graft was left in place. The greater curvature of the stomach was measured to assure an adequate length to reach the neck(1,2). A new tube was fashioned from the greater curvature of the stomach. The left gastroepiploic artery was divided near the splenic artery, and the short gastric vessels were ligated and divided. The gastrocolic omentum was divided, and its vessels were ligated as far as possible from the gastroepiploic arch. A transsection of the great curvature of the stomach was made 4 cm proximal to the pylorus, taking care to avoid any damage to the right

gastroepiploic vessels. A silicone tube (20F) was inserted in the stomach through the opening and placed along the greater curvature from the antrum to the fundus. The new esophagus was fashioned using a double layer anastomosis. The defects of the stomach were closed with continuous hand sutures. An adequate length of isoperistaltic gastric tube was so obtained. The distal end of the GT was anastomosed to the fundus of the stomach. A tunnel is made by blunt dissection dividing the endothoracic fascia very close to the sternum, to place the new tube in a retrosternal manner.

Early postoperative follow-up was uneventful. Oral feeding was started on the 10th postoperative day, and he was discharged on the 14th postoperative day. He has tolerated early normal diets.

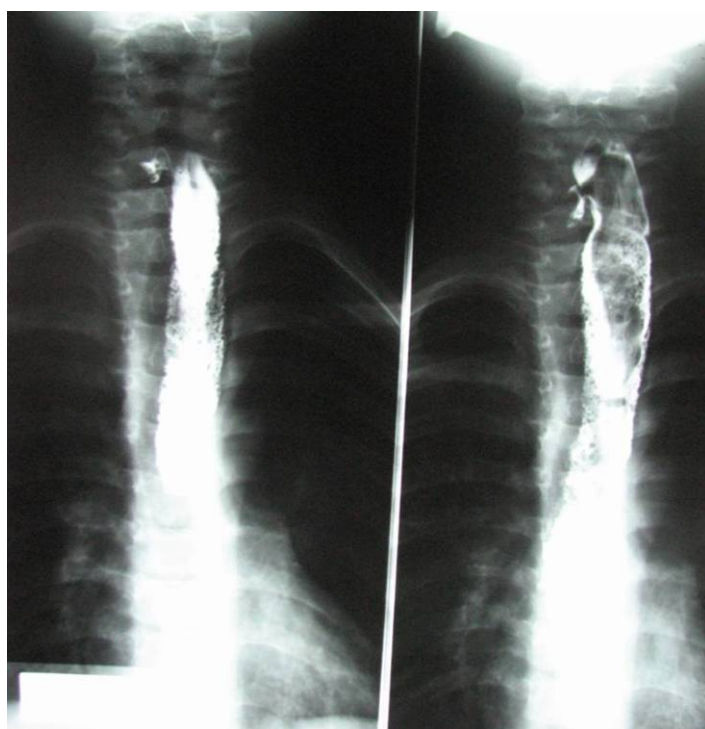


Fig.2. Barium meal – supragenetic diverticula.

After two month a cervical fistula occurs and the children came back to the hospital. With total gastrostomy alimentation, antibiotherapy the fistula closed, but barium meal reveals a stenotic area in the cervical anastomosis with supragenetic diverticula (fig.2).

Because of repeated pneumonia, nocturnal cough, early postprandial vomiting and of partial dysphagia associated with gain lost, in 2003, three year from the second esophagoplasty we performed the surgical cure of the cervical stenosis with the excision of esophageal diverticula, placed at the fifth cervical vertebra. The outcome was good, with no dysphagia, the child growth and development were normal. Oral radiographic contrast studies have been performed at 1 and 3 years of follow-up; neither anastomotic stricture nor diverticula were found.

Discussions

The most frequently possible complications in colonic tube replacement are necrosis of the graft, redundancy of the colonic tube, leakage and stenosis of the esophago-colic anastomosis(4,5).

