

ARTHRITIS (JIA): DIAGNOSTIC AND PROGNOSTIC VALUE – SINGLE CENTER STUDY.

Joanna Lipinska^{1,2}, Marek Kaszkowiak³, Joanna Swidrowska-Jaros¹, Elzbieta Smolewska^{1,2}

¹ Department of Pediatric Cardiology and Rheumatology, 2-nd Chair of Pediatrics, Medical University of Lodz, Poland

² Outpatient Department of Pediatric Rheumatology, Maria Konopnicka' Memorial Hospital, Lodz, Poland

³ Department of Biostatistics and Translational Medicine, Medical University of Lodz, Lodz, Poland

Correspondence to:

Joanna Lipinska MD, PhD.

Department of Pediatric Cardiology and Rheumatology

2-nd Chair of Pediatrics

Medical University of Lodz

36/50 Sporna St.

91-738 Lodz, Poland

Phone/fax: 048-42-617-77-00

e-mail: joanna-lipinska@wp.pl

ORCID: 0000-0002-4418-1718

ABSTRACT:

BACKGROUND: The goal of the study was to assess the diagnostic and prognostic value of survivin in Juvenile Idiopathic Arthritis (JIA).

METHODS: Seventy children with JIA – 59 newly diagnosed and 11 biologically treated (46 girls and 17 boys) aged 1.5-18 years and 29 healthy children as a control group, appropriately matched in terms of sex and age, were included into the study. Concentration of survivin was assessed by an ELISA test in serum and also 18 matched synovial fluid samples collected from JIA patients.

RESULTS: Children with JIA were divided according to the subtype of the JIA. In 65.7% of patients oligoarthritis was diagnosed. The disease activity was established on the basis of JADAS-27 criteria, distinguishing three levels of inflammatory process activity: low, medium and high. The largest group comprised children of low activity (62.9%) of the disease. The serum concentration of survivin was significantly higher in JIA compared to the controls ($p < 0.001$). Higher concentration of survivin correlated positively with the presence of anti-cyclic citrullinated peptide autoantibodies (ACPA) ($p = 0.001$). In all synovial fluid samples the concentration of survivin was higher than in matched serum ($p = 0.003$). Serum survivin concentration did not statistically significantly change according to radiological damage status or active synovitis assessed by joint ultrasonography. Survivin level was independent of the disease duration time and treatment with TNF- α inhibitors in DMARD's non-responders.

CONCLUSIONS: On the basis of the conducted study, it could be concluded that survivin seem to be an independent biomarker that may be helpful in the diagnosis of JIA. Nevertheless, the higher concentration of survivin is being associated with ACPA positivity, survivin can act as a unique biomarker that identifies an additional group of

patients with JIA negative for autoantibodies, even in the early stage of the disease. We have failed to confirm survivin prognostic potential for the active and destructive course of the rheumatoid process, however it is worth to underline that children have better potential for bone regeneration than adults. Survivin measurement should be considered as aiding tool identifying DMARD's non-responders.

KEY WORDS: survivin, juvenile idiopathic arthritis, disease activity, biomarkers, autoantibodies, prognosis, diagnosis, biological treatment, radiological damage

INTRODUCTION:

Juvenile idiopathic arthritis (JIA) is a heterogeneous group of diseases which encompasses all forms of arthritis of unknown aetiology lasting for at least 6 weeks and with onset before the age of 16 years [1,2]. As a result of the lack of pathognomonic features, the diagnosis of JIA is one of exclusion among all possible causes of chronic arthritis in childhood [3,4]. The International League of Associations for Rheumatology (ILAR) has defined seven subtypes of JIA [2]. While there are shared genetic and immunologic features between JIA and rheumatoid arthritis (RA) in adults, only a small subset of JIA patients with polyarticular disease and a positive rheumatoid factor (RF) clinically resemble adult RA patients [5]. Advances in our understanding of the pathogenesis of JIA over the last two decades have revolutionized therapy, reduced morbidity, and improved quality of life for those affected [3,4]. Various autoantibodies have been associated with JIA, including anti-nuclear antibodies (ANA), RF, anti-citrullinated protein autoantibodies (ACPA), and others. The ANA test is not used to diagnose JIA. However, it has important prognostic value with respect to the risk of uveitis. ANA positivity amongst the JIA subtypes is highest in patients with oligoarticular JIA (up to 70%) and is particularly more prevalent in young, female

patients. The prevalence of RF in patients with JIA is very low (<5%), it confers a worse prognosis. In particular, polyarticular patients with positive RF are at higher risk of a more aggressive disease course and bone erosion [3,4]. The identification of ACPA highly specific for adult RA was a milestone for adult rheumatology. ACPA have been shown to predict future risk for developing RA in otherwise healthy individuals. However, as with RF, the sensitivity of ACPA for detecting JIA is low, but in patients with RF presence and polyarticular JIA, these autoantibodies are highly specific and predict a more severe disease course [6]. ACPA positive children with JIA are recommend earlier and more aggressive therapy. Nevertheless, the diagnosis of JIA still depends mainly on clinical characteristics, imaging examination and exclusion of other, more common causes of persistent arthritis and with low serological support [3-6]. Therefore, it is necessary to establish alternate methods or discover new autoantibodies to further improve precise diagnosis at the early stage of the disease.

