UNIVERSITY OF MEDICINE AND PHARMACY

”VICTOR BABEȘ” TIMIȘOARA

FACULTY OF MEDICINE

DEPARTMENT IV

Niculina MANG

SUMMARY OF THE DOCTORAL THESIS

GENETIC AND METABOLIC BIOMARKERS IN PEDIATRIC PATHOLOGY

Scientific Coordinator:

Prof. Univ. Dr. Andrei ANGHEL

TIMIȘOARA

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CONTENT

INTRODUCTION

In fulfilling the millenary aspirations of personalized medicine, in which the establishment of predisposition, diagnosis and therapy will be adapted to the individual profile, biomarkers are an indispensable tool, being called to provide personalized information about the genetic and metabolic profile, reactions to different environmental factors, the modification of some cellular and physiological parameters at different stages of disease and response to the application of therapy.

In this context, the approach to pediatric pathology has new tools to investigate the etiology, diagnosis and predisposition of complex diseases, of which congenital diseases have received special attention lately because, despite advances in medicine and efforts to reduce their incidence, congenital illnesses still have a significant share in infant morbidity and mortality.

On this line of preoccupations, we have also the present study, which purpose and objectives consist in clarification and consolidation of values for new biomarkers in the approach of a complex pediatric pathology, in conditions in which on national plan, there are no known multifactorial approaches for these theme and on international level there are still controversy.

GENERAL PART

Genetic deficiencies and birth defects represent a high percent of the total number of children admissions in hospital. Congenital malformations and chromosomal anomalies are the most frequent causes of infant mortality.

Congenital hypothyroidism is the most frequent endocrine pathology at pediatric age and causes neurological, motor and growth deficiencies, irreversible mental retardation being the most drastic complication.

Neonatal screening programs for HC over the past four decades have been cost-effective diagnosis and benefit from an economic point of view. Affected children are detected very early after birth, before clinical symptoms and signs become evident. Early detection and treatment prevent morbidity, especially neurological disabilities.

Regarding the pathogenesis of congenital hypothyroidism, it has been revealed that genetic deficiencies are detectable not only in patients with dysmorphogenesis, but also in patients with

developmental defects of the thyroid, previously thought to have a sporadic disorder. However, although molecular genetic testing can clarify the cause of dysmorphogenesis in most patients, the molecular basis of congenital hypothyroidism in those with thyroid disorder remains predominantly unknown.

Osteogenesis imperfecta also known as brittle bone disease is a clinical condition with a genetic etiology in matrix formation and bone remodeling, characterized by low bone mass, fragility and bone deformity, low stature, gray or blue sclera, and deafness.

The etiology of the disease is predominantly caused by mutations at the site of COL1A1 and COL1A2 genes. These types of mutations are transcribed in quantitative and qualitative modifications of type I collagen, which is a major component of the extracellular matrix of bone and skin. The other percentage of 10% is caused by other mutations such as dominant or recessive at the site of other non-collagen genes, which are involved either in the post-translational processing of pro-collagen I or in the transport of collagen at the intracellular level, or in specific signaling at the level of osteoblasts.

SPECIAL PART

This PhD thesis aims to contribute to the identification and evaluation of some factors involved in pediatric pathology, especially in the etiology of congenital diseases, biomarker in diagnostics and therapeutic orientation.

A first study seeks to evaluate the effect of thyroid dysfunction on hematologic abnormalities from patients diagnosed with Down syndrome (DS), knowing that DS is associated with multiple complications, among which there is an increased risk for leukemia and thyroid dysfunctions.The present study is a clinical study that aims to examine the quantitative and qualitative distribution of blood cells in patients without leukemia or transient myelopoiesis disorders. At the same time, the effect of thyroid dysfunction on these hematological abnormalities in DS was evaluated.

The second study, focused on the etiology of congenital hypothyroidism and the evaluation of levotiroxine monotherapy investigated the involvement of the polymorphism of the iodotinase 2 deodinase gene.

The third study, dedicated to osteogenesis imperfecta, evaluated the levels of bone metabolism’s biomarkers in the treatment with pamidronate. And the last study regarding juvenile idiopathic arthritis, investigated the possibility of using vitamin D, specifically the plasmatic form 25(OH) D in assessing the severity and duration of this disorder.

