MEDICAL UNIVERSITY
"PROF. PARASKEV STOYANOV" – VARNA
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PEDIATRIC DEPARTMENT

D-r Kalina Binkova Ganeva

POSSIBILITIES OF SOME ECHOCARDIOGRAPHIC TECHNIQUES AND MICRORNAS FOR DETECTING SUBCLINICAL MYOCARDIAL DAMAGE IN CHILDREN AND YOUNG ADULTS WITH BETA-THALASSEMIA MAJOR

ABSTRACT

of a dissertation for the award of an educational and scientific degree "**Doctor**"

Specialty: "**Pediatrics**"

Scientific supervisors:

1. Assoc. Prof. Dr. Petar Atanasov Shivachev, M.D.
2. Prof. Dr. Valeria Ignatova Kaleva, Ph.D.

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- The Department of Pediatrics at the Faculty of Medicine of the Medical University - Varna.
- Department of Clinical and Immunological Laboratory at UMBAL "St. Marina" EAD - Varna.

The dissertation is presented on 130 pages and illustrated with 14 tables, 39 figures, and 2 appendices. 147 literary sources are cited, of which 6 are in Cyrillic and 141 are in Latin.

The dissertation work was discussed and directed for public defence by the Department Council of the Department of Pediatrics at the Medical University - Varna in connection with a report by Prof. Dr Violeta Mihova Iotova, PhD, Head of the Department of Pediatrics at the Medical University - Varna.

The public defence of the dissertation work will be held on 01.06.2023 in the Blackboard system of the Medical University - Varna based on Order No. P-109-187 of Prof. Dr Valentin Lyubomirov Ignatov, MD, Rector of MU-Varna, before a scientific jury composed of:

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The materials on the dissertation are published on the website of the Medical University "Prof. Dr. Paraskev Stoyanov"-Varna and are available at the Scientific Department of the MU-Varna.

CONTENT:

ABBREVIATIONS USED:

BNP	brain natriuretic peptide
BP	blood pressure
BSA	body surface area
BTM	beta thalassemia major
CMP	cardiomyopathy
DecT	deceleration time
ECG	electrocardiography
EchoKG	echocardiography
EF	ejection fraction
Em	tissue diastolic velocity in the mitral annulus
GLS	global longitudinal strain
HbF	fetal hemoglobin
HF	heart failure
HLA	human leucocyte antigen
HR	heart rate
IGF1	insulin growth factor-1
IRT	isovolumetric relaxation time
IVS	interventricular septum
LA	left atrium
LASDs	left atrium diameter in systole
LV	left ventricle
LVDD	left ventricle diastolic diameter
LVM	left ventricular muscle mass
LVMi	left ventricle mass indexed to body surface area
LVPWd	left ventricular posterior wall thickness in diastole
LVSD	left ventricle systolic diameter
MRI	magnetic resonance imaging
NTBI	non-transferrin bound iron
SD	standart deviatrion
SF	shortening fraction
Sm	tissue systolic velocity in the mitral annulus
TCMP	cardiomyopathy due to thalassemia
TGF-β	transforming growth factor-β

I. INTRODUCTION

Thalassemias are a group of inherited diseases characterized by reduced or absent production of the normal globin chains of hemoglobin. Beta-thalassemia major (BTM) belongs to the transfusion-dependent forms of thalassemia, which require regular lifelong hemotransfusions, without which several complications would occur.

Clinical presentation of BTM occurs between 6-24 months of age with progressive anemia, jaundice, and hepatosplenomegaly. Treatment recommendations include lifelong regular blood transfusions every two to five weeks. Blood transfusions ensure normal physical development and prevent several complications, but over time, iron overload occurs from the developing secondary hemochromatosis. The free form of iron, which is highly toxic, is deposited in the internal organs, mainly affecting the heart, liver, and endocrine glands, causing several disorders such as heart failure, liver cirrhosis, and endocrine disorders.

In the past, cardiac complications were the leading cause of death in patients with BTM, and at a young age – around 10. At present, in developed countries, with regular and adequate chelation therapy, good quality and length of life are ensured for these patients, still cardiac complications are the leading cause of morbidity and mortality. There are reports of early deposition of iron in the myocardium - at the age of 7, mainly with limited access to chelation therapy.

In recent years, new methods for evaluating the systolic and diastolic function of the heart, such as tissue doppler and even newer techniques - myocardial strain and strain rate - have entered. Their use has not yet widely used the daily clinical practice of the pediatric cardiologist, given the peculiarity of myocardial dysfunction and the technical specificity and laboriousness. Therefore, at present, there are not much literature data on the assessment of cardiac function among the pediatric population, using the myocardial strain parameter. Children with BTM are a contingent that becomes a target for the pediatric cardiologist usually after 10 years of age. Although the T2* magnetic resonance technique is considered the leading method for assessing myocardial

iron accumulation, the method is still expensive and not widely performed. New echocardiographic methods for evaluating and monitoring cardiac function in these children are more accessible and cost-effective. However, studies in children with BTM, especially those under 10 years of age, are scarce. There are reports of latent systolic, but primarily diastolic dysfunction, assessed by tissue Doppler, in asymptomatic children with BTM, including those under 10. New techniques of myocardial deformation demonstrate regional disturbances in the myocardium, possibly due to the inhomogeneous deposition of iron.

Recently, microRNAs have begun to attract the attention of researchers and clinicians as potential biomarkers for various pathological conditions. Their wide distribution in body fluids, stability, and resistance make them suitable for isolation, identification, and study. In theory, microRNAs possess many specificities required for an ideal biomarker - regulators of several pathological processes, widespread distribution in biological fluids, stability, and persistence. There is sufficient evidence for the involvement of certain microRNAs (microRNA-1, microRNA-21, microRNA-29, microRNA-30, and microRNA-150) in the pathogenesis and progression of heart failure (HF), myocyte hypertrophy and apoptosis, interstitial fibrosis, cardiac remodeling.

In the Center for Coagulopathies and Rare Anemias at UMBAL "St. Marina" in Varna, the medical staff takes care of patients with BTM and beta-thalassemia intermedia from all over North-Eastern Bulgaria. Our daily work with children stimulates us to familiarize ourselves in detail with the disease, the topicality of the treatment, and above all, the modern complications and their timely diagnosis. It is a fact that there is no clinically manifestated complications in childhood, or they are too rare. However, the manifestation of the disease with marked cardiac damage can occur in the most active years of the patients' lives, and the iron deposition is very likely to have started already in childhood. That's why we set ourselves the task of looking for sufficiently sensitive, reliable, and non-invasive methods for early diagnosis of heart damage, before the onset of symptoms, because prevention always remains the best treatment, both for patients and the doctors caring for them. Last but not the least, the methods must be as gentle and non-invasive as possible for children.

II. WORKING HYPOTHESIS

Initial changes in cardiac function in asymptomatic young patients due to BTM disease and myocardial iron accumulation can be identified using modern non-invasive echocardiographic techniques and some microRNAs.

III. AIM OF THE DISSERTATION

To determine whether there are early abnormalities in cardiac function in young BTM patients and whether they can be identified using some modern non-invasive echocardiographic markers and cardiac damage-specific microRNAs.

IV. DISSERTATION TASKS

1. To carry out an assessment of the physical development of children and young adults with BTM, including the anthropometric parameters - height, weight, and body surface area, as well as to clinically assess the cardiovascular system - heart activity, HR, BP. To compare with healthy controls.

2. To perform an echocardiographic assessment of left ventricular cardiac function in BTM patients compared with healthy controls, including:

- Measurement and assessment of cardiac dimensions and systolic left ventricular function - LVDD, LVSD, EF of the LV, SF, LVPWd, and IVS thicknesses, LVM, including indexed to body surface area, LASDs and indexed LA volume to body surface area. Assessment of myocardial deformation by speckle tracking and GLS calculation.

- Assessment of LV diastolic function - measurement of mitral blood flow velocities (E, A, E/A), assessment of tissue myocardial systolic (Sm) and diastolic velocities in medial and lateral mitral valve segments (e', a'), assessment of left ventricular filling pressure - the ratio E/e'.

- To analyze the diagnostic potential of tissue Doppler and GLS for the assessment of early cardiac dysfunction in asymptomatic children and young patients with BTM.

3. To examine a laboratory marker for iron overload in children and young patients with BTM-ferritin.

4. To investigate the expression of specific microRNAs - microRNA-1, microRNA-21, microRNA-29, microRNA-30, and microRNA-150, as markers of HF, remodeling, and fibrosis in BTM patients and healthy controls.

5. To make a comparison between the results of the investigated microRNAs and the echocardiographic parameters of the patients.

6. To look for a correlation between the echocardiographic assessment of cardiac function and myocardial iron accumulation using the MRI T2* technique according to a degree (mild, moderate, severe) in patients over 10 years of age.

7. To propose a protocol for echocardiographic examination and follow-up of cardiovascular status in patients with BTM.

V. MATERIALS AND METHODS

1. Patients and healthy controls.

The dissertation study covers a total of 78 examined persons, of which 27 are children and young patients with proven BTM, with an average age of 15.14 years, who are being treated at the Expert Center for Coagulopathies and Rare Anemias at UMBAL St. Marina, and 51 healthy controls matched for sex and age. Patients visit the Center periodically every 2 to 5 weeks, depending on their clinical condition, for hemotransfusions and chelation therapy.

The research started on 22.07.2019, after receiving permission from the Committee on Ethics of Scientific Research at the University of Medicine - Varna with Decision No. 84 dated 27.06.2019 and covered the period of July 2019 – June 2022.

1.1. Patients:

There are a total of 27 patients, of which 13 are girls and 14 are boys. The mean age was 15.14 years (SD ± 5.83). The selection of patients was carried out using certain inclusion criteria:

- Age: 0-25 years
- Confirmed diagnosis of BTM, through hemoglobin electrophoresis and genetic testing.
- Absence of accompanying pathology affecting the cardiovascular system.

- Signed informed consent from a parent or guardian of persons under 18 years of age.

1.2. Healthy controls:

The study included 51 sex- and age-matched controls who were selected according to the following criteria:

- Age: 0-25 years.
- Absence of underlying heart disease and anemic syndrome.
- Signed informed consent from a parent or guardian of persons under 18 years of age.

2. Research methodology.

The following procedures were performed during the examination of patients and healthy controls:

2.1. Physical examination with measurement of anthropometric parameters: height (in centimeters), weight (in kilograms), and body surface area (BSA), calculated according to the Du Bois formula. Recording of detailed cardiovascular status - heart rhythm, HR, auscultatory findings, BP, peripheral pulsations.

2.2. A 12-lead electrocardiogram using a Shiller brand electrocardiograph, model AT-2 plus.

2.3. Examination of laboratory parameters:

- In patients: complete blood count (pre- and post-transfusion), serum ferritin, separation of serum for the study of microRNA-1, microRNA-21, microRNA-29, microRNA-30, and microRNA-150.
- In controls: complete blood count, separation of serum for the study of microRNA-1, microRNA-21, microRNA-29, microRNA-30, and microRNA-150.

A 5-diff hematology analyzer Sysmex XN 1000 was used to determine the parameters of the complete blood count using the fluorescence flow cytometry method using a semiconductor laser and hydrodynamic focusing. The ferritin test was performed by an immunometric chemiluminescence method on an Immulite 2000 XPi analyzer with an analytical sensitivity of 0.4 ng/ml.

2.4. An echocardiographic examination to assess cardiac structure and function was performed on an Esaote device, model MyLabOmega, manufactured in 2020 with a transducer in the range of 1-9 MHz depending on

the age and physique of the subjects, following the recommendations of the American Association of Echocardiography in childhood. During the study, the following parameters were measured and evaluated:

- **LV dimensions**: thicknesses of the IVS and LVPWd, LVDD, LVSD, and the absolute left ventricular muscle mass was calculated, as well as the muscle mass indexed to the body surface area (LVMi) (Fig. 1).

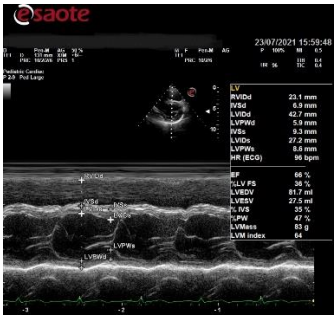

Fig. 1. Measurement of dimensions of LV: LVDD, LVSD, IVS, LVPWd; measurement of LVM and LVMi; calculation of EF and FS.

- **LA volume and indexed left atrial volume** to body surface area (LAVi) (Fig. 2).

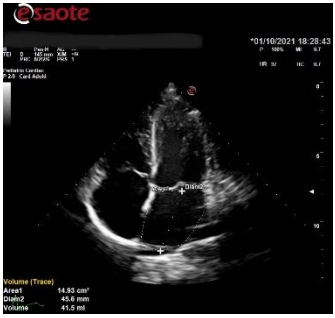

Fig. 2. Measurement of LA volume.

- **Assessment of LV systolic function**. The most commonly used parameters for the assessment of global left ventricular systolic function are EF and FS. Calculation of the GLS, myocardial deformation parameter, was performed using the speckle-tracking technique,

following the recommendations of the American Association of Echocardiography (Fig. 3).

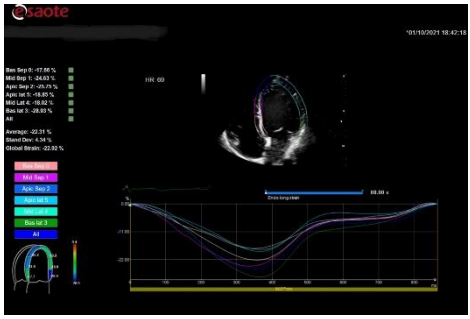

Fig. 3. Calculation of GLS.

- **Assessment of LV diastolic function**. Measurement of peak early diastolic velocity (E-wave), peak late diastolic velocity during atrial contraction (A-wave), and deceleration time (DecT). The E/A ratio was calculated (Fig. 4). Using tissue Doppler, systolic myocardial velocity (s'), early and late diastolic myocardial velocity (e', a') were measured, respectively, in the medial and lateral segments of the mitral valve annulus. The ratio E/e' was calculated (Fig. 5, Fig. 6).

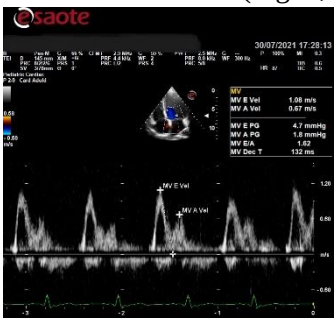

Fig. 4. Measurement of transmitral blood flow by pulse wave doppler; calculation of E/A ratio and DecT.