Survivin is an anti-apoptotic protein, an oncoprotein, known as a tissue marker of cancer. During recent years, the role of survivin in non-malignant cells was intensively explored. Survivin has been shown essential for differentiation, growth, and regeneration of healthy tissues. Due to survivin roles in apoptosis and proliferation, it plays important roles in pathogenesis of autoimmune diseases [7]. At preclinical phase of RA, high levels of survivin correlate with cytokines assuring formation of aggressive Th1 and Th17 cells. In patients after RA diagnosis, survivin predicts joint destructive course of the disease and resistance to anti-rheumatic treatment [8-10]. Survivin has been suggested as a predictive marker of severe course of adult RA and could be used for preclinical recognition of the disease. Survivin-positive patients have poor outcomes if treated with methotrexate (MTX) monotherapy. Serum? survivin concentration decrease is associated with better clinical response to treatment [11].

With the continuous development of medicine, the inflammatory process in JIA patients can be alleviated by regular treatment but still cannot be completely cured. Therefore, exploring the pathogenesis of rheumatoid process is of high importance for developing precise, personalized treatments, and new drug targets. The aetiology of RA is still not clear and JIA the more/especially because of the heterogeneity of the disease phenotype. Unfortunately, because there are far fewer patients with JIA than with RA, much of our knowledge about the role of RF, ACPA, and other autoantibodies in inflammatory arthritis is derived from the adult literature. This is clearly a limitation, since these studies only pertain to a small subset of JIA patients overall, specifically polyarticular children RF positive.

Taking advantage of the recent reports in adults with RA, we designed this study aiming to validate the diagnostic and prognostic utility of survivin in patients with JIA.

MATERIAL AND METHODS:

PATIENTS:

Seventy children diagnosed with JIA according to the 2001 Edmonton ILAR classification criteria [12, Petty 2001] (51 girls and 19 boys) aged 1.5-18.0 years (median 10.25 years) were included into the study. Patients with JIA biologically treated were significantly older (Me: 17.0 vs 9.0 years) and had longer disease duration time (Me: 84.0 vs 3.0 months) than the biologically naïve group with JIA. Additionally, 29 healthy children as a control group, appropriately matched in terms of sex and age, took part in the analysis. Children with JIA were divided according to the subtype of the disease. In the majority of the study group - 65.7% - oligoarthritis was diagnosed and 32.9% children have polyarticular subtype of the JIA. In the study group there was only one child with systemic disease. The type of onset was defined according to ILAR criteria (2001) [12]. The activity of the disease was established on the basis of 27-joint

Juvenile Arthritis Disease Activity Score (JADAS-27) [13]. Low, intermediate, and high stages of the disease activity have been distinguished accordingly.

Patient characteristics was presented in Table 1.

Table 1. Characteristics of the study group.

Main group of children with JIA (59/70 – 84.3%) was biologically naïve and had not been treated with disease modifying anti-rheumatic drugs (DMARDs) yet. However, most of them were occasionally taking non-steroid anti-inflammatory drugs (40/59 – 67,7%). Additionally, a group of 11/70 (15.7%) patients with JIA had been already treated at the time of inclusion into the study were recruited. All of them (11) were taking DMARDs (8 - methotrexate, 3 - methotrexate with sulfasalazine) and TNF- α inhibitors (3 - adalimumab and 8 – etanercept). Seven out of these eleven patients (7/11 – 63.6%) had history of intraarticular or systemic glucocorticoids (methylprednisolon), and all 11 were occasionally taking non-steroid anti-inflammatory drugs.

The study protocol was approved by regional Medical University of Lodz, Poland ethical committee [No RNN/58/13/KB].

At enrollment, all patients and their parents gave their written informed consent to participate in the study.

METHODS:

Serum samples were obtained simultaneously with routine laboratory examinations, including red blood cells (RBC), white blood cells (WBC), and platelets (PLT) counts, as well as erythrocyte sedimentation ratio (ESR) (cut off value <12mm/h) or C-reactive protein (CRP) (cut off value < 5mg/L). ESR was assessed by Westergren method and

CRP level using the immunoturbidimetric method. Additionally, ACPA, ANA and RF were routinely assessed using standard methods.

Synovial fluid, if available, was obtained during diagnostic or/and therapeutic puncture of the swollen joint.