The main objectives proposed and achieved during the PhD program were as follows:

1. Designing clinical trials, setting trial batch and control batch, developing criteria for inclusion and exclusion from the study, elaboration of the patient's consent card, obtaining the opinion of the ethics committee.
2. Establishment and optimization of laboratory methods for analysis of anthropometric, physiological and biochemical parameters, as well as methods of analysis of blood biomarkers.
3. Establishing batches of patients and control groups throughout the study period. Collection, conservation and analysis of biological samples.
4. Centralization of results, statistical analysis and interpretation of the obtained data.
5. Elaborate the final conclusions and establish the biomarker value of the cellular profile in Down's syndrome, the deiodinase polymorphism in the case of congenital hypothyroidism, vitamin D in juvenile idiopathic arthritis and the markers of bone metabolism in the case of pamidronate treatment in osteogenesis imperfecta.
6. Dissemination of results through publications in specialized journals and participation in conferences with subjects related to the present study.

RESULTS AND DISCUSSION

Patients with DS have had a higher neutropenia and lymphopenia than DS patients associated with hypothyroidism and hypothyroidism patients. Surprisingly, patients with DS have shown a significant degree of eosinopenia in peripheral blood. Interestingly, hypothyroidism had an attenuating effect on various cell lines in blood samples. The findings of this study are consistent with previous data from the literature on DS-associated changes in the haemoleucogram. Our study also shows changes in total blood cell counts in patients with DS without leukemia in combination with hypothyroidism.

CBC significant differences between the three group of patients using Z score (P value < 0,05)

Blood cells	**DS-ht vs ht**			**DS vs DS-ht**			**DS vs ht**		
	Z score (P value)	%DS-ht	%ht	Z score (P value)	% DS	%Ds-ht	Z score (P value)	%DS	%ht
Eosinophils	↓ 2.11 (0,034)	46,2	11,8	--	--	--	↓ 3.09 (0,002)	70	11,8
Neutrophils	--	--	--	↑ 2.88 (0,003)	50	0	↑ 2.66 (0,007)	50	5,9
Lymphocytes	--	--	--	↓ 2.29 (0,022)	50	7,7	↓ 3.22 (0,001)	50	0

ht: hypothyroidism; DS-ht: Down syndrome with hypothyroidism; DS: Down syndrome without hypothyroidism; ↑: increased CBC count; ↓: decreased CBC count; --: no significant differences.

The mitigating effect of thyroid dysfunction on changes in various blood cell lines in the DS context is a new observation that deserves further analysis in larger studies.

Investigating the prevalence of polymorphism of Ala92Thr iodothyronine deiodinase gene 2 in children with congenital hypothyroidism in the West of Romania and its impact on TSH level, demonstrated that AA genotypes were significantly more prevalent in patients with high TSH after levotiroxine treatment (p = 0.044) than genotypes TT and AT.

Specific allelic discrimination of homozygous genotypes (AA and TT) and heterozygote (AT) in the patient population by Real Time PCR with Taqman probes

This correlation is explained by a functional impairment of D2 deiodinase produced by AA polymorphism, impairment that will reduce T4 → T3 conversion and under these conditions even if the T4 plasma level is normal, decreasing the amount of T3 (thyroid hormone effector) will affect restoration complete thyroid function. The effect will be more pronounced in the brain where more than 80% of T3 is the result of deiodinase 2 activities, and this will affect the differentiation process of thyroid hormone-mediated brain tissue.

The results of the study show that for the polymorphism of the Ala92Thr D2 gene, the AA genotype may be unfavorable to etiology in CH patients treated with levotiroxine, and therefore polytherapy could be considered a better approach in these patients.

Investigating the effects of anti-resorptive therapy with pamidronate in the treatment of children with osteogenesis imperfecta aimed to promote the experience in the treatment of this pathology, in conditions where there is no consensus regarding the optimal form, dose or duration of bisphosphonate therapy.