The necrosis of the graft is the most serious complication of colonic tube esophagoplasty that can lead to severe mediastinitis, sepsis and even death. To avoid this type of complication we must have a careful identification

of the blood supply and using a double blood supply from the left colic and the marginal paracolic arcade. Also the graft must be closely analyzed in the cervical area before the esocolic anastomosis is made. We must be extremely careful not to twist the vascular pedicle during his passage behind the stomach and through the, mediastinum. With the same importance as to prevent is to recognize the necrosis of the graft, any leakage on the mediastinal tube, associated with fever and radiologic signs of mediastinal collection must be considered a sign of graft necrosis. Once the diagnostic is made the oral intake must be stopped, with antibiotherapy, fluid resuscitation. If the outcome is not good, the fever and general signs worsen immediate intervention should be considered with total excision of the necrotic tissue. In any case a new method of esophagoplasty must be performed several months after(6).

Leakage and stenosis of the esophago-colic anastomosis is avoided by careful dissection of the esophagus avoiding injury to the blood supply and wide anastomosis. The incidence of leakage should be extremely low as the proximal esophagus is healthy. Once a cervical fistula occurs, the healing of it will leave behind a cervical stenosis that will require further dilatation or surgical correction.

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GIANT PEDICULATE LIPOMA OF THE ANTERIOR NECK

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Abstract

Background: Lipomas are common benign soft tissue tumors that are found rarely on the anterior part of the neck. Surgical interventions in these tumors are very challenging because of the proximity of the blood vessels and the vagus nerve and this knowledge of the anatomy and meticulous surgical technique are essential.

Case presentation: A male patient of about 5 1/2 years old with a large asymptomatic mass, with which occupied the base of neck involving the whole anterior part of the neck just like the crest of a turkey mimicking an hygroma, this require a total excision followed by reconstruction and drainage with two tubes.

Conclusion: Giant pediculate lipom of the anterior neck just like the neck crest of a turkey mimicking an hygroma is an extremely rare case. The high resolution MRI provides an accurate cost effective preoperative investigation method. Surgical operation of this nature should be performed by an experienced surgeon and care should be taking to protect the carotid arteries the jugular vein and the vagus nerve.

Key words: pediculate lipom of the anterior neck, surgical treatment.

Background

Lipomas are the most common encountered benign mesenchymal tumors, arising in any location, where fat is normally present^{1,2}. The occurrence in the head and neck is relatively rare³. Most commonly at the posterior subcutaneous neck. Surgical intervention here is challenging because of the proximity of the blood vessels and the nerve and thus the knowledge of anatomy and meticulous surgical technique are essential. Here we describe a rare case of giant pediculate lipoma of the anterior part of the neck that was successfully managed surgically.

Case presentation

A 5,1/2 years old male child and his mother presented in our department with a large anterior neck mass requesting surgical excision for a cosmetic better neck appearance. Parents had been aware of the relatively fast going painless swelling for the past 5 years, parents are poor, with less education and living in isolated part of the village and never sought medical advice. Clinical examination revealed a mobile, soft and tender mass measuring 11/9,5 cm in size and located at the anterior part of the neck. The surface off the mass was smooth and the underlying skin was normal without sign of decoloration or tumor adhesion (Figure 1).



Fig. 1. Pediculate lipom of the anterior neck – clinical aspect.

The vagus nerve function was intact. The high resolution MRI showed an adipose tissue signal density

mass well encapsulated measuring 11,9,5 cm (Figure 2).

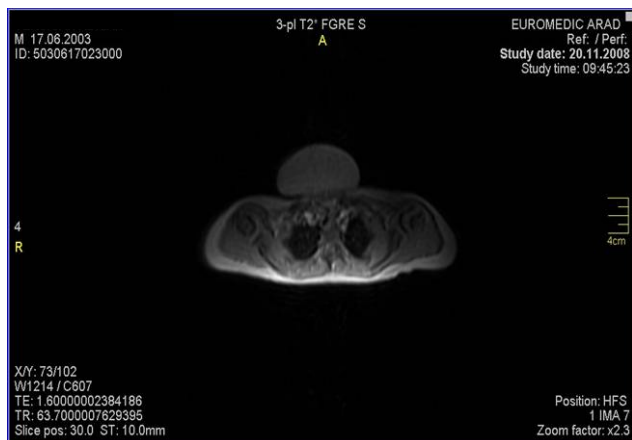


Fig. 2. Pediculate lipom of the anterior neck – MRI aspect.

General anesthesia with orotracheal intubation. Transverse incision about 12cm wide, meticulous dissection of encapsulated lipoma, enucleation of the tumor mass,

followed by excision of the excess skin. Hemostasis by electrocoagulation, drainage tube.