Measurements of serum and synovial fluid survivin:

Blood and synovial fluid samples were centrifuged and stored at -80 °C. Concentration of survivin was assessed by a sandwich enzyme-linked immunoassay (ELISA test) in blood and 18 matched synovial fluid samples collected from swollen knees of JIA patients and in sera of children from control group by commercially available Kit (rabbit anti-human survivin; R&D, no DSV00, Lille, France). The sensitivity of the assay was 4.45 pg/ml, with the cut-off at 9.96 pg/ml. The intra-assay precision for serum – 4.5-5.5% and inter-assay precision – 5.7-9.5%. All serum and synovial fluid samples were tested twice.

Joint ultrasonography and hands' X-ray:

Affected joint ultrasonography assessment was performed at the time of JIA diagnosis, by a clinician experienced in musculoskeletal ultrasonography. Synovitis detected by ultrasonography was graded as mild, moderate, or severe (score from 0 to 3). Power Doppler ultrasonographic signal (PDUS) was scored on semiquantitative four-grade scale: 0 no signs of vascularization, 1 = mild (presence of single/vessel dots), 2 = moderate (presence of confluent vessel dots in less than half of the synovial area), and 3 = marked (presence of confluent vessel dots in more than half of the synovial area) (Table 1.) [14].

A Philips CX50 CompactXtreme ultrasound system and a 5–12 MHz linear transducer were used in this study.

Conventional plain-film radiographs of both hands and wrists of all children with JIA included into the study were obtained. The radiographs were scored using the Steinbrocker assessment method, with a global damage score to hands and wrists on a four-point scale from I (minimal damage) to IV (severe damage) as previously described (Table 1.) [15].

STATISTICAL ANALYSIS:

Continuous data were presented as median with interquartile range and categorical data were presented as number with respective percentage. The differences for continuous variables were tested with the Kruskal-Wallis ANOVA, Wilcoxon signed-rank test or U Mann-Whitney rank sum test. Nominal variables were analyzed using Chi² test or Fisher test when appropriate. Correlations analyses were performed with Spearman rank test. Additionally, receiving operating characteristic (ROC) curve was used for determining best cut-off value for the survivin concentration in JIA diagnosis. Sensitivity and specificity were calculated for selected cut-off value with <http://vassarstats.net/> online tool. The area under the curve (AUC) was calculated to evaluate the diagnostic value of survivin. All tests were two-tailed and performed at the 0.05 level of significance. The statistical analyses were carried out with the Statistica 13.1 (Tibco, Tulsa, OK, USA) and VassarStats tool (<http://vassarstats.net/>).

RESULTS:

Majority of the newly diagnosed children with JIA comprised with oligoarticular subtype of JIA (41/59 - 69.5%), whereas 18/59 (30.5%) patients represented polyarticular JIA onset type and none among newly diagnosed had systemic JIA. In whole study group, the largest cohort of children with JIA had low disease activity (44/70 - 62.9%)

established on the basis of JADAS-27 criteria. There were 17/70 (24.3%) children with JIA and medium and only 9/70 (12.9%) with high disease activity.

The concentration of survivin was significantly higher in sera of children with JIA compared to the controls (Me: 23.14 pg/ml (IQR 17.37 – 35.31) vs Me: 10.11 pg/ml (IQR 5.24 – 14.10); $p < 0.001$).