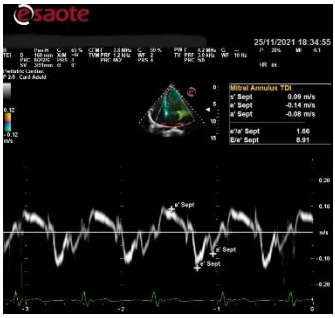

Fig. 5. Tissue Doppler measurement of tissue myocardial velocities in the medial mitral annulus.

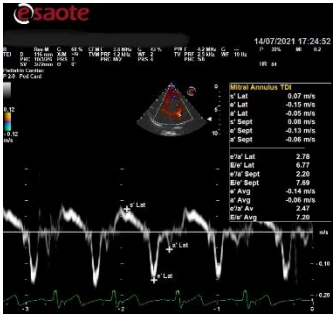

Fig. 6. Tissue Doppler measurement of tissue myocardial velocities in the lateral mitral annulus.

3. Examination of non-coding small ribonucleic acids (microRNA).

The following microRNAs were selected for the purposes of the study: microRNA-1, microRNA-21, microRNA-29, microRNA-30, and microRNA-150. The microRNAs were selected based on the information available in scientific circles about their involvement in cardiac pathology - acute cardiac injury, cardiac remodeling, and fibrosis. The study was approved and financed by the Science Fund under project №19007 - "Heart damage in young patients with beta-thalassemia major".

The material used for microRNAs research is blood serum. Isolation of microRNAs was performed from 200 μl of serum, using a ready-made commercial miRNeasy Serum/Plasma Kit (50), catalog №217184 (QIAGEN, Germany), according to the manufacturer's protocol. Each of the samples was then subjected to reverse transcription using the off-the-shelf commercial

miScript II RT Kit (50), catalog №218161 (QIAGEN, Germany), according to the manufacturer's protocol.

Subsequently, each of the samples was subjected to real-time quantitative polymerase reaction (RealTime PCR) using a ready-made commercial kit miScript SYBR Green PCR Kit (200), catalog number №218073 (QIAGEN, Germany) and ready-made primers miScript Primer Assay (100), catalog №218300 (QIAGEN, Germany) according to the manufacturer's protocol.

The relative concentration of the studied target microRNAs was calculated by the ΔΔCt method, compared to a reference microRNA - normalization control C. elegans miR-39 compared to a reference sample - represented by the geometric mean value of Ct of all studied samples, calculated using Microsoft Office Excel 2016 and presented as relative amount to the reference sample.

4. Statistical methods.

The following methods were used for the statistical processing of microRNAs: non-parametric descriptive statistics and non-parametric comparison of main trends between groups (patients and controls) with Mann-Whitey test using GraphPad Prism version 9.3.0 64-bit on Windows 10 OS. The statistical significance of the results was set at $p<0.05$.

For the dissertation, the following statistical methods were used:

- Statistical grouping of data.
- Descriptive methods.
- Statistical hypothesis testing.
- Correlation analysis.

VI. RESULTS AND DISCUSSION.

1. Analysis of demographic, anthropometric, and hemodynamic parameters of patients with beta-thalassemia major and comparison with the control group.

The present study included a total of 78 individuals, of which 27 were children and young patients diagnosed with BTM, and 51 healthy controls. The

analysis of the demographic and anthropometric parameters in the BTM patients and the control group showed the following results:

1.1. BTM patients had a mean age of 15.15 years (±5.83), and healthy controls 14.45 years (±5.73), respectively. The difference is not significant with p=0.613.

1.2. In the distribution by gender in both groups, the male gender prevails: in patients with BTM 52%, and healthy controls – 55% (fig. 7).

1.3. In terms of body surface area, BTM patients had a smaller BSA (1.42 m^2 ± 0.37) compared to healthy controls (1.48 m^2 ± 0.44), this difference not being significant (p=0.527) (table 1).

Parameter	*Group*	*n (%)*	*Average value*	*Standard deviation (±SD)*	*p-value / p=0.05/*
Age (y)	Patients	27	15,15	5,83	p=0,613
	Controls	51	14,45	5,73	
Sex - women	Patients	13 (48%)			
	Controls	23 (45%)			
BSA ($м^2$)	Patients	27	1,42	0,37	p=0,527
	Controls	51	1,48	0,44	

Table 1. Demographic parameters of the studied persons.

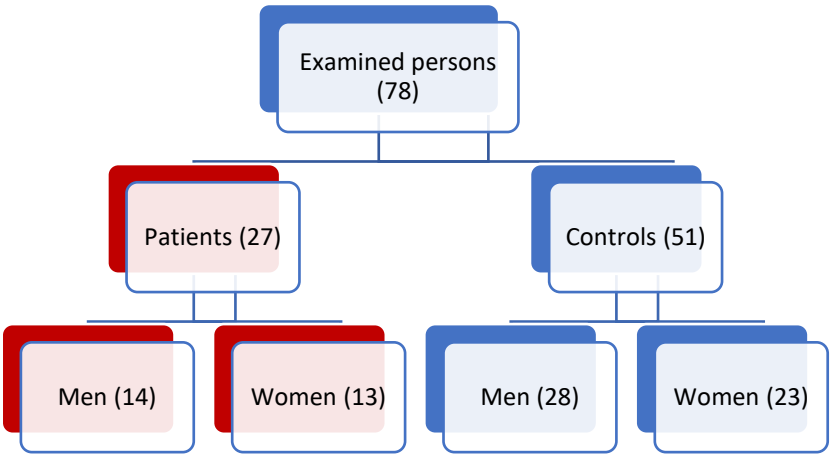

Fig. 7. Distribution of the examined persons by gender.

Smaller body surface area in BTM patients has also been reported by other authors (*Wood et al, 2003; Farmakis et al, 2004; Noori et al, 2006; Westwood et al, 2007*). Delay in physical development is a common and well-known pathology in BTM. Children grow relatively well until about 9-10 years of age, after which there is a slowdown in growth rate with late puberty and lower final height (*Skordis, Kyriakou, 2011*). The pathogenesis of growth disorders is multifactorial, with iron deposition in endocrine glands with subsequent iron toxicity considered to be the main cause (*Skordis, Kyriakou, 2011; Pemde et al, 2011*). Several reports have reported a correlation between short stature in BTM patients and high serum ferritin levels (*Rathaur et al, 2020; Hashemi et al, 2011, Moiz et al 2018*). Additional factors include chronic hypoxemia, nutritional deficiencies, chronic liver damage, and dysregulation of the growth hormone-insulin-like growth factor (IGF1) (*Skordis, Kyriakou, 2011; Moiz et al, 2018*). According to different studies, short stature is observed in 33.11% to 65.71% of patients (*Pemde et al, 2011; Hashemi et al, 2011; Rathaur et al 2020*), and late puberty in up to 71% (*Najafopour et al 2008*). All this shows that patients with BTM should be monitored regularly for endocrine disorders, especially after 10 years of age.

In the present study, we obtained the following results from the clinical examination of patients with BTM and healthy controls regarding heart rate and arterial pressure (table 2):

Parameter	*Group*	*n (%)*	*Average value*	*Standard deviation (±SD)*	*p-value /p=0.05/*
Heart rate	Patienst	27	83,5	11,24	p=0,195
	Controls	51	79,47	13,67	
Systolic BP (mmHg)	Patients	27	103,7	12,78	p=0,062
	Controls	51	109,5	12,96	
Diastolic BP (mmHg)	Patients	27	66,56	9,13	p=0,070
	Controls	51	70,67	9,52	
Average BP value (mmHg)	Пациенти	27	78,78	9,84	p=0,054
	Контроли	51	83,53	10,38	

Table 2. Hemodynamic parameters of the subjects with BTM.

Mean HR in patients was higher (84/min ± 11) compared to healthy controls (79/min ± 14), but the difference was not significant (p=0.195) (fig. 8, fig. 9). The study reported lower mean values in BTM patients for both systolic (patients: 103.7 ± 12.78 mmHg; controls: 109.5 ± 12.96 mmHg) and diastolic BP (patients: 66 .56 ± 9.13 mmHg; controls: 70.67 ± 9.52 mmHg) compared to the control group, but the difference was not significant (p=0.070). Accordingly, the mean BP values among patients were lower compared to healthy controls (p=0.054) (fig. 10). 66.6% of BTM patients had low-for-age blood pressure values compared to 43% of healthy controls. BP is classified as low or normal based on the reference values presented by the European Association of Hypertension.

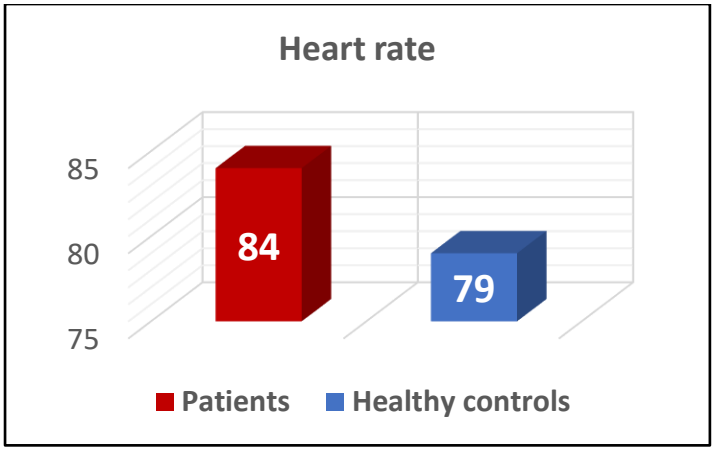

Fig. 8. Mean values of heart rate in patients with BTM and healthy controls.

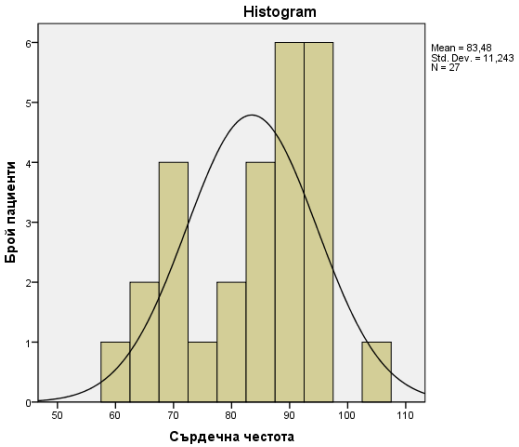

Fig. 9. Histogram demonstrating heart rate distribution in BTM patients.

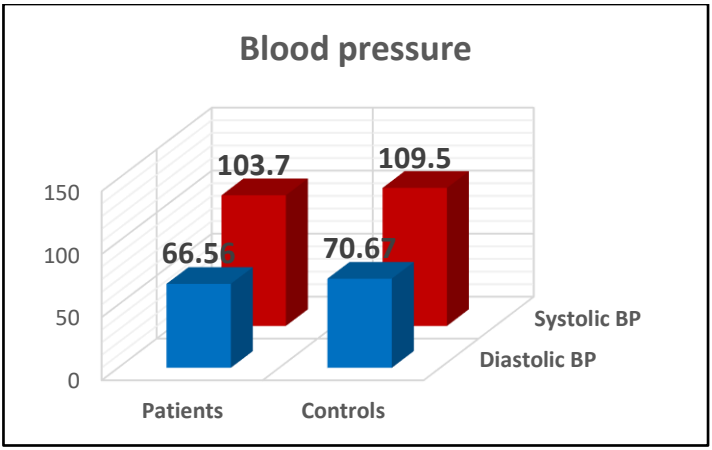

Fig. 10. Mean BP values in BTM patients and healthy controls.

It is known that beta-thalassemia is a chronic high-output condition in which chronic anemia and reduced oxygen delivery to tissues are compensated by an increased cardiac output, via an increase in HR and stroke volume in patients (*Metivier et al, 2000*). Therefore, it is not a surprise that patients with BTM have a higher HR. Although we did not find a statistically significant difference, the recorded HR in patients was higher. Spirito et al. (1990) also found no significant differences in HR between BTM patients and controls (73 ± 11/min and 74 ± 12/min). Veglio et al. (1998) investigated variations in HF and BP in young BTM patients aged 18. The average HR in the patients they studied was 75/min (66-86/min) without a significant difference, but they found significantly higher values during the night, compared to healthy controls. Our results for HR are closer, in absolute value, to those reported by Westwood et al. (2007). They conducted a detailed MRI study of 205 young BTM patients with a mean age of 28 ± 7 years. They aimed to analyze left ventricular volumes, function, and hemodynamics in patients who were shown not to have myocardial iron overload yet (i.e., MRI T2* > 20 msec), thereby excluding the toxic effect of iron on the cardiovascular system. The average value of HR in the patients was 81.7 ± 16.4/min. ($p<0.001$). Aessopos et al. (2004) also studied a large group of 202 young BTM patients aged 27.3 ± 6.3 years and recorded a mean HR of 84 ± 12/min, significantly higher compared to healthy controls ($p<0.001$).

Variations in the average values of HR presented by different authors, are observed, which is not surprising, considering the factors that influence it, such as the age of patients, hemoglobin level at the time of the study, emotional state, etc. However, it is interesting that the patient cohort of Spirito et al. from 1990 with a mean age of 17 ± 5 years, and Veglio et al. from 1998 – mean age of 18 years, demonstrate mean HR values of almost 10 beats per minute lower, compared to the results presented by us and other authors. The mean age of the patients studied by us is closer to the age of the patients of Spirito et al. and Veglio et al. than to the patients of Westwood et al. and Aessopos et al. We could not find a logical explanation for this values discrepancy, considering the study was performed after blood transfusion in all patients in order to avoid the influence of anemic syndrome on the assessment of hemodynamics.

There are not several reports that present specific BP values in children with BTM. Spirito et al. (1990) reported mean systolic values of 109 ± 10 mmHg and mean diastolic values of 69 ± 7 mmHg, both values being significantly lower ($p<0.02$) compared to healthy controls. The mean age of the patients they studied was 17 ± 5 years. Although the difference we found between the BP of BTM patients and healthy controls was not significant our results for both systolic and diastolic values, were again close to those presented by Aessopos et al. (2004), who reported a mean systolic BP in their patient group (mean age 27.3 ± 6.3 years) of 102 ± 5 mmHg and a mean diastolic BP of 65 ± 4 mmHg, respectively, with significant differences from healthy controls ($p<0.001$). In the patients studied by Veglio et al. (1998), the average systolic values were 106 mmHg (97 - 114) mmHg, and the average diastolic values were 74 mmHg (65 - 83) mmHg, and almost half of the patients had circadian rhythm disorder with an unsatisfactory drop in blood pressure during the night hours.