Treatment with pamidronate administered by intravenous infusions (0.5 mg / kg - first dose, followed by the next dose of 1 mg / kg / cycle) over a period of 3-4 hours, every 3 months for 3 years) demonstrated good tolerance without adverse effects or events without affecting puberty. The evaluation of the treatment was assesed based on clinical parameters (height increase, mobility, DMO, decrease in pain and fracture number), specific biomarkers (serum alkaline phosphatase and bone specific alkaline phosphatase, osteocalcin and serum beta-C-telopeptides (bCTx)); the evaluation of the improvement of the quality of life. Treatment proved effective, with patients reaching a normal height based on gender and their subsequent age.

Other effects described in our patients were: increased mobility and DMO highlighted by Z score in DXA assessment, decreased pain and fracture rate and improved quality of life.

Investigation of bone-specific biomarkers showed that bone-specific alkaline phosphatase has got a good correlation between clinical aspects and treatment progress ($p < 0.05$) as well as osteocalcin and serum beta-C-telopeptides (bCTx), whereas phosphatase serum alkaline has not correlated with either clinical progression or the number of fractures.

Investigation of assessing vitamin D as a biomarker in the etiology and evolution of juvenile idiopathic arthritis corresponds to research trends in this field because despite the lower levels of vitamin D in children with juvenile arthritis the interpretation of the results is problematic and controversial because there is no accepted definition of deficiency vitamin D in this category and consequently the standardization of vitamin D levels in the pediatric population and especially in juvenile arthritis becomes a requirement.

The results of the study are similar to those in the literature: 66% of children in the AJI group have vitamin D deficiency and 27% vitamin D insufficiency, accounting for 93% of the study group with levels below normal vitamin D values.

Also, statistically significant correlations ($p<0.05$) were obtained between the severity of the disease (the number of articulations with obvious radiological lesions) and the duration of the disease.

Regarding the types of AJI, the most affected by the deficit of vitamin D, are the systemic polyarticular forms and those forms which are associated with a positive rheumatoid factor. Due to these reasons, especially these types of AJI should be routinely screened for the identification of vitamin D deficiency and as a consequence might be treated with supplemental vitamin D administration.

This study was a complex approach to the factors involved in the etiopathogenesis and management of pediatric diseases. An association was demonstrated between the response to therapy in congenital hypothyroidism and the particular polymorphism of D2 deiodinases, between pamidronate therapy in osteogenesis imperfect and biomarkers of diagnosis, monitoring and prognosis as well as, between vitamin D3 levels and the etiology and management of juvenile idiopathic arthritis.

CONCLUSIONS

1. The study aims to contribute to the identification and evaluation of some factors involved in pediatric pathology, especially in the etiology of congenital diseases, biomarkers in diagnosis and therapeutic orientation.

2. The objective of the study was to establish the biomarker value of deiodinase polymorphism in the case of congenital hypothyroidism, vitamin D in juvenile idiopathic arthritis and bone metabolism markers in pamidronate treatment in osteogenesis imperfecta.

3. Investigating the prevalence of the polymorphism of Ala92Thr iodothyronine deiodinase gene 2 in children with congenital hypothyroidism in the West of Romania and its impact on TSH

level demonstrated that AA genotypes were significantly more prevalent in patients with high TSH after levotiroxine treatment ($p = 0.044$) than genotypes TT and AT.
This correlation is explained by a functional impairment of D2 deiodinase generated by AA polymorphism, which will reduce T4 → T3 conversion and under these conditions even if the T4 plasma level is normal, the decrease in T3 (thyroid hormone effector) will affect recovery complete thyroid function.The effect will be more pronounced in the brain where more than 80% of T3 is the result of deiodinase 2 activity, and this will affect the differentiation process of thyroid hormone-mediated brain tissue. Our results suggest that for the Ala92Thr D2 gene polymorphism, AA genotype may be unfavorable to euthyroidism in CH patients treated with levotiroxine, therefore polytherapy could be considered a better approach in these patients.