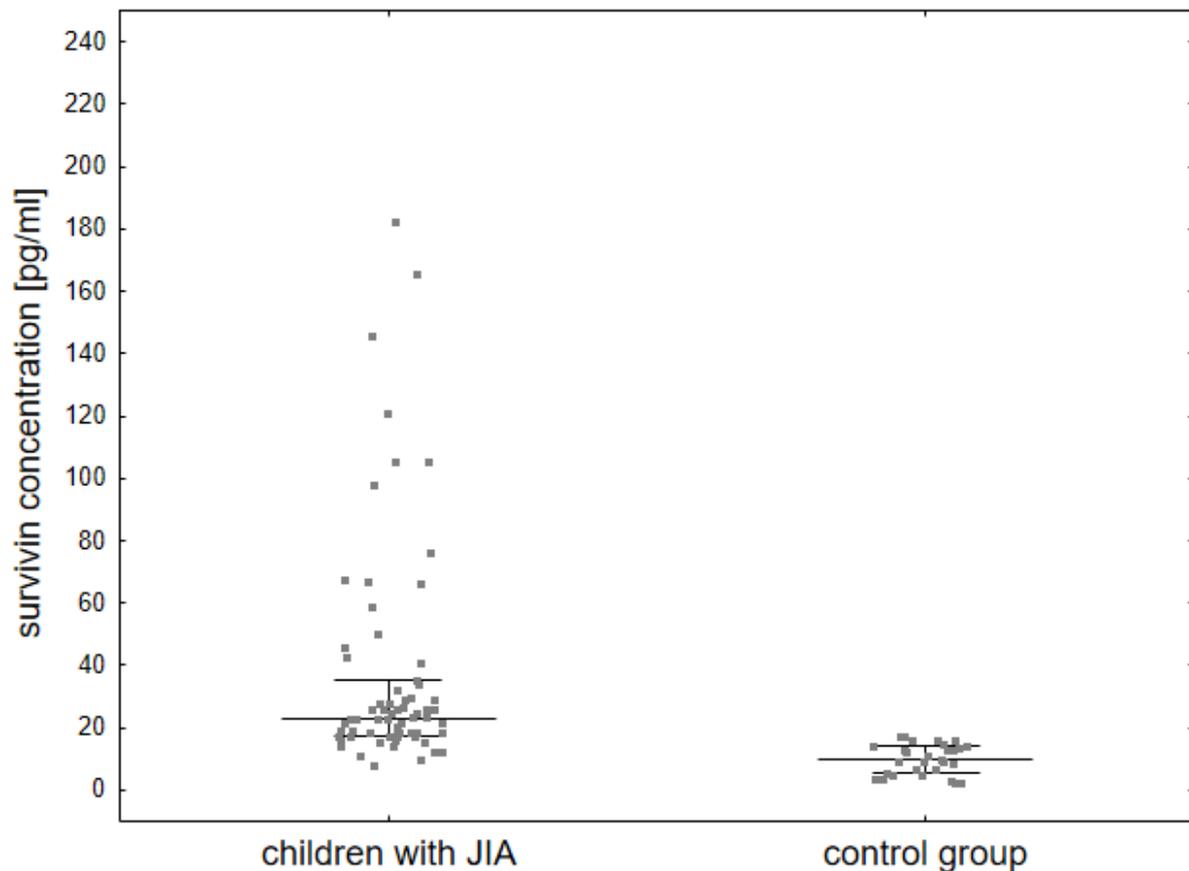


Figure 1. Serum survivin concentration (median and IQR) in children with JIA and in control group.

*Patient with value 537.82 pg/ml was not shown in the graph but contributed to median and IQR calculations.

There was no statistically significant difference in serum survivin level between children with JIA newly diagnosed comparing to children with JIA biologically treated (Me: 22.61 pg/ml (IQR 17.37 – 35.31) vs Me: 24.72 pg/ml (IQR 19.46 – 40.64); $p=0.68$).

In all but one (17/18 – 94.4%) synovial fluid samples, the concentration of survivin was higher than in matched blood (Me: 94.15 pg/ml (IQR: 54.87 – 165.91) vs 23.135 pg/ml

(IQR: 17.37 – 35.31); $p=0.003$) (Figure 2). In that girl with polyarticular JIA onset type and high disease activity and 2 months of disease duration survivin concentration in the blood was the highest from the whole study group (537.82 pg/ml), and the concentration in synovial fluid was above the median (127.2 pg/ml). Surprisingly, there was no significant difference in synovial fluid survivin level between newly diagnosed and biologically treated children with JIA (Me = 125.59 (IQR: 76.8 - 187.3) vs Me: 56.3 (IQR: 44.2 - 94.2); $p = 0.143$).