These results are not surprising considering the altered hemodynamics of the patients. Chronic anemia and the consequent increased cardiac output with an increase in HF and stroke volume lead to a fall in peripheral vascular resistance (*Metivier et al, 2000*).

2. Analysis and discussion of morphological echocardiographic parameters of patients with beta-thalassemia major and healthy controls.

The summary results for left ventricular diameters, left ventricular muscle mass, indexed left ventricular muscle mass, and indexed left atrial volume are presented in table 3.

Parameter	*Group*	*n (%)*	*Average value*	±*SD*	*p-value*
LVDD (mm)	Patients	27	47,30	8,11	p=0,271
	Controls	51	45,51	5,96	
LVSD (mm)	Patients	27	29,26	5,62	p=0,224
	Controls	51	27,84	4,42	
LVM(g)	Patients	27	158,37	67,69	**p=0,031**
	Controls	51	126,41	57,21	
LVMi	Patients	27	107,15	26,29	**p=0,000**
	Controls	51	81	22,03	
LAVi (ml/m^2)	Patients	27	32,19	7,87	**p=0,000**
	Controls	51	22,69	9,24	

Table 3. Echocardiographic parameters in patients with BTM and healthy controls.

2.1. According to our results, in patients with BTM, both end-diastolic (patients: 47.3 ± 8.11 mm; controls: 45.51 ± 5.96 mm) and end-systolic (patients: 29.26 ± 5.62 mm; controls: 27.84 ± 4.42 mm) **LV diameter** was greater compared to healthy controls, but without significant difference (p=0.27; p=0.224) (fig. 11). Only in 1 of the patients (3%) **increased LV size** were found compared to the reference values for age.

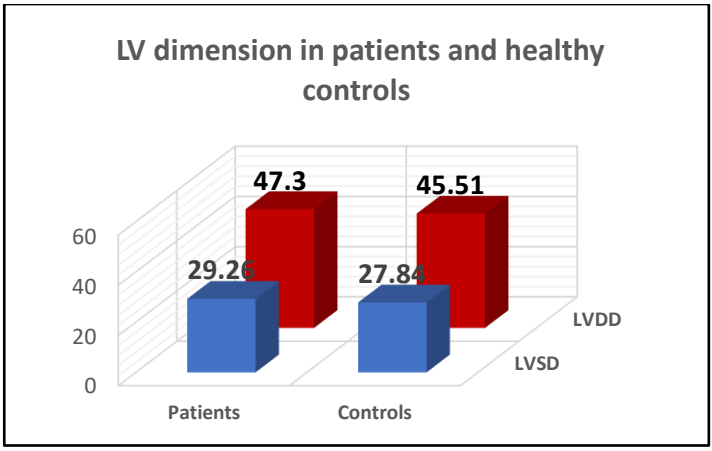

Fig. 11. Comparative analysis of LV dimensions in BTM patients and healthy controls.

Spirito et al. (1990) reported, close to our results, end-diastolic and end-systolic LV dimensions in their patients with BTM – 46 ± 5 /30 ± 4 mm. Similar results are the ones presented by Veglio et al. (1998): 48 mm (42 - 53) - for end-diastolic and 32 mm (29 - 37) - for end-systolic diameter. A Bulgarian study of children with BTM from 2011, conducted by Dr Chakarov, also reported similar left ventricular end-diastolic dimensions, respectively 46.5 ± 6.52 mm. After a detailed analysis of the existing literature databases, these groups of patients were selected because of their similar age to our patients: the patients of Spirito et al. had a mean age of 17 ± 5 years, the cohort of Veglio et al. - 18 years, and for Dr Chakarov - 17.8 ± 7 years. This fact is taken into consideration due to the intensive growth of patients in this age group and the large variations in dimensions of the heart cavities that can be observed.

The increased size of LV in patients with BTM has a logical explanation, similar to HR and BP, and it is related to the changed hemodynamics in this condition. BTM represents a chronic, high-output state as a compensatory response of the cardiovascular system to chronic anemia (*Wood et al, 2005*). The long-term consequences are increased left ventricular dimensions and heart muscle mass. This fact has been proven several times by many of studies, including not only echocardiographic but also magnetic resonance assessment

of the dimensions of the cardiac cavities, cardiac volumes, as well as the cardiac index (*Wood et al, 2005; Aessopos et al, 2004; Westwood et al, 2007*).

2.2. The **mean left ventricular muscle mass** in our BTM patients (158.37 ± 67.69 g) was greater than in healthy controls (126.41 ± 57.21 g), the difference being statistically significant in this case (p=0.031) (fig. 12).

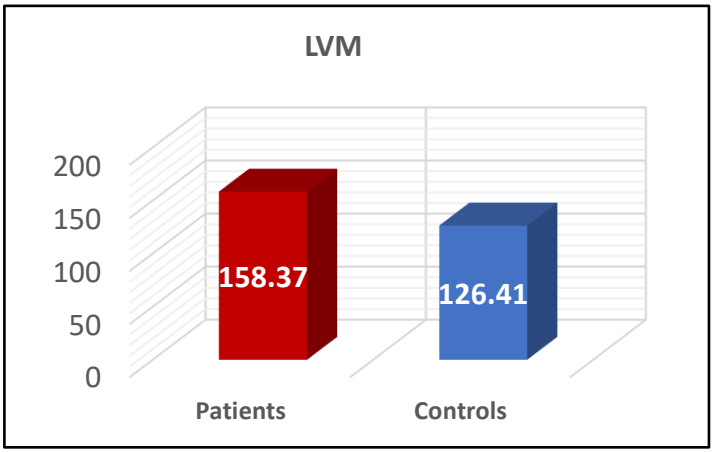

Fig. 12. Comparative analysis of LVM in BTM patients and controls.

Given the increases in end-diastolic and end-systolic LV diameter, the increased LVM among patients with BTM is surprise. Dr Chakarov reported similar to our results. The children with BTM studied by him had absolute values of LVM of 140.07 ± 52.10 g. Our results differ significantly from the one reported by Spirito et al. (1990). In their group of patients, the mean LVM was 111 ± 41 g. We were unable to find a logical explanation for this results difference, given the fact that the patients of Spirito et al. are examined nearly 30 years before the Bulgarian children. The patients had been given chelation therapy only with Deferoxamine in the form of an 8-12hour subcutaneous infusion, in contrast to our patients who conducted therapy with modern oral chelators.

2.3. The ratio of **indexed left ventricular muscle mass to body surface area (LVMi)** in BTM patients was also significantly greater

(**p=0.000**) compared to healthy controls (107.15 g/m2 vs. 81 g/m2) (Fig. 13) . When compared with reference values, it was found that 25 of the patients (**92.6%**) had evidence of **left ventricular hypertrophy** (table 4, fig. 14).

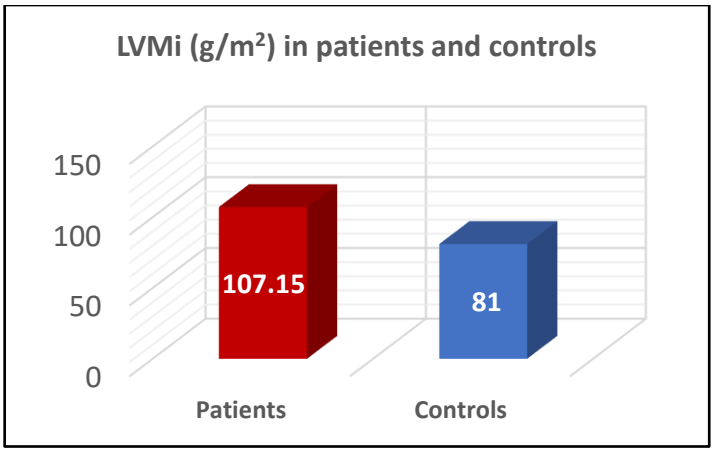

Fig. 13. Comparison of LVM indexed to body surface area (LVMi) in BTM patients and healthy controls.

Patients with BTM	N	Percent
With LV hypertrophy	25	92,6%
Without LV hypertrophy	2	7,4%
Total	27	100%

Table 4. Number of BTM patients with evidence of left ventricular hypertrophy according to increased LVMi.

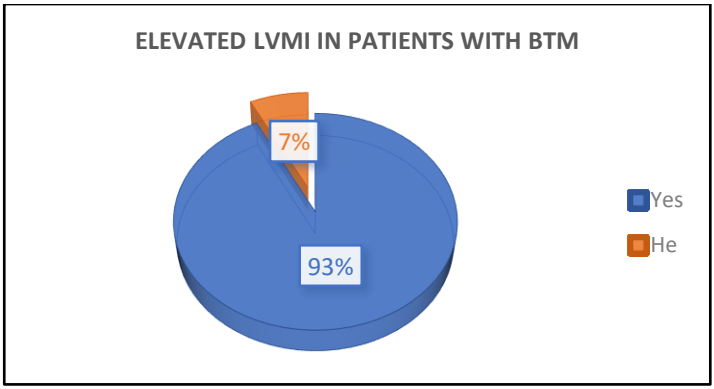

Fig. 14. Schematic representation of patients with left ventricular hypertrophy according to LVMi.

Increased indexed LVM to body surface area in BTM patients has been reported by many authors (*Spirito et al, 1990; Aessopose et al, 2004; Bossi et al, 2003; Westwood et al, 2007; Casale et al, 2015*). Casale et al. (2015), who studied children < 18 years of age with BTM, found increased LV end-diastolic and end-systolic volumes. In contrast to ours, their studied patients demonstrated lower LVMi values. They divided BTM patients into three groups depending on the myocardial iron burden. Quite logically, patients with data on homogeneous iron deposition in the myocardium, also demonstrated the greatest LVMi, respectively 67 ± 13.9 g/m^2. The significant discrepancy could be explained by the age difference in the studied patients. Our patient group also includes young adults up to 25 years of age, which invariably affects both body surface area and LVM.

Similar to our results were reported by Bosi et al. (2003), who conducted a detailed study of 197 young patients with BTM without evidence of cardiac dysfunction. LVMi in their patients was 99.2 ± 22.1 g/m^2, with correspondingly larger left ventricular end-diastolic and end-systolic volumes.

Significantly, but not unexpectedly, evidence of left ventricular hypertrophy was found in 92.6% of our patients, according to age-referenced values. It is known that hemodynamic changes induce cardiac enlargement and left ventricular hypertrophy. Thus, the increase in LVM with preserved systolic function in patients with BTM is interpret as a compensatory mechanism.

2.4. In **55.6%** of patients, an **increased indexed volume of LA to the body surface (LAVi)** was found (Fig. 15). LAVi showed

significantly higher values in patients (32.19) compared to healthy controls (22.69) with **p=0.000** (fig. 16).

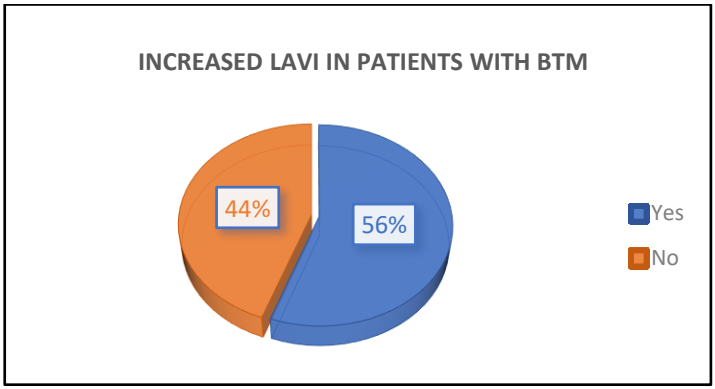

Fig. 15. Schematic presentation of patients with BTM with increased sizes of LA according to LAVi.

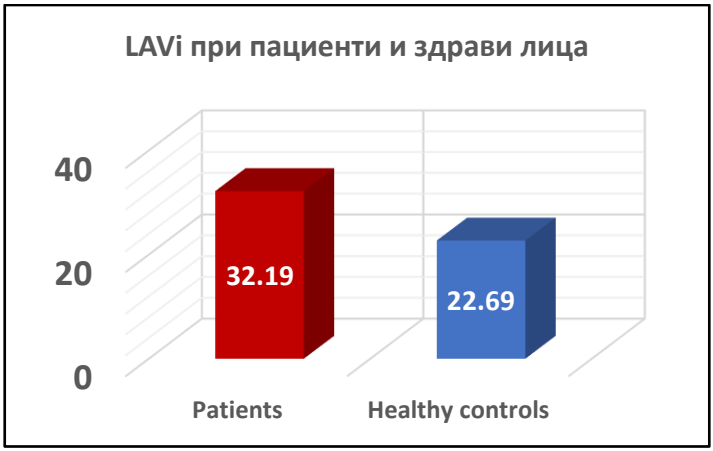

Fig. 16. Comparison between LAVi in BTM patients and healthy control.

Increased volumes of LA in patients with BTM have been reported by a number of authors (*Spirito et al, 1990; Kremastinos et al, 1993; Aessopos et al, 2004*). In the young patient group of Spirito et al. (1990) LA was enlarged compared to healthy controls, although the difference was not significant. Our

results are close to the values presented by Kremastinos et al. (1993) – 32.19 ± 7.87 vs 28 ± 8, while the age of the studied patients was similar – 15.15 ± 5.83 years vs 15.1 ± 3.2 years. In both studies the difference in LAVi between BTM patients and healthy controls was significant.

The detailed MRI study of children with BTM by Casale et al. (2015) compared LV dimensions in patients with cardiac fibrosis and those without fibrosis. The authors found significantly larger sizes of LA in children with cardiac fibrosis.

Shabanian et al. (2010) conducted a magnetic resonance imaging study of 42 young BTM patients aged < 21 years with preserved EF of the LV with the aim of establishing the prognostic role of the EF of LA for the early detection of myocardial iron overload. They divided patients into two groups depending on the presence or absence of myocardial iron overload assessed by T2* MRI. The authors found increased LA volume indexed to body surface area, both in patients with iron overload and in those without, although the difference was not significant - 40.17 ± 15.16 and 43.25 ± 15.92. Curious are their results regarding the left atrial ejection fraction index. With a sensitivity of up to 93% and a specificity of 74%, this parameter can be prognostic for establishing critical iron overload. Bulgarian study of adult patients with beta-thalassemia major by Assoc. prof. M. Dimova from 2017 demonstrated a larger LA volume, compared to healthy controls, with a lower LA ejection fraction. In addition, Assoc. prof. Dimova established a correlation between the indexed volume of the LA and NT-proBNP values, which confirms the prognostic role of the increased dimensions of the LV for future heart damage.