Fig. 3. Resected lipoma.



Fig. 4. Postoperative aspect.

The histological finding revealed fibrolipoma, a histological variant of lipoma. The patient had a very good recovery with satisfying neck contour and intact blood vessels and vagus nerve. Tumor recurrence was not observed 6 months after surgery.

Discussion

Lipomas are benign mesenchymal tumors histologically similar to mature adipose tissue, but the presence of fibrous capsule helps to differentiate them from simple fat aggregations⁴. Only 25% of lipomas arise from the head and neck. Lipomas of the anterior neck are extremely rare, they may extend posteriomedially between

the sternocleidomastoidian and digastric muscles sometimes. They may extend to the deep parotid lobe in very rare case.

CT scan or MRI may be helpful in further assessment and diagnosis⁵. Ultrasonography has been used as an initial imaging study in cases suspected to have head and neck lipomas^{6,7}. Compared with CT scan and MRI, ultrasonography is quick, easy, less cost by with the use of right frequency transducers may be more suitable for imaging superficial structures. However the soft tissue characterization is less specific with ultrasonography than with CT scan or MRI.

On CT scan, lipomas have typical characteristics of homogeneous masses with few septations, MRI can also accurately diagnose lipomas preoperatively. Moreover, the margin of a lipoma is clearly defined by MRI as a black rim, enabling lipomas to be distinguished from surrounding adipose tissue, a distinction that cannot be made from CT images. In this reported case, however the high resolution MRI provided enough information with respect to the preoperative planning and contributed to the diagnosis.

Although MRI may prove to be better diagnostics tool regarding tumor margin characteristics, the cost of MRI is nearly three times that of CT and so we believe that although MRI is highly useful, the CT scan with specific radiodensity recording is the preferred operative investigation⁸.

Fine needle aspiration requires an experienced cytologist but it still has a significant false negative rate⁹.

Consecutive follow up might be a valid option for patients with anterior neck lipomas surgical interventions here is challenging and may be reserved for patients with cosmetics and pressure effects. Possible postoperative morbidities such as vascular injury, vagus nerve dysfunction, scar and asymmetric contour must be explained to the patient before operation.

Conclusion

Giant pediculated lipoma of the anterior neck is extremely rare. Although MRI may provide better tumor margin characteristics, the CT scan with specific radiodensity recording is the preferred preoperative investigation. Surgical management of this tumor is challenging and should be performed by an experienced surgeon due to the need for meticulous dissection with respect to the underlying blood vessels and nerve.

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ENCHONDROMATOSIS-OLLIER DISEASE-CASE REPORT

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Abstract

Enchondromas are common intraosseous, usually benign cartilaginous tumors, that develop in close proximity to the growth plate cartilage. When multiple enchondromas are present, the condition is called enchondromatosis also known as Ollier disease. Clinical manifestations often appear in the first decade of life. Ollier disease is characterized by an asymmetric distribution of cartilage lesions and these can be extremely variable. Clinical problems caused by enchondromas include skeletal deformities, limb length discrepancy and the potential risk for malignant change to chondrosarcoma. The condition in which multiple enchondromatosis is associated with soft tissue hemangiomas is known as Maffucci syndrome. The diagnosis is based on clinical and conventional radiological evaluations. Histological analysis has a limited role and is mainly used if malignancy is suspected.

Key words: Ollier disease, enchondromatosis, multiple enchondromatosis, dyschondroplasia

Definition

Enchondromas are common benign, usually asymptomatic cartilage tumors, which develop in the metaphyses and may become incorporated into the diaphyses of long tubular bones, in close proximity to the growth plate cartilage.

Enchondromatosis or Ollier disease is defined by the presence of multiple enchondromas and characterized by an asymmetric distribution of cartilage lesions that can be extremely variable in terms of size, number, location, evolution, age of onset and diagnosis and requirement for surgery.

The condition in which multiple enchondromatosis is associated with soft tissue hemangiomas is known as Maffucci syndrome.

Epidemiology

The estimated prevalence of Ollier disease is 1/100.000. Maffucci syndrome has indeed a lower prevalence.

Solitary enchondromas are most commonly discovered between 20 and 40 years of age but Ollier disease tends to present before 10 years old.