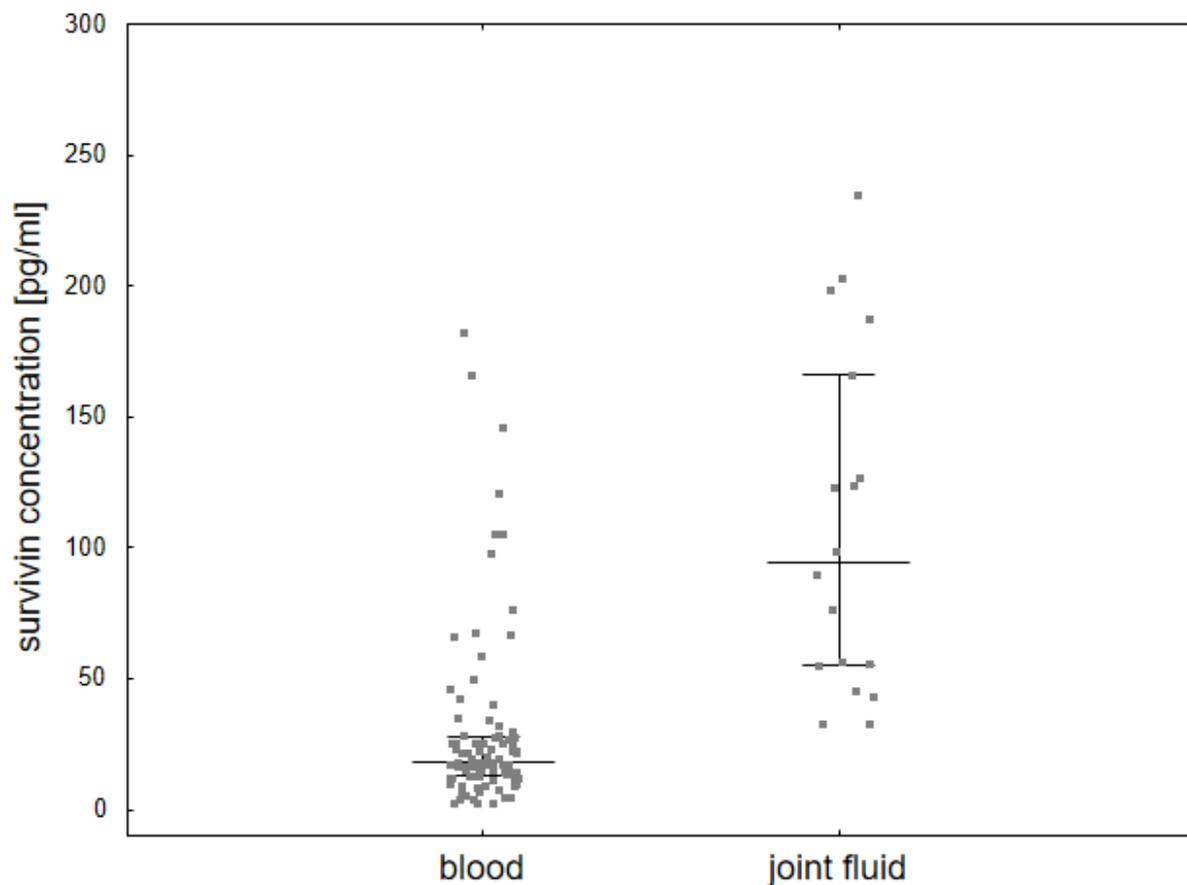


Figure 2. Serum and joint fluid survivin concentration (median and IQR) in children with JIA. Patient with in-blood value 537.82 pg/ml was not shown in the graph but contributed to median and IQR calculations

Different JIA onset types or disease duration time and disease activity according to JADAS-27 did not influenced significantly survivin concentration in blood and joint fluid. Additionally, the survivin concentration did not statistically significantly change

according to radiological damage status based on hands X-ray or active synovitis grade of the affected joints assessed by joint ultrasonography (Table 2).

Table 2. Median survivin concentration [pg/ml] according to laboratory tests and clinical characteristics in serum (a) and synovial fluid (b). *IQR – interquartile range*

Statistically higher serum survivin concentration was observed among children with JIA and presence of anti-CCP antibodies (ACPA) than in anti-CCP negative patients (Me: 66.98 pg/ml (IQR 25.77 – 105.47) vs Me: 22.61 pg/ml (IQR 17.37 – 27.89) ; $p=0.001$) (Figure 3). Nonetheless, there was no such difference between synovial fluid survivin concentration and anti-CCP (ACPA) positivity ($p = 0.1976$).

No dependence was observed of survivin concentration due to RF and ANA positivity neither in serum nor in synovial fluid of children with JIA.