LA dilatation is due not only to iron deposition but also to increased LV diastolic pressure due to diastolic dysfunction (*Kostopolou et al, 2014*). A number of authors found that a dilated LA is a very early sign of cardiac damage (*Kostopolou et al, 2014; Pepe et al, 2017*), and is also a prognostic risk factor for future arrhythmias such as atrial flutter, atrial fibrillation and intra-atrial re-entry tachycardia (*Pepe et al, 2017; Russo et al, 2016, 2019*).

3. Systolic function in patients with beta-thalassemia major.

3.1. The mean EF in BTM patients was 68.19% (SD ± 5.25). Compared to healthy controls (69.61% ± 4.85), no significant difference was found (p=0.235). EF ≤ 59% was recorded in 2 of the patients (7.9%) (table 5, fig. 17, fig. 18).

Parameter	***Group***	***n (%)***	***Average value***	***±SD***	***p-value***
EF (%)	Patients	27	68,19	5,25	p=0,235
	Controls	51	69,61	4,85	
GLS (%)	Patients	27	-21,35	2,97	p=0,276
	Controls	51	-20,64	2,05	

Table 5. Ejection fraction and GLS in BTM patients and healthy controls.

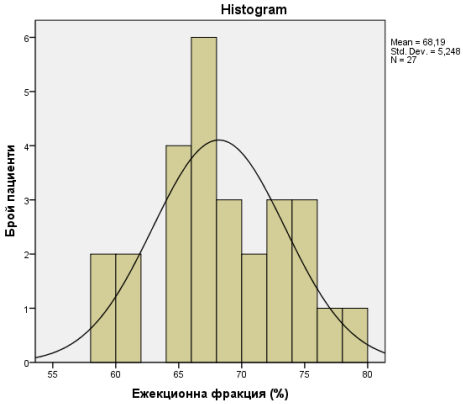

Fig. 17. Mean EF values among BTM patients.

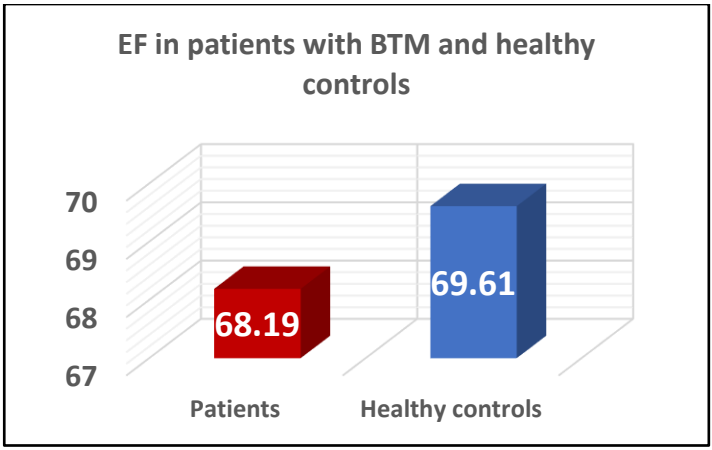

Fig. 18. Comparison of EF values in BTM patients and healthy controls.

It is well known that LV EF is a marker of global systolic function, and its decline is a terminal phase in the progression of TCMP (*Murphy and Oudit, 2010*). Our results of preserved EF confirm the above statement, considering the lack of clinical manifestation of cardiac damage in the patients. In the two patients with lower EF values, a slightly increased LAVi was also found, with values of 35.06 and 34.79, respectively.

Dr Chakarov's study of Bulgarian children with BTM found EF < 65% in 29% of patients. There is no data, however, on how many of them have a pathological decrease in systolic function, i.e. EF < 59% (*Chakarov, 2011*). In a magnetic resonance imaging study, Casale et al (2015) divided children with BTM into 4 main groups: without evidence of myocardial iron overload, with heterogeneous deposition but with MRI T2* > 20 msec., with heterogeneous deposition and MRI T2* < 20 msec. and with homogeneous myocardial iron deposition. The trend they report is a progressive decrease in EF from the first to the last group, i.e. with the severity of myocardial iron overload. Although the difference was not significant, the trend was obvious with EF values in groups, respectively: 62%, 59.5%, 59%, and 59.5%. However, EF cannot be a reliable indicator for early cardiac dysfunction, because of its late decrease in the evolution of cardiac damage. Another interesting result in their study was

the lower recorded EF, although again non-significant, in children with evidence of cardiac fibrosis.

3.2. The average value of **GLS** in BTM patients was -21.35% (SD ± 2.97) (table 5). When compared with healthy controls (-20.64% ± 2.05), the difference was not significant (p=0.276) (fig. 19, fig. 20). 7 out of 19 patients **(37%) under 18 years of age** had GLS values below the average for the corresponding age (GLS < -20.15%), while **50% of patients over 18 years** had values below the average (GLS < -19.7%).

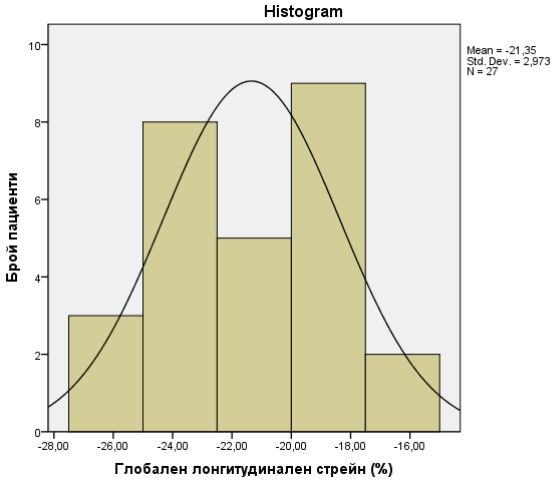

Fig. 19. Distribution of mean GLS values in BTM patients.

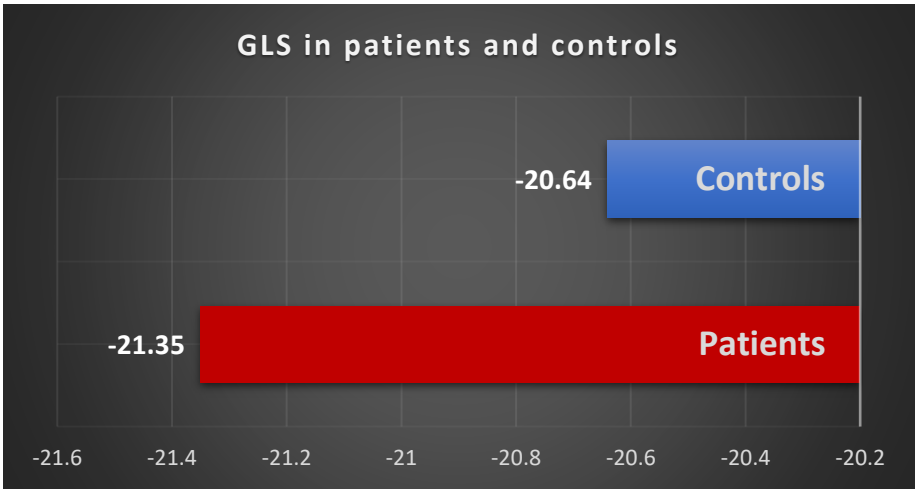

Fig. 20. Comparative presentation of mean GLS values in BTM patients and healthy controls.

At the same time, pathologically low GLS < -15.30% was measured in only 1 patient up to 18 years of age. He also had a slightly increased E/e' ratio of 8.66, as well as an increased left atrial dimension with LAVi of 43.8. Although he did not meet all the criteria for diastolic dysfunction, the patient still had evidence of elevated LV diastolic pressure as well as LA dilatation. Unfortunately, due to severe claustrophobia, no MRI T2* of the heart was performed until now.

In one of the patients over 18 years of age, a lower-limit GLS -16.65% was registered. However, he also had evidence of severe myocardial iron overload with MRI T2* 8.5 msec, as well as left atrial enlargement with LAVi 37.03 (fig. 21).

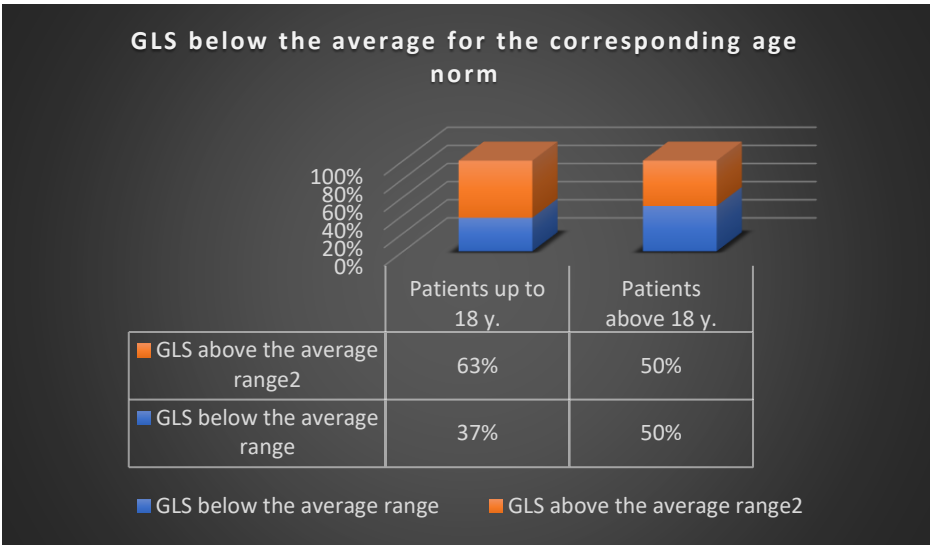

Fig. 21. Patients with BTM with GLS values below the average for the corresponding age norm.

The available data in the literature regarding GLS in children with BTM are currently quite scarce, given that the technique is still new and has not been used widely in the routine examination of pediatric cardiologists. The mean GLS values we measured were within the reference range. Our results are similar to those presented by El-Shanshory et al. (2020), who studied 140 children with BTM with a mean age of 10.9 ± 3.7 years. The authors conducted a combined MRI and echocardiographic examination using the speckle tracking technique with the aim not only to assess cardiac function but also to make a comparison between the different techniques. The measured GLS values in their patients without iron overload were not statistically different from those with myocardial iron overload and were -21.23 ± 2.68% and -21.375 ± 2.06%, respectively. In addition, the values are within the reference range.

Pizzino et al. (2017) studied elderly patients with BTM (mean age 37.4 ± 10 years) and found mean GLS values of -20.6 ± 2.8%. They also found a correlation between GLS and MRI T2* values, with GLS being lower in patients with myocardial iron overload and values < -19.5% being sensitive to

severe myocardial burden. Parsaee et al. (2018) also found a correlation between GLS and MRI T2*. The patients they studied were again adults (30.79 ± 9.37 years) and divided into two groups depending on the presence or absence of myocardial iron overload. Mean GLS in patients without myocardial iron overload was -19% (-20% to -18%), and in those with iron overload was significantly lower ($p<0.0001$) with a mean value of -17% (-19% to - 16%). According to them, values of GLS < -18% are predictive of myocardial iron overload. Rezaeian et al. (2020) divided their patient (mean age 24.6 ± 6.2 years) into 2 groups, with myocardial iron overload (MRI T2* < 20 msec) and without (MRI T2* > 20 msec), respectively. The mean value of GLS in patients with myocardial damage was -12.90 ± 4.18%. What is interesting in their study is that even patients with MRI T2* > 20 msec had significantly lower GLS compared to healthy controls. According to the authors, these results show that even a minimal degree of iron deposition in the myocardium can affect myocardial function and strain.

4. Doppler examination and assessment of diastolic function in patients with beta-thalassemia major and healthy controls.

It is well known that in the evolution of TCMP, a disturbance in diastolic function occurs initially, and systolic dysfunction with a decrease in EF is already the final stage of heart damage. Several authors have investigated the early disturbances in diastolic function in young patients and children with BTM in an attempt to detect the initial changes. However, some controversies still exist, specifically in patients with BTM, due to the initially altered hemodynamics of the disease, as well as the young age and pronounced left ventricular relaxation.

We found no significant differences in **the ratio of fast (E) to slow (A) ventricular filling (E/A)** when measuring transmitral blood flow in BTM patients and healthy controls. Mean values in patients and controls were 1.94 ± 0.36 and 1.98 ± 0.37, respectively, with not a significant difference ($p= 0.678$) (table 6, fig. 22). The measured ratio is within the reference range.

Parameter	*Group*	*n (%)*	*Average value*	*±SD*	*p-value*
E/A	Patients	27	1,94	0,36	p=0,678
	Controls	51	1,98	0,37	
DecT (msec)	Patients	27	145,48	30	p=0,293
	Controls	51	154,67	39,40	
s'(cm/sec)	Patients	27	8,41	1,248	**p=0,020**
	Controls	51	7,61	1,484	
e` sept. (cm/sec)	Patients	27	13,11	1,81	p=0,919
	Controls	51	13,16	1,91	
e` lat. (cm/sec)	Patients	27	17,74	3,02	p=0,654
	Controls	51	17,43	2,82	
E/e`	Patients	27	6,69	1,21	**p=0,001**
	Controls	51	5,76	1,01	

Table 6. Comparative results of parameters of diastolic function in patients and healthy controls measured by pulse and tissue Doppler.

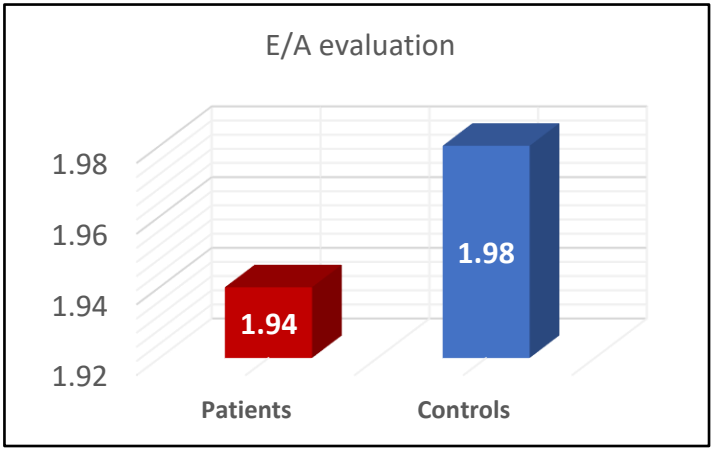

Fig. 22. E/A ratio of transmitral blood flow in BTM patients and healthy controls.