4. Investigating the effects of anti-resorptive therapy with pamidronate in the treatment of children with imperfect osteogenesis aimed at promoting the experience in the treatment of imperfect osteogenesis in conditions where there is no consensus regarding the optimal form, dose or duration of bisphosphonate therapy. Treatment with pamidronate administered by intravenous infusions (0.5 mg / kg - first dose, followed by the next dose of 1 mg / kg / cycle) over a period of 3-4 hours, every 3 months for 3 years) proved to have a good tolerance without adverse effects and without affecting puberty. The evaluation of treatment was based on clinical parameters (elevation of height, mobility, DMO, decrease in pain and number of fractures), characteristic biomarkers (serum alkaline phosphatase and bone specific alkaline phosphatase, osteocalcin and serum beta-C-telopeptides (b-CTx)) and the evaluation of the improvement of the quality of life.Treatment proved to be effective at patients with normal height based on gender and their subsequent age. Other effects described in our patients were: increased mobility, DMO highlighted by Z score in DXA assesment, decreased pain and fracture rate and improved quality of life.

Investigation of bone-specific biomarkers has shown that bone-specific alkaline phosphatase exhibits a good correlation between clinical and developmental progress($p<0.05$) as well as osteocalcin and serum beta-C-telopeptides(b-CTx) over time serum alkaline phosphatase did not correlate with either clinical progression or the number of fractures.

5.Investigating the possibility of assesing vitamin D as a biomarker in the etiology and evolution of juvenile idiopathic arthritis corresponds to the research trends in the field, because despite the lower levels of vitamin D in children with juvenile arthritis the interpretation of the results is problematic and controversial because there is no accepted definition of deficiency vitamin D in this category and as a result standardization of vitamin D levels in the pediatric

population and especially in juvenile arthritis becomes a requirement. At the same time, there is no study on the effect of vitamin D supplementation on the activity or outcomes of juvenile arthritis.

Our results are consistent with the data presented in the literature, even more dramatic: 66% of children in the AJI group have vitamin D deficiency and 27% vitamin D deficiency, accounting for 93% of the study group with levels below normal vitamin D levels. ($p<0.05$) between the severity of the disease (the number of joints with obvious radiological lesions) and the duration of the disease. In terms of all the AJI forms, the most affected ones by the vitamin D deficiency are the systemic polyarticular forms and those associated with positive rheumatoid factor, and for this reason these conditions should undergo a routine screening to identify vitamin D deficiency and consequently treated by supplemental vitamin D administration.

6. This study was a complex approach to the factors involved in the etiopathogenesis and management of pediatric diseases. An association was demonstrated between the response to therapy in congenital hypothyroidism and the particular polymorphism of deiodinases, between pamidronate therapy in ostegenesis imperfecta and biomarkers of diagnosis, monitoring and prognosis, as well as in the investigation of the possibility that vitamin D could become an important biomarker in the etiology and management idiopathic juvenile arthritis.

7. The results of the study have obvious practical applications in investigation and therapy.

In case of substitution treatment of congenital hypothyroidism, in cases where the normalization of serum T4 levels after levotiroxine substitution therapy is not followed by a normalization of the high level of TSH, it is necessary to analyze the polymorphism of the Ala2Thr D2 gene, and in the existing AA profile requires an introduction to the substitution treatment and the T3 hormone until the TSH level is normalized.

In case of osteogenesis imperfect treatment, administration of pamidronate given by intravenous infusions (0.5 mg / kg - first dose, followed by the next dose of 1 mg / kg / cycle) is recommended over a period of 3-4 hours every 3 months for 3 years) over a longer period of 3 years, and treatment monitoring should take into account, in particular, the normalization of bone-specific alkaline phosphatase.

In the etiological analysis and treatment of juvenile idiopathic arthritis, a necessary element is the investigation of serum vitamin D, serum form 25 (OH) D3, and in case of insufficiency or deficiency, vitamin D supplementation treatment is instituted until values are normalized. Further studies will be able to determine if treatment schemes should include also vitamin K. Due to the demands of bone tissue during the period of growth, monitoring of vitamin D levels should be extended at least until the growth process ceases.

The experimental design and methodology used to investigate the gene polymorphism of deiodinases in congenital hypothyroidism can also be used in other diseases where there are high levels of TSH concomitantly with normal T4 values, with the exclusion of other possible etiologies.