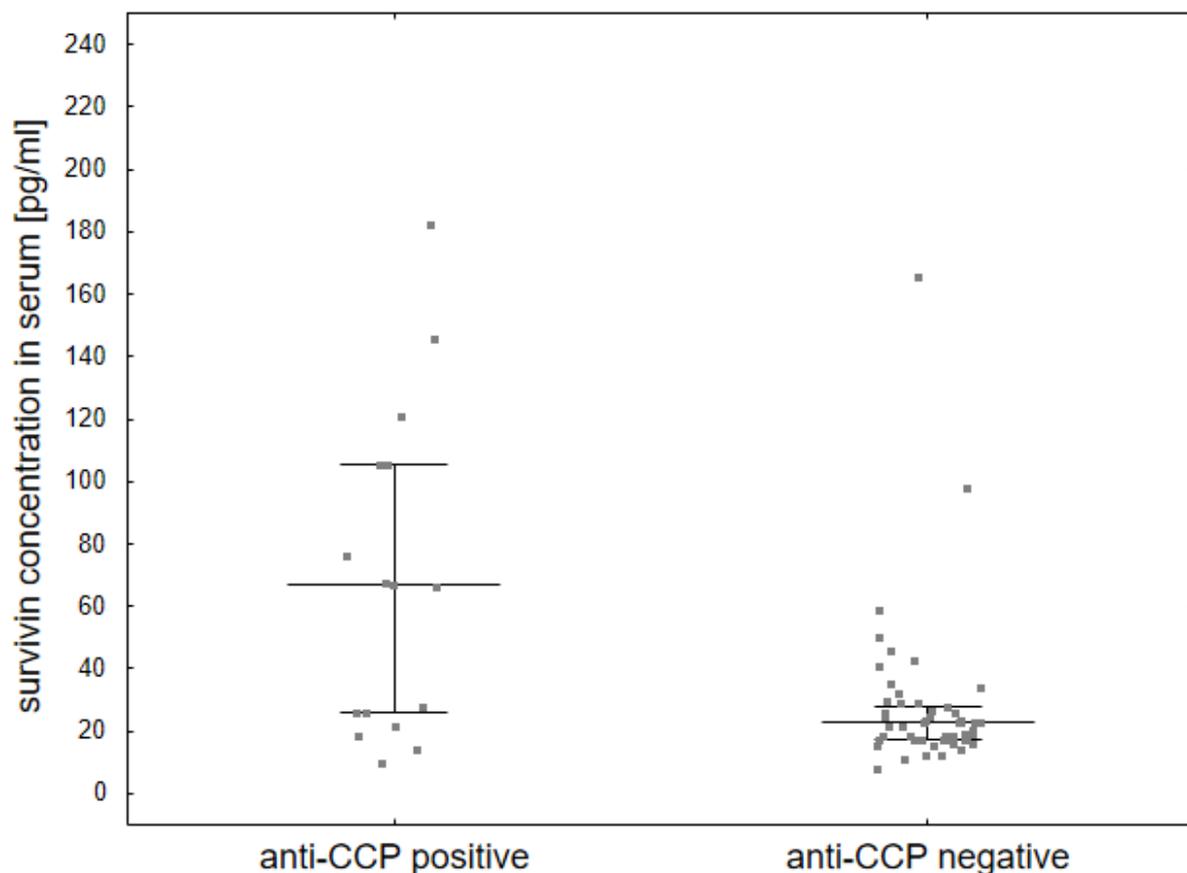


Figure 3. Median serum survivin concentration (median and IQR) according to anti-CCP (ACPA) positivity in children with JIA.

* Patient from anti-CCP (ACPA) positive group with value 537.82 pg/ml was not shown in the graph but contributed to median and IQR calculations.

According to ROC analysis the cut-off for survivin concentration was calculated at 17.37 pg/ml. The area under the curve (AUC = 0.945 (95CI: 0.905 – 0.985)) confirmed the ability of survivin to distinguish children with JIA with sensitivity of 0.843 (95CI: 0.732 – 0.915) and specificity of 0.931 (95CI: 0.758 – 0.988); Youden Index value = 0.77 (Figure 4).

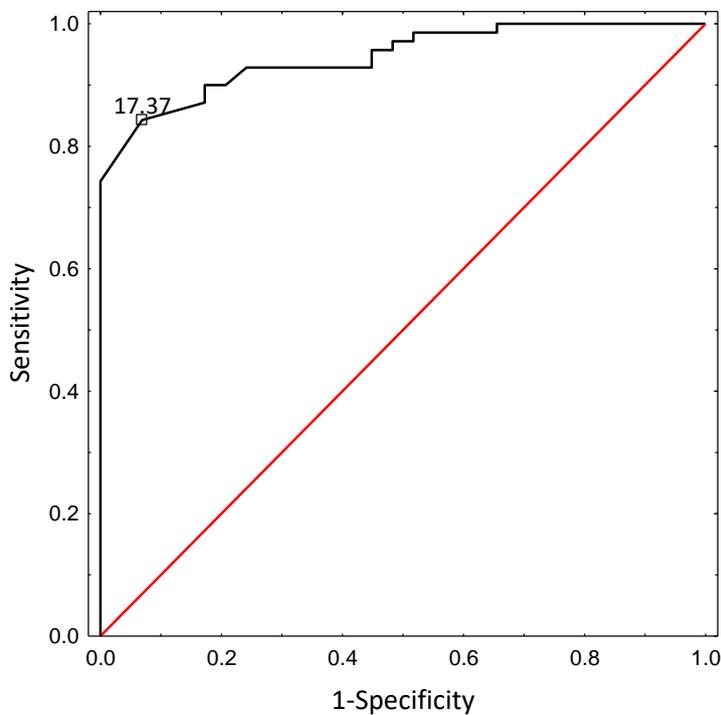


Figure 4. Receiver operator characteristics (ROC) analysis of survivin concentration in children with JIA (cut-off = 17.37 pg/ml).

According to the established cut-off value at the 17.37 pg/ml in total, 59/70 (84.3%) children with JIA were survivin positive, including almost 91% (10/11) of children with JIA, who were biologically treated. However, also 2/29 (6.9%) children from the control group were survivin positive, but the positive concentrations in their sera were just at the cut-off level (both exactly 17.37 pg/ml).

In group of survivin positive JIA children the majority (38/59; 64.4%) represents oligoarthritis, 20/59 (33.9%) - polyarthritis and only one child had systemic JIA.

There was no statistically significant difference in survivin positivity according to: gender, age, JIA onset subtype, disease duration time, disease activity, radiological damage or ultrasonographic synovitis grade (Table 3).