The established **deceleration time** in our patients with BTM was 145.48 msec (SD ± 30) and in healthy controls 154.67 msec (SD ± 39.40), with no significant difference (p=0.293) (table 6, fig. 23).

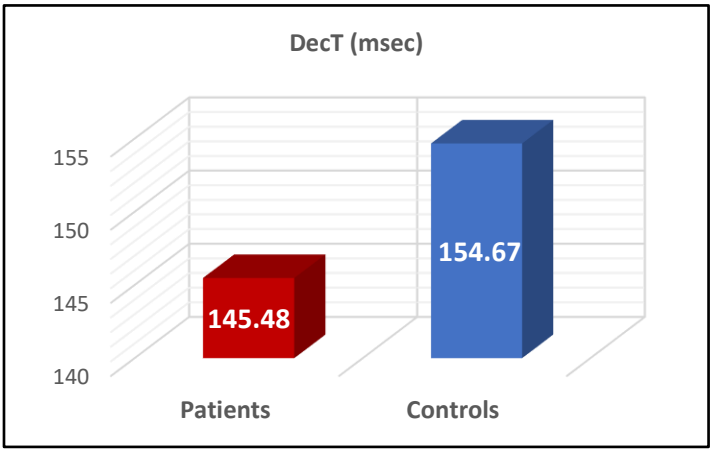

Fig. 23. Comparative presentation of deceleration time in the measurement of transmitral blood flow in BTM patients and healthy controls.

When measuring **systolic myocardial velocities (s')** by tissue Doppler in our study, we obtained the following results. The mean systolic tissue myocardial velocities in the BTM patients were 8.41 cm/sec (SD ± 1.248) and for the controls, respectively 7.61 cm/sec (SD ± 1.484), and this difference was statistically significant with p=0.020 (table 6, fig. 24).

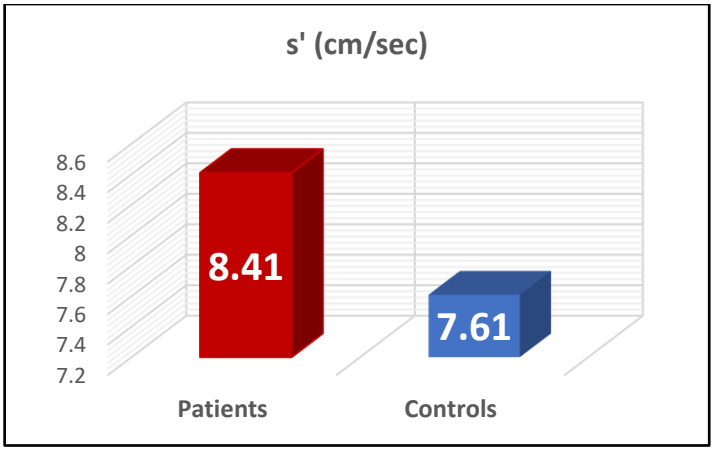

Fig. 24. Comparison of mean systolic tissue myocardial velocities in BTM patients and healthy controls.

Measurement of early diastolic myocardial velocity in the medial segment of the mitral annulus (**e' septum**) showed a non-significant difference with p=0.919 between BTM patients (13.11 ± 1.81) and healthy controls (13.16 ± 1.91) (table 6, fig. 25). Only in 2 patients (7.41%) was registered a pathological decrease in tissue diastolic myocardial velocities.

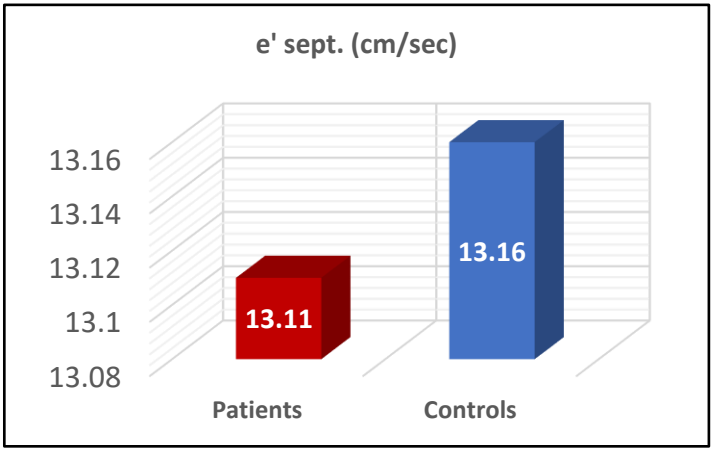

Fig. 25. Tissue velocities in the medial mitral annulus in BTM patients and healthy controls.

Measurement of early diastolic myocardial velocity in the lateral segment of the mitral valve annulus (**e' lateral**) also showed a non-significant difference with p=0.654 between BTM patients (17.74 ± 3.02) and healthy controls (17.43 ± 2.82) (table 6, fig. 26). Again, in the same two patients (7.4%), a pathological decrease in tissue diastolic myocardial velocities was registered in the lateral mitral segment.

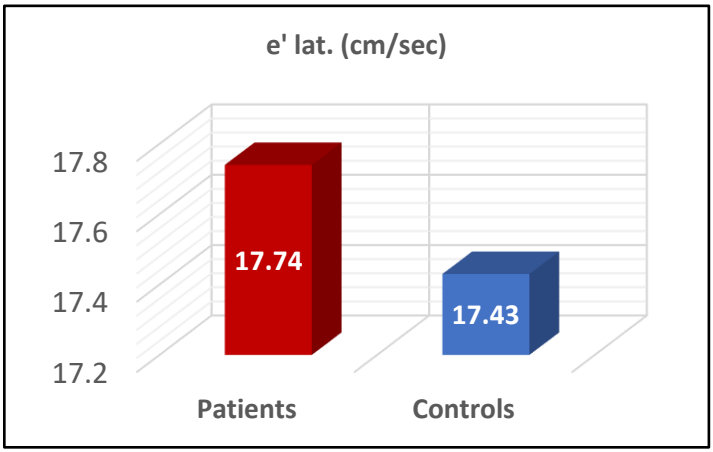

Fig. 26. Tissue velocities in the lateral mitral annulus in BTM patients and healthy controls.

When calculating the mean values of the **E/e' ratio** in patients with BTM and healthy controls, a significant difference was found with **p=0.001** (6.69 ± 1.21 vs. 5.76 ± 1.01) (table 6, fig. 27, fig. 28). Our patients demonstrated higher values of the E/e' ratio, which is indicative of LV filling pressure, compared to healthy controls. But only in 3 patients (11%) a pathologically increased E/e' ratio was registered (fig. 29). In contrast, none of the healthy controls showed evidence of elevated E/e'.

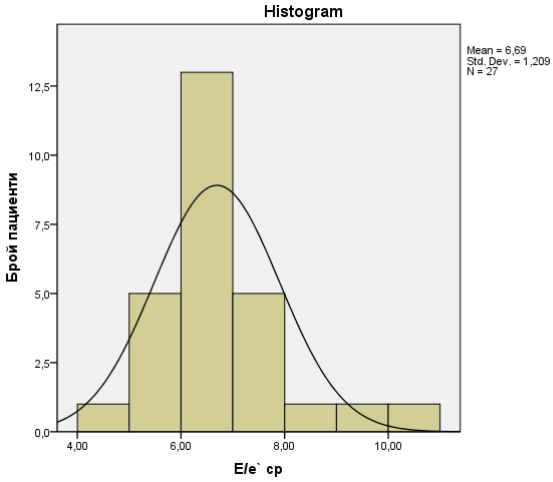

Fig. 27. Schematic presentation of mean values of the E/e' ratio among patients with beta-thalassemia major.

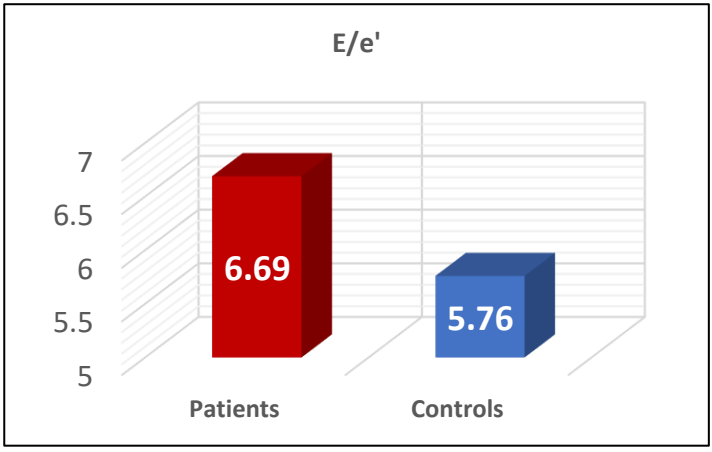

Fig. 28. Comparison of mean E/e' values in BTM patients and healthy controls.

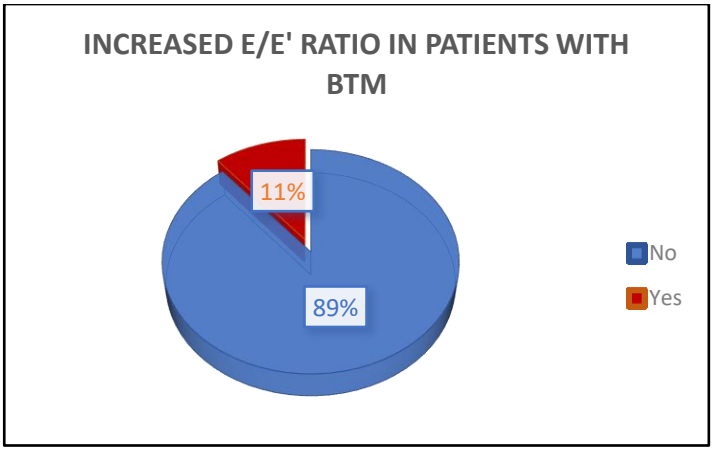

Fig. 29. Schematic presentation of patients with BTM with increased E/e' ratio.

Similar to our results, in terms of E/A ratio have been reported by many other authors, and most of them also not finding a significant difference compared to healthy controls (*Kremastinos et al, 1993; Iarussi et al, 2003; Ucar et al, 2009; Garadah et al, 2010; Abdelmoktader et al, 2013*). Iarussi et al. (2003) investigated the characteristics of transmitral blood flow in their patients before blood transfusion and after blood transfusion. They found that before hemotransfusion, the fast (E) and slow left ventricular filling (A) velocities were accelerated compared to healthy controls, while after hemotransfusion no difference was reported. Thus, the authors confirm that the changes in transmitral blood flow are due to hemodynamic state of increased preload rather than to an impairment in left ventricular relaxation.

We found that our results differ substantially from those obtained in the first study of diastolic function in young BTM patients reported by Spirito et al. (1990). They reported mean E/A values of 2.7 ± 0.7, which was significantly greater than in healthy subjects, and the authors interpreted this as early diastolic dysfunction. Because Spirito et al.'s patient cohort is very similar in age to our patients, we further analysed the results attempting to explain the discrepancies. Transmitral blood flow and the E/A ratio depend largely on hemodynamic characteristics, left atrial diastolic pressure, and left ventricular relaxation. In our patients, the mean heart rate was 10 beats per minute higher, compared to

the patients of Spirito et al., respectively: 83.5 ± 11.24/min and 73 ± 11/min, and the mean BP was lower: 103.7/66.56 mmHg vs 109/69 mmHg. This shows that the hemodynamic changes with hyperdynamic circulation are probably more pronounced in our patients compared to Spirito et al. and does not explain the existing differences.

Shortening of DecT is associated with severe diastolic dysfunction and high left ventricular filling pressure. Given the reference transmitral blood flow velocities (E/A ratio), regardless of the tendency for DecT shortening in our BTM patients, we could not interpret it as pathological. Similar to our results were reported by other authors who examined echocardiographically cardiac function in children and young patients with beta-thalassemia major: Iarussi et al. (2002) – mean DecT 144 ± 40 msec., Abdelmoktader et al. (2013) – 122 ± 27 msec. and Garadah et al. (2010) – 170.83 ± 20.023 msec. Most of them also found no significant difference compared to healthy controls.

Significantly shortened DecT, was reported by Spirito et al. (1990), Ucar et al. (2009), and Kremastinos et al. (1993). According to Spirito et al., the change in mitral blood flow characteristics is due to initial disturbances in cardiac function before occurrences of impaired systolic function due to iron overload. In contrast, Kremastinos et al. (1993) interpreted their results as a consequence of the hyper-flow condition.

The results of transmitral blood flow characteristics in our patients showed a normal left ventricular filling pattern, with no evidence of impaired left ventricular relaxation. But, as it is well known, the assessment of diastolic function based only on transmitral blood flow is insufficiently informative.

We found no decrease in tissue systolic myocardial velocities in BTM patients, but even a significant increase compared to healthy controls. However, measured tissue systolic myocardial velocities in our patients were within the reference range, and this is not a surprise, given preserved systolic function. Similar to our results reported Ragab et al. (2015), Ucar et al. (2009), and Abdelmoktader et al. (2013), respectively, systolic myocardial velocities of: 7.90 ± 0.87 cm/sec; 9.53 ± 1.59 cm/sec; 7.3 ± 2.2 cm/sec (69,70,143). Unlike us, they did not find significant differences with their healthy controls except

for Abdelmoktader et al., who reported significantly lower systolic myocardial velocities in their BTM patients compared to healthy subjects. Comparing, however, the absolute mean values indexed to body surface area according to the current recommendations of Choi et al. from 2016, the results presented by Abdelmoktader et al. are within the reference range for age.

Iarussi et al. (2003) measured, by tissue Doppler, the myocardial velocities of their patients before hemotransfusion and after hemotransfusion. They found no differences in systolic velocities pre- and post-transfusion with values of 10 ± 2 cm/sec, respectively. This again confirms that myocardial tissue velocities are independent of LV preload, which is particularly important for assessment of cardiac function in BTM patients.