Males are affected twice as often as females.

Etiology and pathogenesis

Endochondral bone ossification is a highly regulated process, which requires the progression of undifferentiated

mesenchymal cells into hypertrophic chondrocytes and the subsequent replacement of a cartilaginous matrix by mineralized bone. Enchondromas develop in the metaphysis of long tubular bones in close proximity to the growth plate. Consequently, it was proposed that they result from abnormalities in signaling pathways controlling the proliferation and differentiation of chondrocytes, leading to the development of intraosseous cartilaginous foci.

Genetics

Ollier disease and Maffucci syndrome are usually non-familial disorders and both disorders thus appear to occur spontaneously and are not inherited. The irregular distribution of the lesions in Ollier disease strongly suggests that it is a disorder of endochondral bone formation that occurs due to a post-zygotic somatic mutation that results in mosaicism.

Although an identical heterozygous mutation in the PTHR1 gene has been identified, other mutations involving this gene were identified. These studies suggest that the cause of Ollier disease is heterogeneous and raise the possibility that two or more genetic mutations are required to develop the disease.

Additional mutational events may underly progression from enchondromas to tumors.

Histopathology

Macroscopic examination of enchondromas usually shows multiple oval shaped or round cartilaginous nodules in osseous portions of bone. The individual nodules are limited at their periphery by woven or lamellar bone and are separated from each other by intertrabecular marrow spaces.

The cartilaginous tumor matrix is usually solid, with myxoid changes, which manifest as fraying of the matrix. Enchondromas are characterized by the presence of a striking heterogeneity and diversity in the degree of cellularity and chondrocyte phenotype. This heterogeneity depends to some extent on factors such as localization and the patient's age.

In part, due to this important cellular heterogeneity the distinction between benign enchondromas and malignant chondrosarcomas by histochemical criteria is difficult. The histological criteria for malignancy that are used for conventional chondrosarcoma can not be used in Ollier disease because of the increased cellularity and therefore the distinction between enchondroma and grade I chondrosarcoma in the context of enchondromatosis is extremely difficult or even impossible.

Classification

There are six types of enchondromatosis but three are more common:

- In Ollier disease there are multiple enchondromas that are mostly unilateral or unevenly distributed throughout the metaphases of the long bones, sparing the cranium and spine.

- In Maffucci syndrome the enchondromas occur with multiple cutaneous hemangiomas that appear as soft tissue calcifications or phleboliths on x-ray.

- If there is symmetrical involvement throughout the body including the cranium, hands and feet the condition is known as generalised enchondromatosis.

Clinical description

Clinical manifestations in Ollier disease often appear in the first decade of life and usually start with the appearance of palpable bony masses on a finger or a toe, an asymmetric shortening of an extremity with a limping gait and osseous deformities eventually associated with pathologic fractures. Upon physical examination, enchondromas present on the extremities are usually visible as masses embedded within phalanges, metacarpal and metatarsal bones. The masses increase in size as the child grows along with asymmetrical shortening of a limb and either genu valgum or most commonly genu varum deformities. Enchondromas frequently affect the long tubular bones, particularly the tibia, the femur and/or the fibula. Flat bones, especially the pelvis, can also be affected. The lesions are usually asymmetrically distributed, exclusively or predominantly affecting one side of the body.

Affected bones are often shortened and deformed.

Bone shortening may be the only clinical sign of the disease and these bone shortenings are often associated with bone bending and curving and may lead to limitation in articular movement.

Forearm deformities are frequently encountered and these are similar to those observed in hereditary multiple exostosis (HME). Ulnar shortening is usually more relevant than shortening of the radius.

The trunk is usually not affected except for costal enchondromas and secondary scoliosis resulting from pelvic imbalance.

During childhood, the lesions are subjected to pathologic fractures.

Diagnostic methods

The diagnosis of Ollier disease is based on clinical and conventional radiological evaluations.

Histological analysis has a limited role and is mainly used if malignancy is suspected.

Additional investigations such as scintigraphy,

Ultrasound, magnetic resonance imaging (MRI) are not useful for establishing the diagnosis and they are indicated for the evaluation and surveillance of lesions that become symptomatic (pain, increase in size).