Table 3. Clinical characteristics of children with JIA divided by *survivin* status.

Nineteen out of 59 survivin positive children with JIA (32.2%) were also positive for RF and/or anti-CCP. Only 2 patients with positive survivin – 2 (3.4%) was recognized also by simultaneous presence of RF and anti-CCP antibodies (ACPA).

DISCUSSION:

Suppression of apoptosis has been suggested as a key mechanism supporting selection and accumulation of distinct lymphocyte subsets in chronically inflamed joints [7 - 9, 11, 16 - 18]. Indeed, at preclinical phase of RA, high levels of survivin correlate with cytokines assuring formation of aggressive Th1 and Th17 cells, that would not normally be expected to survive for a prolonged time within inflamed joints unless their death was actively inhibited. In the patients early after RA diagnosis, positivity of survivin predicts joint destructive course of the disease and resistance to anti-rheumatic treatment [7 – 9]. To our best knowledge there is only one study assessing the survivin in sera of children with JIA [19]. However, in our paper we correlate the survivin level not only with laboratory data, but also with radiological status of the affected joints and with synovitis grade evaluated by ultrasonography (PDUS) of the inflamed joint. Moreover, this is the first paper that compares survivin concentration in serum and available matched synovial fluid of children with JIA. In the current study

we demonstrate that concentration of survivin is notably increased in sera of children with JIA compared to healthy controls (23.135 vs 10.11 pg/ml; $p < 0.001$), what is in line with the previous observations in adults with RA and children with JIA [8, 16 - 20]. Additionally, we show that gender, age and different JIA onset types do not influence significantly survivin concentration in blood, as well as, in joint fluid. It has been speculated that survivin is produced and secreted locally in the inflamed joints [7, 18, 21]. Thus, we confirm results obtained in adults with RA, indicating, that there is strong positive correlation between survivin level in serum and matched synovial fluid [7, 18, 21]. Additionally, our present study demonstrates that higher survivin concentration is detected in joint fluid than in matched serum. Nevertheless, the grade of synovitis evaluated by ultrasonography (PDUS) of the inflamed joint does not influence the survivin concentration in synovial fluid of our study group. Interestingly, in our study we do not register higher survivin concentration in children with JIA and worse radiological joint destruction. This could be explained with high number of children with newly diagnosed JIA and the fact that radiological damage in children contrary to adults is rarely observed at the early stage of the JIA, as children have potential for bone regeneration [2,4,6]. Thus, we failed to confirm survivin prognostic potential for the active and destructive course of the rheumatoid process [7, 11, 21-23]. Surprisingly, we do not support the prior correlation of high survivin level with higher disease activity found in adults with RA and by Galeotti et al. in children with JIA [8, 11, 16, 17, 19]. Almost 63% of our study group consisted of children with oligoarticular JIA, mostly with low disease activity and short duration time of JIA symptoms. On the other side, it is confirmed that survivin level is irrespective of disease duration time. It has been postulated, that rheumatoid process starts years before clinical symptoms and may be identified by autoantibody measurement. Previous studies advocated survivin to

provide insight in the pre-antibody process. Some authors suggest that survivin occurs at the earlier phase of disease development followed by autoantibody production [8, 17]. Our results indicate that survivin concentration is independent of the disease duration time – could be increased in sera of newly diagnosed children at the beginning of the disease and after years, even in patients who had been biologically treated. However, some authors noticed significant decrease in survivin levels from baseline over 24th months [11]. The combination of various markers increases further risk of rheumatoid process and may assist its preclinical diagnosis. The other important finding of this study is the fact that anti-CCP (ACPA) positive children with JIA have increased level of survivin in blood comparing to ACPA negative patients, what supports previous studies in adults with RA [8]. However, other authors noticed no dependence of survivin presence due to ACPA or RF positivity in RA patients [11]. They also underlined that neither the presence of RF or ACPA, nor the combined multi-biomarker disease activity score supported discrimination in the disease outcome achieved by survivin measurements. Surprisingly, we found no significant dependence between anti-CCP antibodies positivity and higher survivin level in synovial fluid of JIA patients. At the cut off (17.37 pg/ml) established according to ROC analysis survivin positivity was found in majority of children with JIA, including all but one child biologically treated. Thus, we confirm the ability of survivin to distinguish children with JIA with sensitivity of 0.843 and of specificity 0.931. The numbers are similar to that obtained by other researchers [8, 16]. However, also two (6.9%) children from our control group were survivin positive, but the survivin concentration in their sera was just at the cut-off value (both exactly: 17.37 pg/ml). Similar percentage of the survivin positivity in the control groups received other authors - 5.2% - 6.6% [11, 19, 21]. It could be speculated that it is possible that these children in the future could present