Significantly lower, compared to our systolic tissue velocities were reported by Garadah et al. (2010) – 4.82 ± 1.2 cm/sec. Given that the patient cohort was very similar in age to ours (15.7 ± 8.9 years and 15.15 ± 5.83 years, respectively), we tried to find a plausible explanation for these differences. Their patients received regular hemotransfusions every 3 weeks, but chelation therapy was with Desferrioxamine by 8-12-hour subcutaneous infusions at least once a year, suggesting an increased risk of myocardial iron overload. In addition, the authors also found a restrictive pattern of left ventricular diastolic filling, which makes plausible the decrease in systolic myocardial velocities in their patients, in contrast to ours.

The velocity of the e' wave is relatively independent of preload and afterload and objectively reflects LV relaxation in early diastole. The lateral e' wave has higher normal values than the septal e' wave due to the influence of the right ventricle on the interventricular septum (*Vitlianova et al. 2018*). In most of our patients with BTM, we found normal values of early diastolic myocardial velocities. Patients in whom a decrease in velocities was registered also demonstrated disorders in other parameters of diastolic function. The first patient had pathologically low GLS (-15.3%), increased E/e' ratio (8.66), as well as increased LAVi (43.8). In the second patient, an increased E/e' (10.41) was registered, as well as an increased LA, respectively LAVi – 53.77. Similar to our results, without significant differences compared to healthy controls, were also reported by other authors - Iarussi et al. (2003) and Ucar et al. (2009),

respectively with values: 12 ± 5 cm/sec and 14.48 ± 2.18 cm/sec (for the medial segments), 20.66 ± 3.01 cm/sec (for the lateral segment). Significantly lower early diastolic myocardial velocities were recorded by Garadah et al. (2010) (4.31 ± 2.7 cm/sec for the medial segment), Abdelmoktader et al. (2013) (8.1 ± 3.3 cm/sec for the medial segment and 9.1 ± 5.4 cm/sec for the lateral segment) and Ragab et al.(2015) (11.5 ± 2.2 cm/sec for the medial segment and 12.9 ± 1.85 cm/sec for the lateral segment). The authors commented on these results as a marker of early diastolic dysfunction in their patients.

Ragab et al. (2015) and Ucar et al. (2009) reported similar to our E/e' ratio results, 8 ± 2.09 and 5.88 ± 1.26, respectively, but with no significant difference compared to healthy subjects. In comparison, Garadah et al. (2010) reported a pathologically increased ratio (15.027 ± 2.291) that was also significantly higher in their patients than in healthy subjects, the main reason being, according to them, a decrease in tissue diastolic myocardial velocities. Furthermore, the authors found that the E/e' ratio had a positive correlation with serum ferritin levels.

Summary of the results of diastolic function in patients with BTM

According to the recommendations of the European Association of Cardiovascular Imaging and the American Society of Echocardiography, the assessment of diastolic cardiac function is performed based on several indicators: tissue early diastolic myocardial velocities in the medial and lateral mitral annulus, the E/e' ratio, indexed to body surface left atrial volume (LAVi), deceleration time, tricuspid regurgitation jet velocity. To define an impaired function, more than half of the parameters need to be out of the reference values. In our patients, we found the following results regarding diastolic function assessed by pulse and tissue Doppler:

- The E/A ratio in all patients is within normal values.
- Deceleration time is also within reference range regardless of the tendency to shorten.
- We did not detect a pathological decrease in tissue systolic myocardial velocities.
- We did not find a pathological decrease in tissue diastolic myocardial velocities, except for 2 patients.

- E/e' ratio in BTM patients is significantly increased compared to healthy controls.

In conclusion, the results of transmitral blood flow parameters measured by pulsed Doppler showed a normal left ventricular filling pattern, with no evidence of impaired left ventricular relaxation. From tissue Doppler measurements a trend towards an increase in left ventricular end-diastolic pressure was observed. **Although our results do not cover all the criteria for diastolic dysfunction, the increased ratio of indexed left atrial volume to body surface area (LAVi) in 56% of our patients, as well as the tendency of rising in left ventricular end-diastolic pressure, indicate initial changes in diastolic function with an increased risk of impairment in the future.**

5. Ferritin testing in patients with beta-thalassemia major.

The mean values of serum ferritin in BTM patients were 1461.37 ng/ml (SD ± 876.4 ng/ml). The maximum measured value was 3991 ng/ml, and the minimum was 611 ng/ml, respectively (fig. 30).

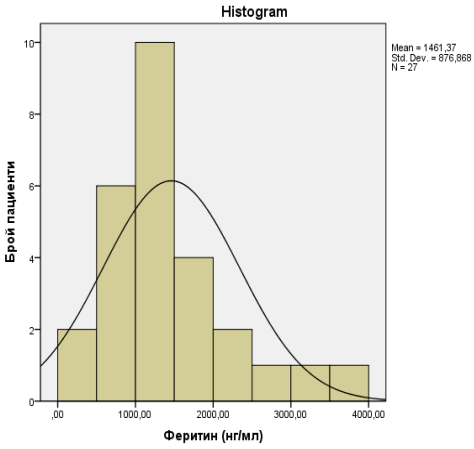

Fig. 30. Histogram presenting the distribution of ferritin values in BTM patients.

Although it is known, that serum ferritin levels do not correlate with myocardial iron deposition (*Anderson et al, 2001; Pepe et al, 2017, Demirkan*

et al, 2020), several studies have reported an increased risk of cardiac complications at high levels of ferritin in the blood (*Olivieri et al, 1994; Borgna-Pignatti et al, 2004*). When interpreting and comparing its values among patients from different studies, it is necessary to take into account the factors that determine its elevated levels: the frequency of hemotransfusions, the type of chelation therapy performed, and last but not least, the cooperation of the patients.

A retrospective study by Olivieri et al. from 1994 demonstrated a better prognosis regarding cardiac complications in patients with BTM and serum ferritin < 2500 ng/ml. The fact that the examined patients were born in the period 1954-1975 cannot be ignored, and their chelation therapy consisted of 10-12 hourly subcutaneous infusions of Deferoxamine that the patients administered themselves. This is emphasized given the inevitably arising doubt about the collaboration of the patients. Ten years later, Borgna-Pignatti et al. confirmed the prognostic value of serum ferritin at cut-off values of 2500 ng/ml, but according to their data, total ferritin was prognostic even at values > 1000 ng/ml. In addition, a proportion of patients with BTM were switched to an oral chelator - Deferiprone, due to the lack of cooperation or the toxic effect of Deferoxamine. Held in 2015, a large multiparametric MRI study only in children with BTM determined upper limit values of serum ferritin > 2000 ng/ml, above which the risk of cardiac complications increases (*Casale et al, 2015*). More than half of the studied children were on oral chelation therapy with Deferiprone and Deferasirox. This information is useful for the pediatric population, among whom the performance of cardiac MRI for the assessment and monitoring of myocardial iron overload is complicated by the young age of the patients, the lack of collaboration, as well as technical and financial reasons.

Interestingly, the mean ferritin values of our patients are very close to the group of patients of Casale et al., who were without myocardial iron overload confirmed by MRI T2* - 1461 ng/ml versus 1459 ng/ml. Aessopos et al. (2004) studied older patients (27.3 ± 6.3 years) and found that ferritin values in the group with marked cardiac damage were lower compared to those without cardiac involvement (1699 ± 298 ng /ml vs 1866 ± 518 ng/ml), and all of the patients had good compliance to the treatment. Thus, the authors concluded serum ferritin does not correlate with left ventricular contractility, and the

values differences may be due to the fact that ferritin is also an acute-phase protein. Nevertheless, it is an indisputable fact that elevated ferritin levels in the human body will inevitably affect the development of secondary hemochromatosis.

Significantly higher ferritin values and their association with cardiac function were reported by El-Shanshory et al., who studied 140 children with BTM in Egypt (*El-Shanshory et al, 2020*). Mean ferritin levels were 4657.12 ± 6835 ng/ml, with 32% of children having myocardial iron overload and 20% having severe overload and T2* < 10 msec. Even patients without evidence of iron overload had ferritin values > 2000 ng/ml (2245.17 ± 1401.89 ng/ml). The children were hemotransfused every 2 or 4 weeks, and Deferasirox was the main chelator given alone or in combination with Deferoxamine. It is likely that other factors, such as patient cooperation, are relevant to the effect of treatment in their patients.

High range of serum ferritin weas also reported in a Bulgarian study among children with BTM by Dr. I. Chakarov in 2011. The mean reported values were 2579.49 ± 1521.75 ng/ml, regardless of whether patients used modern chelation therapy with the three main types of chelators – Desferal, Exjade, Ferriprox, alone or in combinations. One of the reasons, according to the author, is the suboptimal chelation in many of patients. The logical consequence is also not to be neglected - 29% of the children had an EF of LV < 65%.

Taking into account the above data, we believe that our results of examining ferritin levels among our BTM patients are not only indicative of adequate treatment, but are also a predictor of reduced cardiovascular risk in the future.

6. Analysis of microRNAs in patients with beta-thalassemia major and healthy controls.

The study of specific microRNAs was performed in all BTM patients and in 23 healthy controls. 5 types of microRNAs associated with vascular damage, cardiac fibrosis, and cardiac remodelling were investigated. Due to the

experimental nature of the study and the lack of sufficient scientific information worldwide regarding the normal expressions of microRNAs in human serum, the determination of reference values is not possible at this stage.

When examining **miRNA-1 (RQ has-miR-1-3p)** in our study, only 7 of the patients (25.9%) were found to express it, with a mean value of 2.17 (SD ± 6.99). In healthy controls, it was expressed in 9 of the subjects (mean value 0.48 ± 1.11). The difference between the groups is not insignificant - p=0.21 (table 7, fig. 31).

Micro RNA	***Group***	***n***	***MicroRNA expression***	***Average value***	***SD***	***P Value***
RQ has-**miR-1-3p**	Patients	27	7	2,1720	6,99201	p=0,2105
	Controls	23	9	0,4788	1,10826	
RQ has-**miR-21-5p**	Patients	27	27	1,2599	0,64147	p=0,3635
	Controls	23	23	1,1000	0,62009	
RQ has-**miR-29b-3p**	Patients	27	27	1,1368	0,77123	p=0,4307
	Controls	23	23	2,0307	3,39450	
RQ has-**miR-30a-5p**	Patients	27	27	1,0148	0,66806	**p=0,0298**
	Controls	23	23	1,5563	0,99742	
RQ has-**miR-150-5p**	Patients	27	27	42,0431	30,03246	**P<0,0001**
	Controls	23	23	13,7688	24,04979	

Table 7. Analysis of microRNAs in BTM patients and healthy controls.

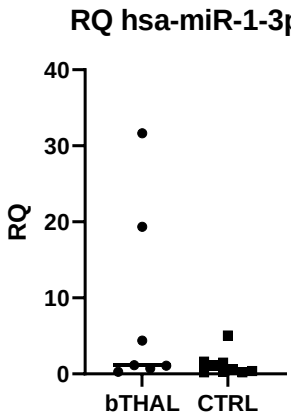

Fig. 31. Schematic presentation of RQ has-miR-1-3p expression in BTM patients and healthy controls.

MicroRNA-21 (RQ has-miR-21-5p) was detected in the blood of all subjects, with mean values in BTM patients and healthy controls being, respectively: 1.2599 ± 0.64 vs. 1.10 ± 0. 62. No significant difference was found between the groups (p=0.3635) (table 8, fig. 32).

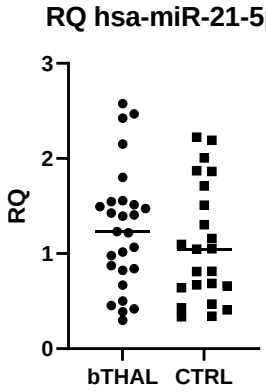

Fig. 32. Schematic presentation of RQ has-miR-21-5p expression in BTM patients and healthy controls.

Expression of **miRNA-29 (RQ has-miR-29b-3p)** was found in all BTM patients and healthy controls examined in our study. The difference between the mean values in BTM patients and controls was found to be insignificant with p=0.4307 (table 7, fig. 33).

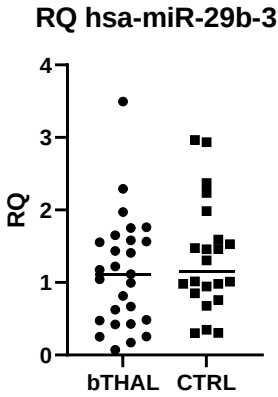

Fig. 33. Schematic presentation of RQ has-miR-29b-3p expression in BTM patients and healthy controls.

Examination of **miRNA-30 (RQ has-miR-30a-5p)** revealed that it was expressed in the blood of all subjects in our study. In BTM patients, the amount of RQ has-miR-30a-5p was significantly lower (mean value 1.0148 ± 0.66) compared to healthy controls (1.55563 ± 0.99). **The difference between the groups is statistically significant with p=0.0298** (table 7, fig. 34).

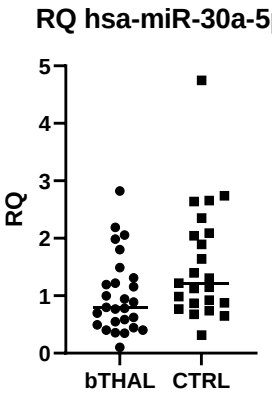

Fig. 34. Schematic presentation of RQ has-miR-30a-5p expression in BTM patients and healthy controls.

In all of the subjects we studied, **microRNA-150 (RQ has-miR-150-5p)** was expressed. For patients with BTM, the mean values were 42.0431 ± 30.032, **significantly higher compared to healthy controls**, respectively 13.7688 ± 24.049 ($p<0.0001$) (table 7, fig. 35).

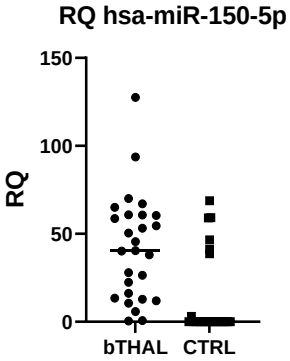

Fig. 35. Schematic presentation of RQ has-miR-150-5p expression in BTM patients and healthy controls.

As already mentioned, it is essential to distinguish disease-specific microRNAs from those with altered expression following cardiac injury, with

the potential to become future markers. Thanks to the recently published data on the expression of all types of microRNAs in children and adults with BTM, it is clear that **RQ has-miR-1-3p** is not specific to the disease itself and, without complications, is not detected in the serum of patients (*Wang et al, 2021; Das et al, 2021*). This prompted us to search international and worldwide online databases in an attempt to find other reports or publications on this specific microRNA, specifically in BTM patients. At present, we were unable to find information on whether RQ has-miR-1-3p has been studied in BTM patients.