Biopsy of suspicious lesions may be required.

Radiography

Enchondromas are rarely observed at birth, although the lesions are most likely already present. X-ray show multiple, radiolucent, homogenous lesions which run parallel with long bone axis. The lesions usually calcify with time and become diffusely punctated or stippled, a light trabeculation may be visible. Enchondromas are frequently assembled as clusters, thus resulting in the metaphyseal widening. When localized at the bone border, the enchondromas produce a typical notch-like image.

The lesions are almost exclusively localized in the metaphysis of long bones and in the small bones of the hands and feet. They are initially localized close to growth plate cartilage and then migrate progressively towards the diaphysis. The epiphyseal region next to an affected metaphysis may show irregularities. In the hands, the lesions almost never affect all metacarpal bones and phalanges.

Signs of malignant transformation should be looked for, as it is a major complication of enchondromatosis. These signs include cortical erosion, extension of the tumor into soft tissues and irregularity or indistinctness of the surface of the tumor.

Enchondromas tend to be well circumscribed and to show a uniform pattern of mineralization, whereas chondrosarcomas show poor demarcation and the presence of unmineralized parts.

Differential diagnosis

The differential diagnosis may include:

- Hereditary multiple exostosis –HME is an autosomal dominant disorder characterized by multiple bone tumors capped by cartilage, that occur mostly in the metaphyses of long bones.

- Other rare forms of chondromatosis which include metachondromatosis, spondyloenchondrosia and genochondromatosis type I and II

- Polyostotic fibrous dysplasia

- Diaphyseal aclasis

- Kaposi sarcoma

- Klippel-Trenaunay syndrome

- Weber-Parks syndrome

Treatment

There is no medical treatment for Ollier disease.

Surgery is indicated in enchondromatosis complicated by pathological fractures, growth defect or malignant transformation.

Complications

Besides asymmetrical growth, the condition might be complicated by pathological fractures and malignant change as chondrosarcoma and osteosarcoma.

In Ollier disease, about 25% of cases will undergo malignant change by the age of 40.

Prognosis

The prognosis for Ollier disease is difficult to assess. Early onset disease seems to have a more severe course. Research has shown that patient with numerous lesions may



[Figure-2] Roentgenogram illustrating enchondromas involving the superior metaphyseal and diaphyseal segments of the right femur.



[Figure-3] Roentgenogram revealing enchondromatous involvement of right distal tibia, metatarsal bones and proximal falanges.

Prevention

Despite the universal acceptance that Ollier disease carries a high risk of malignant change the data from the literature about systematic screening for early diagnosis are scarce. One such paper advised periodic surveillance of the brain and abdomen for occult malignant lesions in patients with enchondromatosis (12), but failed to be more specific the optimal screening frequency.

Another article emphasized the association with an increased risk of malignancy including intracranial

chondrosarcomas, and labelled early diagnosis and screening patients with Ollier disease as being of a crucial relevance (13). But then again, the optimal screening frequency is a subject that has been conspicuously omitted. It did state that the treatment of choice for intracranial cartilaginous tumors is complete surgical excision, but this is fraught with technical difficulties. An alternative therapeutic option to be considered would be proton-beam therapy.

Conclusions

Ollier disease is an extremely rare, non-hereditary skeletal condition. There is no medical treatment for this disease, with surgical treatment only intervening in the unfortunate instance of a complicated enchondromatosis. The evolution of most enchondromas enters a steady state after puberty as cartilage is replaced by bone, nonetheless

around 25% of lesions will undergo malignant transformation by the age of 40.

We recommended clinical follow-up once a year untill puberty, a deadline by which ossification is completed

Thereafter a long term follow up until the age of 40, once a year or every 2 years for early detection of a malignant change.

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MANUSCRIPT REQUIREMENTS

The manuscript must be in English, typed single space, one column on A4 paper, with margins: top – 3 cm, bottom – 2,26 cm, left – 1,5 cm, right – 1,7cm. A 10-point font Times New Roman is required.

The article should be organized in the following format: Title, Names of all authors (first name initial, surname), Names of institutions in which work was done (use the Arabic numerals, superscript), Abstract, Keywords, Text (Introduction, Purpose, Materials and Methods, Results, Discussions and/or Conclusions), References, and first author's correspondence address.