Since the concentration of RQ has-miR-1-3p is increased under myocardial stress, the fact that it is not expressed in all BTM patients may be explained by the absence of acute cardiac injury at the time of the study in patients as well as in healthy controls. The presence of RQ has-miR-1-3p necessitated a more in-depth analysis of the patients in which it was expressed. Characteristics of patients with BTM in which increased expression of RQ has-miR-1-3p was found:

- a patient with a severe degree of iron deposition in the myocardium, respectively MRI T2* 8.14 msec and lower-limit GLS values (-18.66%).
- a patient with a lower-limit value of the ejection fraction (EF 59%) and increased LA.
- 4 patients with GLS below 19.5%.
- 4 patients with increased LA sizes (increased LAVi).

The results show that, although not significantly elevated, the expression of RQ has-miR-1-3p in BTM patients may be associated with pre-existing cardiac damage as well as to predict future deterioration in cardiac function. In 2021, Badacz et al. investigated the potential role of certain microRNAs as a risk factor for cardiovascular events. Their study included 142 adult patients who had experienced an acute myocardial infarction or ischemic stroke. They found increased expression of miR-1-3p and, according to the authors, this specific microRNA may be associated with an increased risk of future cardiovascular events. Of course, larger clinical trials involving more patients are needed to confirm this.

RQ has-miR-21-5p is also one of the microRNAs whose expression has not been documented in BTM patients (*Wang et al, 2021*). According to some

publications, the increased concentration is associated with the activation of fibroblasts and subsequent cardiac fibrosis. On the other hand, a 2019 study by Moghaddam et al. on the cardioprotective role of microRNAs found that the increased expression of RQ has-miR-21-5p suggests a cardioprotective effect by stimulating cardiomyocyte proliferation, suppressing cardiomyocyte apoptosis, and reducing cardiac fibrosis, improving cardiac function after acute myocardial infarction. As it appears the study of microRNAs is still an experimental process, and there is a lack of clarity regarding their specific function.

Similar to RQ has-miR-1-3p, we were unable to find information in the literature on whether RQ has-miR-21-5p has been studied in BTM patients. Although there was no statistically significant difference, the measured mean values of RQ has-miR-21-5p in BTM patients in our study were higher compared to those in healthy controls. Due to the lack of sufficient information in the scientific literature, we could not correctly interpret the results.

Although we found no significant difference, mean **RQ has-miR-29b-3p** values in BTM patients were lower in comparison to healthy controls. RQ has-miR-29b-3p is also widely distributed in cardiac fibroblasts and is involved in the processes of cardiac remodelling and fibrosis (*Tijsen et al, 2012*). But, in contrast to RQ has-miR-21-5p, the decreased expression of RQ has-miR-29b-3p contributes to the development of cardiac fibrosis (*van Rooji et al, 2008*).

The colleagues of Wang et al. (2021) investigated all types of microRNAs, that are expressed in children and adult BTM patients and reported an increased amount of RQ has-miR-29b-3p in both children and adult patients. The increased amount of RQ has-miR-29b-3p in the serum of their patients is more likely to be due to the underlying disease, BTM. But in the study by Wang et al. there is no assessment of cardiovascular status in BTM patients and controls, so no comparison of their cardiac function could be made. In contrast, in our study, we can compare the obtained results for RQ has-miR-29b-3p and cardiac function in BTM patients. Assessed by conventional echocardiographic methods, systolic and diastolic cardiac function in our patients did not demonstrate significant disturbances at this stage. The impossibility of comparing the obtained results for this microRNA with the "gold standard" for

cardiac fibrosis assessment - MRI of the heart - was considered a shortcoming. The conclusion is that, although not significant, the decreased expression of RQ has-miR-29b-3p in our patients may be a risk for the development of cardiac fibrosis, and regular monitoring of cardiac function in these patients is necessary.

Analysing the currently available scientific data, we found that **RQ has-miR-30a-5p** is not specific for BTM disease and that its expression is not increased in healthy people. Different microRNA subtypes of the microRNA-30 family are widely distributed in cardiomyocytes. Duisters et al. (2009) demonstrate that microRNA-30 (miR-30) is involved in pathological cardiac remodelling and fibrosis by directly targeting values of connective tissue growth factor. Decreased expression of miR-30 stimulates the production of connective tissue growth factor, thereby contributing to increased cardiac fibrosis. I.e. miR-30 levels are significantly lower in the pathological cardiac hypertrophy leading to HF. Furthermore, Duisters et al. emphasized that the decrease in miR-30 was observed very early in the developing of left ventricular hypertrophy, before the appearance of clinical symptoms. On the other hand, increased expression of miR-30c downregulates levels of connective tissue growth factor and suppresses collagen production accordingly. Yang and Zhao (2022) also confirmed the role of miR-30 in cardiac fibrosis. They found that increased expression of miR-30a-5p suppressed proliferation and collagen formation in cardiac fibroblasts in diabetic cardiomyopathy. Maciejak et al. (2018) found an association between miR-30a-5p and miR-150-5p and left ventricular dysfunction. They investigated the prognostic role of specific microRNAs in adult patients who had an acute myocardial infarction, following them for 6 months and considering the risk of developing HF. Interestingly increased values of miR-30a-5p and miR-150-5p, as well as miR-29b-3p were found in patients who subsequently developed heart failure.

The fact that significantly lower expression of RQ has-miR-30a-5p was found in our BTM patients compared to healthy controls warrants close monitoring of their cardiac function, given the risk of future cardiac damage.

As it appears, the involvement of specific microRNAs (RQ has-miR-21-5p, RQ has-miR-29b-3p, and RQ has-miR-30a-5p) in processes of cardiac

remodelling and fibrosis is still not fully understood, and probably there are additional factors responsible for these complex processes of heart damage. Larger clinical trials involving more patients are needed to confirm the potential applicability and reliability of microRNAs as biomarkers.

Wang et al. (2021), who investigated the expression of microRNAs in children and adults with BTM identified 4 types of microRNAs, that were expressed simultaneously in both children and adult patients. **RQ has-miR-150-5p** is one of them, along with RQ has-miR-29b-3p, which shows that these microRNAs are probably specific to the disease itself. MiR-150 has been found to have a role in normal erythropoiesis. Since it suppresses α-globin synthesis during erythrocyte differentiation and accordingly erythropoiesis, its expression is normally decreased. This means that the increased expression of miR-150 in BTM patients leads to suppression of α-globin synthesis and, accordingly, to suppression of fetal hemoglobin (HbF) synthesis. Clinical studies suggest that increasing the amount of HbF can reduce disease severity (*Saki et al, 2016*). This is how the ideas for targeted pharmacological therapy, by means of microRNAs, arise to stimulate the production of HbF. Of course, many large studies need to be conducted to achieve this goal.

It has already been mentioned that the team of Maciejac et al. (2018) found that increased expression of miR-150-5p was associated with left ventricular dysfunction. In contrast to them, the collective of Devaux et al. (2013) reported opposite results. They investigated microRNAs that play a role in left ventricular remodelling after acute myocardial infarction, focusing on mir-150. The authors found that its expression was decreased in patients with cardiac remodelling compared to those without remodelling. Furthermore, they found a correlation between miR-150 and levels of NT-pro-BNP, which is considered the gold standard in cardiac damage biomarkers. They even found that miR-150 could outperform NT-proBNP in predicting left ventricular remodelling.

There appears to be conflicting data regarding the role of microRNA-150 in left ventricular remodelling. It has been confirmed that this microRNA plays a role in the process of erythropoiesis and that its expression is increased in BTM patients. The increased amounts in our patients confirm this fact. Whether

high levels of microRNA-150 will be a harbinger of future cardiac complications could not be interpreted correctly, given the available information in scientific circles at this time.

In summary, the microRNAs study in BTM patients and healthy controls found the following:

- In the available scientific literature, we did not find a study of **RQ has-miR-1-3p** in BTM patients. Increased expression in some of our patients could be associated with future cardiovascular damage, necessitating even more strict follow-up of patients.
- Scientific information on **RQ has-miR-21-5p** in BTM patients is also scarce. Although we did not find statistical significance, in our BTM patients, we recorded a higher level of expression of this microRNA. Due to the conflicting scientific data regarding its association, both with the processes of cardiac fibrosis and with cardioprotection and suppression of cardiomyocyte apoptosis at this stage, we could not predict the effect of its increased expression in patients.
- We found decreased expression of **RQ has-miR-29b-3p** in the serum of our BTM patients. Since this microRNA is associated with the processes of pathological cardiac fibrosis, it could increase the risk of cardiac complications.
- We also found significantly lower expression of **RQ has-miR-30a-5p** in our BTM patients compared to healthy controls. This microRNA is not specific for BTM. It has a role in cardiac remodelling, and despite conflicting scientific information about its function, we would associate these results with increased cardiovascular risk.
- The significantly higher expression of **RQ has-miR-150-5p**, in our patients with BTM confirms the literature data. We could not exclude the possibility of increased cardiovascular risk because of the information about its role in cardiac dysfunction, as well.

7. Correlation analysis of some echocardiographic parameters and certain microRNAs.

7.1. RQ Has-miR-150-5p and some echocardiographic parameters.

The results of the correlation analysis are presented in table 8.

Parameter	n	Pearson correlation	Sig (2-tailed)
RQ has-miR-150-5p/LVMi	27	-0,182	0,364
RQ has-miR-150-5p/ЕФ на ЛК	27	0,126	0,530
RQ has-miR-150-5p/GLS	27	0,160	0,425
RQ has-miR-150-5p/LAVi	27	-0,440	**0,022**

Table 8. Results of Pearson's correlation analysis between RQ Has-miR-150-5p and some Echocardiographic parameters.

a) **Has-miR-150-5p and LVM/BSA**.

Both parameters are quantitative, and located on an interval statistical scale. In this regard, the Brave-Pearson correlation coefficient is considered appropriate. After applying correlation analysis, a weak feedback relationship r = -0.182 is found. The obtained correlation coefficient cannot be accepted as statistically reliable (p=0.364> α=0.05).

b) **Has-miR-150-5p and EF of the LV.**

Correlation analysis found a weak, straight relationship r = 0.126. The obtained correlation coefficient cannot be accepted as statistically reliable (p=0.530> α=0.05).

c) **Has-miR-150-5p and GLS.**

The correlation between the parameters found a weak, straight relationship r = 0.160. The obtained correlation coefficient cannot be accepted as statistically reliable (p=0.425> α=0.05).

d) **RQ Has-miR-150-5p and LAVi.**

Correlation analysis found a **moderate, inverse relationship** r = -0.440. The obtained correlation coefficient **can be accepted as statistically reliable and significant** (p=0.022< α=0.05).

e) **Has-miR-150-5p and LVM**.

Given the fact that one variable is quantitative, located on an interval scale, and the other variable is qualitative, on a nominal scale, a non-parametric correlation coefficient Eta is applicable. It can characterize the strength and direction of the relationship, but given its nature, its statistical significance cannot be tested. A weak, direct relationship was found between RQ Has-miR-150-5p and LV hypertrophy (r = 0.094) (table 9).

Directional Measures

			Value
Nominal by Interval	Eta	MiRNA150 Dependent	,094
		LV hypertrophy Dependent	1,000

Table 9. Correlation analysis between Has-miR-150-5p and left ventricular hypertrophy.

f) **Has-miR-150-5p and increased LAVi.**

A moderate, straight relationship between RQ Has-miR-150-5p and the increased indexed left atrial volume (r = 0.311) was established during the correlation (table 10).

Directional Measures

			Value
Nominal by Interval	Eta	MiRNA150 Dependent	,311
		Increased LA/BSA (ml/м^2) Dependent	1,000

Table 10. Correlation analysis between microRNA-150 and increased LAVi.

The correlation analysis performed between **RQ Has-miR-150-5p** and the echocardiographic parameters of the patients found:

- Moderate, inverse relationship between **RQ Has-miR-150-5p and LAVi**. The obtained correlation coefficient can be accepted **as statistically reliable and significant** ($p=0.022< \alpha=0.05$).
- Moderate, straight association between **RQ Has-miR-150-5p and increased LAVi**.
- A weak, direct association was found between **RQ Has-miR-150-5p and LV hypertrophy.**

7.2. RQ Has-miR-30a-5p and some Echocardiographic parameters.

The correlation between RQ has-miR-30a-5p and certain echocardiographic parameters is presented in table 11.

Parameter	n	Pearson correlation	Sig (2-tailed)
RQ has-miR-30a-5p/LVMi	27	0,125	0,535
RQ has-miR-30a-5p/ЕФ на ЛК	27	0,144	0,474
RQ has-miR-30a-5p/GLS	27	-0,088	0,661
RQ has-miR-30a-5p/LAVi	27	-0,158	0,430

Table 11. Results of Pearson's correlation analysis between RQ Has-miR-30a-5p and some Echocardiographic parameters.

a) **RQ has-miR-30a-5p** and **LVM/BSA.**

The study found a weak, straight relationship r = 0.125. The obtained correlation coefficient cannot be accepted as statistically reliable ($p=0.535> \alpha=0.05$).

b) **RQ has-miR-30a-5p** and **EF of the LV.**

The correlation performed found a weak, straight relationship with r = 0.144. The obtained correlation coefficient cannot be accepted as statistically reliable (p=0.474> α=0.05).

c) **RQ has-miR-30a-5p** and **GLS**.

Correlation analysis found a weak, inverse relationship between the parameters with r = -0.088. The obtained correlation coefficient cannot be accepted as statistically reliable (p=0.661> α=0.05).

d) **RQ has-miR-30a-5p and LAVi.**

The analysis found a weak, inverse relationship with r = -0.158. The obtained correlation coefficient cannot be accepted as statistically reliable (p=0.430> α=0.05).

e) **RQ has-miR-30a-5p and patients with increased LAVi.**

The data are presented in table 12.

Directional Measures

			Value
Nominal by Interval	Eta	MiRNA30 Dependent	,234
		Increased LAVi Dependent	1,000

Table 12. Correlation analysis between microRNA-30 and LAVi.

A **weak, straight correlation** was found between **RQ has-miR-30a-5p and increased LAVi** (r = 0.234).

f) **RQ has-miR-30a-5p and left ventricular hypertrophy**.

The data are presented in table 13.

Directional Measures

			Value
Nominal by Interval	Eta	MiRNA30 Dependent	,105
		LV hypertrophy Dependent	1,000

Table 13. Correlation analysis between RQ has-miR-30a-5p and left ventricular hypertrophy.

The correlation performed revealed a **weak, direct relationship** between **RQ has-miR-30a-5p and LV hypertrophy** (r = 0.105).

The correlation analysis performed between **RQ has-miR-30a-5p** and the echocardiographic parameters of the patients found:

- **Weak, direct association** between **RQ has-miR-30a-5p** and increased indexed left atrial volume.
- **Weak, direct association between RQ has-miR-30a-5p** and LV hypertrophy.

8. **Assessment of myocardial iron deposition in patients with beta-thalassemia major using MRI T2* technique**.

An MRI was performed on 19 of the BTM patients. In the remaining 8 patients, the study was not performed for the following reasons: age under 10 years, presence of a cochlear implant, or claustrophobia. Assessment of myocardial iron deposition is classified as follows:

- T2* > 20 msec - no iron accumulation;
- T2* 14 - 20 msec – slight degree of iron accumulation;
- T2* 10 - 14 msec – moderate degree of iron accumulation;
- T2* < 10 msec – severe degree of iron accumulation.

In 2 of the patients (10.5%), severe myocardial iron overload was detected with T2* values of 8.5 msec and 8.14 msec, respectively. No patient was found to have mild or moderate iron accumulation. The patient who had an MRI T2*

of 8.5 msec also had a low GLS (-16.65%) as well as an increased LA (LAVi 37.03). The second patient, with MRI T2* 8.14 msec, had borderline GLS (-18.66%). However, probably due to the small number of patients, we did not find a correlation between GLS and MRI T2* (fig. 36, fig. 37).

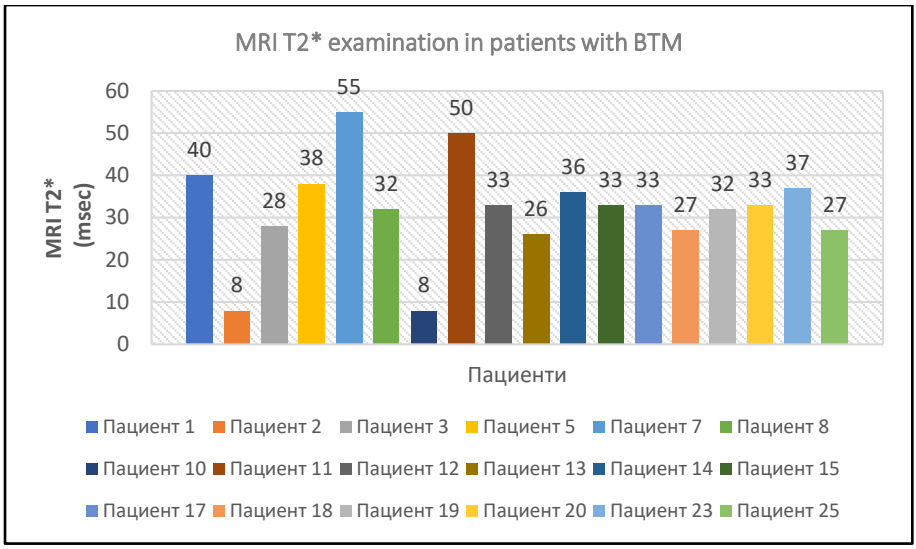

Fig. 36. Schematic presentation of the measured MRI T2 values in all of the studied BTM patients.*

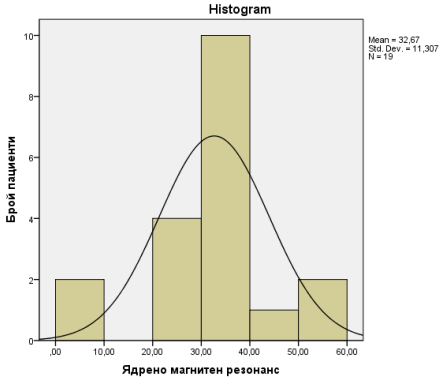

Fig. 37. Schematic presentation of mean MRI T2 values in the studied BTM patients.*

Pizzino et al. (2017) found a significant correlation (with p=0.001) between GLS and MRI T2* values. Patients with BTM, myocardial iron overload, and MRI T2* < 20 msec also demonstrated lower GLS. On the other hand, the authors did not find a positive correlation between MRI T2* and EF or diastolic function, specifically E/e'. In conclusion, the authors emphasize that MRI remains the most accurate non-invasive method for assessing iron accumulation, but GLS can identify patients with severe myocardial burden. Similar to the authors above, Parsaee et al. (2018) also found a statistically significant correlation (p<0.0001) between MRI T2* and LV GLS. A significant finding is that GLS can identify myocardial damage due to iron deposition while LV EF is still preserved, i.e. this parameter (GLS) can be used as a predictor of impairment in left ventricular function in the early stages of heart damage in patients with BTM, even with normal EF.

El-Shanshory et al. (2020) studied 140 children with BTM and found a positive correlation only between MRI T2* and the myocardial performance index assessed by tissue Doppler. They also found significantly lower global, but also circumferential and radial strain, compared to healthy controls. They also confirm that LV EF is a parameter that is impaired in the final phase of the disease. In 32% of their patients, the authors found myocardial iron overload, with 20% having MRI T2* < 10 msec (severe overload), and all children had normal EF assessed by conventional echocardiography.

9. Development of a protocol for echocardiographic examination in children and young patients with beta-thalassemia major.

Based on the obtained results and analyses, we propose the following protocol for the echocardiographic follow-up of children and young patients with beta-thalassemia major.

PROTOCOL FOR ECHOCARDIOGRAPHIC EXAMINATION IN CHILDREN WITH BETA-THALASSEMIA MAJOR

Patient's name: **Age:**

Weight: kg. **Height:** cm. **BSA** m^2

1. Morphological echocardiographic parameters:

PARAMETER	VALUE	NORM
1. LVDD (mm.)		Z-score indexed to BSA
2. LVSD (mm.)		Z-score indexed to BSA
3. LVMass (g.)		
4. LVMi (g/m^2)		$>45 g/m^2$
5. LAVi (ml/m^2)		BSA $<1\ m^2$: 31,5±5,5 ml/m^2; BSA $>1\ m^2$: 26±4,2 $ml/m^{2;}$

2. Evaluation of systolic function:

PARAMETER	VALUE	NORM
1. EF (%)		59-83%
2. GLS (%)		(-16.7%) – (-23.6%)

3. Evaluation of diastolic function.

PARAMETER	VALUE	NORM
1. E/A		
2. DecTime (cm/sec.)		
3. e' sept. (cm/sec.)		

4. e' lat. (cm/sec.)		
5. E/e'		<8 –norm; 8-14 – borderline; >14 – pathology

VIII. CONCLUSION FROM THE DISSERTATION:

1. BTM patients have a **smaller body surface** area compared to healthy controls, but the difference is not significant.

2. BTM patients have **higher heart rates and lower blood pressure values** compared to healthy controls, without the difference being significant.

3. Patients with BTM have **significantly greater left ventricular muscle mass in absolute value** as well as indexed to body surface area (LVMi).

4. Patients demonstrate **statistically significant increased indexed left atrial volume (LAVi).**

5. Children and young adults with BTM from the Center for Coagulopathies and Rare Anemias have **preserved LV systolic function assessed by ejection fraction.** Although the mean GLS values measured were within the reference range, 37% of children under 18 years and 50% of young patients over 18 years showed a trend towards values below the mean for age in the GLS measurement. In patients with low GLS, there are initial disturbances in cardiac function, as well as evidence of myocardial iron overload. In these same patients, LV EF was within normal limits.

6. Patients with BTM have no reduction in either systolic or diastolic tissue myocardial velocities. When assessing diastolic function, only **the E/e' ratio showed statistically significantly higher values** compared to healthy controls.

7. We found no correlation between GLS and MRI T2* in BTM patients, possibly due to the insufficient number of studies.

8. In the study of microRNAs in BTM patients, the following results were registered: the increased expression of RQ has-miR-1-3p in some patients; higher expression of RQ has-miR-21-5p; lower expression of RQ has-miR-29b-3p; significantly lower expression of RQ has-miR-30a-5p as well as significantly higher expression of has-miR-150-5p compared to healthy controls.

9. A moderate, inverse relationship was found only between **has-miR-150-5p and LAVi**, and between **Has-miR-150-5p and increased LAVi**. A weak, direct association was found between **RQ has-miR-30a-5p and increased LAVi,** as well as between **RQ has-miR-30a-5p and LV hypertrophy**.

IX. CONCLUSION:

In recent years, we have witnessed incredible progress in the therapy of a number of rare diseases, including beta-thalassemia major. Thanks to new medications and their easier administration, survival and life quality of patients with BTM have improved dramatically.

However, despite the undeniable progress, the leading causes of morbidity and mortality in these patients remain cardiovascular diseases. The fact cannot be ignored that in Europe, regardless of the quality of care that patients receive, about ¼ of them have evidence of myocardial iron overload. Considering the relatively short period from the appearance of clinical symptoms to the manifestations of cardiac damage, we set ourselves the task of looking for the earliest signs and manifestations of cardiac dysfunction, starting, of course, from childhood. Using modern echocardiographic techniques, as well as innovative laboratory markers, we tried to find a sufficiently sensitive, specific, maximally non-invasive, and safe way to promptly diagnose early cardiac changes, before their unfolded clinical manifestation.

Our study is a "case-control" type, covering children and young patients with BTM up to 25 years of age, who are treated at the Center for Coagulopathies and Rare Anemias at UMBAL "St. Marina" in the city of Varna.

The only study in our country, conducted by Dr. Chakarov in 2011, found that nearly 1/3 of Bulgarian children with BTM have reduced systolic left ventricular function, which is a late manifestation of thalassemic cardiopathy.

The goal we set in the current dissertation work is to determine whether there are early changes in cardiac function in young asymptomatic patients with BTM, and whether they can be established using modern echocardiographic techniques and parameters and some microRNAs compared to healthy controls that matched in gender and age to our patients.

Our result confirmed the literature data that patients with BTM have higher heart rates and lower systolic and diastolic blood pressure values. On the other hand, they have significantly greater left ventricular muscle mass in absolute value, as well as indexed to body surface area. These changes can be explained by the altered hemodynamics due to the disease itself and are expected.

At the time of the study, all our patients had no obvious cardiovascular symptoms. In our study, the currently most widely used parameter of systolic LV function, EF, demonstrated normal values. We aimed to evaluate cardiac function also through the new deformation techniques. Mean GLS values measured in BTM patients were found to be comparable to those of healthy controls, although there was a trend towards lower mean values with increasing age. In patients with low GLS, there are evidence of initial heart disorders, while at the same time EF is within normal limits. Regardless of the small number of studied patients, we can conclude that GLS, as an echocardiographic parameter, can identify early heart disorders, before the decrease in EF and the appearance of clinical symptoms, therefore we recommend its use in routine clinical practice.

Although not all criteria of the current recommendations for impaired diastolic function were covered, we observed changes in some of the parameters that favour early diastolic dysfunction. A significantly higher E/e' ratio, as well as data on increased LA, are indicative of an increase in left ventricular end-diastolic pressure. This confirms the role of tissue Doppler in the early assessment of diastolic dysfunction.

For the first time, we investigated in Bulgaria some microRNAs associated with cardiac damage in patients with BTM. Significantly lower expression of RQ has-miR-30a-5p, as well as significantly higher expression of has-miR-150-5p, could be associated with increased cardiovascular risk in the future. In support of this are the established links of these microRNAs with the increased LA and LVH. Our next task is to follow patients in the future to test this hypothesis. Since the research of microRNAs is still in the experimental phase, in order to confirm their reliability in clinical practice, it is necessary to conduct larger clinical studies covering a large number of patients.

With the current dissertation work, we were able to confirm the hypothesis that we set for ourselves. Changes in cardiac function due to the disease and iron deposition in the myocardium in patients with BTM can be observed from an early age and identified by modern echocardiographic methods including tissue Doppler and new deformation techniques. Based on these results, we recommend their use in the routine clinical follow-up of patients with beta-thalassemia major, which should begin in childhood, because prophylaxis and prevention of complications remain the best approach both for patients and for us medical professionals.

IX. CONTRIBUTIONS:

A detailed assessment of the cardiovascular status was carried out in children and young patients with a diagnosis of beta-thalassemia major, who are treated at the Center for Coagulopathies and Rare Anemias at UMBAL St. Marina in the city of Varna.

1. CONTRIBUTIONS OF ORIGINAL CHARACTER:

- For the first time in Bulgaria, new echocardiographic parameters related to myocardial deformation are used to detect early cardiac dysfunction in children and young patients with beta-thalassemia major, with the development of a protocol for echocardiographic examination.
- For the first time in our country, specific microRNAs associated with heart damage in children and young patients with beta-thalassemia major are being studied.

2. CONTRIBUTIONS OF CONFIRMATORY CHARACTER.

- We have confirmed the role of tissue Doppler in the early assessment of diastolic dysfunction in children and young patients is BTM.
- We confirmed that GLS contributes to the identification of early cardiac disorders with greater sensitivity than ejection fraction before the appearance of clinical symptoms.
- We found that BTM patients have changes in the expression of specific microRNAs, which could be future biomarkers for early cardiac damage.

Limitations of the study

As a limitation, we consider the relatively small number of BTM patients included in the present study. Beta-thalassemia major is classified as a rare disease with a frequency of <5 per 10 000 people in Europe, and 3.66 per 100 000 people in our country alone. Scientific publications and reports concerning the topic under consideration also cover small cohorts of young patients with this disease.

X. PUBLICATIONS AND CONTRIBUTIONS RELATED TO THE DISSERTATION WORK:

PUBLICATIONS:

1. Ganeva K., Shivachev P., Petrova K., Changes in cardiovascular function due to iron overload in young thalassemia major patients – what we have learned so far. New methods for early diagnosis. Pediatrics. 2021; Supplementum: 22-26.

2. Ganeva K., Micro-RNAi and cardiac health. Their role as a potential biomarker for heart disfunction. Journal of the Union of scientists - Varna. Medicine and ecology series.2021;26(1):5-9.

PARTICIPATIONS IN CONFERENCES:

1. K. Ganeva. Cardiac status in young patients with beta-thalassemia major. Modern non-invasive methods for early assessment. First

working meeting Rare benign hematological diseases. 18-20.03.22, Sliven.

2. Ganeva K, Shivachev P, Kaleva V, Preliminary results of research of cardiac status in young patients with beta thalassemia major using modern non-invasive methods, XVII National Congress of Cardiology, 2022, Albena. A poster

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