JIA symptoms as survivin occurs before clinical manifestation, even in the pre-antibody period. Our results sustain the earlier conclusions in adults and children that: age, gender, JIA onset subtype, disease duration time and presence of RF are similar in survivin positive children with JIA, as compared with those survivin negative [11, 19, 21]. The combined presence of survivin and autoantibodies was found in surprisingly small group of our children with JIA. One third (19/59 – 32.2%) of our survivin positive study group was recognized positive for RF or ACPA and only two children were simultaneously positive for both RF as well as ACPA. These results are similar to other papers. Obtained data show increased risk for development of JIA irrespective of ACPA status. This provides further support to the hypothesis that survivin is a unique biomarker that recognize the additional group of patients with JIA negative for autoantibodies. We assess the survivin levels also in group of children with JIA biologically treated. Nevertheless, that group of patients was small (11/70 – 15.7%), it was noticed that high survivin concentration and positivity is independent of TNF- α treatment, as we observe no difference in survivin level between patients newly diagnosed and biologically treated with TNF-inhibitors, what is in line with previous observations in adults with RA [7,11,18,21]. However, our biologically treated patients with JIA were previously treated unsuccessfully with DMARDs for a long time (above one year).This findings provides further support to the hypothesis that high survivin concentration or survivin positivity is associated with poor response to DMARDs treatment, so one could ask if the obtained results are sufficient to justify the prior conclusions that survivin is the marker of DMARDs non-responders [11,23,24]. Nevertheless, the observations assessing the influence of treatment on survivin release are conflicting. In the previous studies in adults with RA it was indicated that in survivin positive methotrexate non-responders, anti-TNF treatment appeared to be

less successful than combination of synthetic disease-modifying drugs [11]. The authors speculated that poor response to anti-TNF treatment and lack of a direct correlation between serum survivin and inflammatory markers or disease activity suggested independent mechanism of survivin release. The process triggering and abrogating survivin release in RA could therefore pave a way to efficient therapeutic control of the disease [11]. On the other hand, some researchers proved that survivin concentration is decreased in TNF- α treatment responders. Some authors showed that TNF- α failed to induce survivin expression by peripheral blood mononuclear cells. In contrast, neutralization of IL-6 could reduce survivin expression – these observations support the regulatory role of survivin in pathogenesis of arthritis [7, 18, 21] suggesting that level of survivin to be a transient phenomenon in the course of RA, however the results may be affected by DMARDs treatment. Moreover, they indicate that comprehensive measurement of survivin and IL-6 could serve as a convincing biomarker of clinical response to TNF- α -therapy patients with RA [23,24]. The SWEFOT trial demonstrates that survivin is a changeable serological marker with a notable connection to disease outcome. A decrease of serum survivin levels is shown to be an overall consequence of antirheumatic treatment, an serological conversion to survivin-negative occurred in about a half of the survivin-positive patients. Thus, the monitoring of survivin levels assists in prognosis and treatment decisions for patients with early RA [11]. Therefore, measuring survivin adds new dimension to early recognition and monitoring of patients at risk of developing rheumatoid process [17]. We are aware of the limitations of our work. First of all, small group of children with JIA biologically treated - only with TNF- α inhibitors. These study group patients had long disease duration time, with DMARDs treatment failure before implementation of TNF-

α inhibitors. Additionally, only two subtypes of JIA onset are properly represented, and there is small number of joint fluid samples available.

CONCLUSIONS:

On the basis of the conducted study, it could be concluded that survivin seem to be an independent biomarker, irrespective of disease duration time, that may be helpful in the diagnosis of JIA. Nevertheless, the higher concentration of survivin is being associated with ACPA positivity, survivin can act as a unique biomarker that identifies an additional group of patients with JIA negative for autoantibodies even in the early stage of the disease. We have failed to confirm survivin prognostic potential for the active and destructive course of the rheumatoid process, however it is worth to underline that children have better potential for bone regeneration than adults. Survivin measurement should be considered as aiding tool identifying DMARDs non-responders and biomarker probably not dependent of treatment with TNF- α inhibitors.

ABBREVIATIONS:

ANA – antinuclear antibodies; anti-CCP = ACPA - anti-cyclic citrullinated peptide autoantibodies; AUC - area under the curve; CRP - C-reactive protein; DMARDs – disease modifying antirheumatic drugs; ESR - erythrocyte sedimentation ratio; ILAR - The International League of Associations for Rheumatology; IQR – interquartile range; JADAS-27 - 27-joint Juvenile Arthritis Disease Activity Score; JIA - juvenile idiopathic arthritis; MTX – methotrexate; PDUS - Power Doppler ultrasonographic signal; (PLT - platelets count; RA – rheumatoid arthritis; RBC - red blood cells; RF - rheumatoid factor; ROC - Receiver operator characteristics; TNF- α – tumor necrosis factor- α ; WBC - white blood cells

DECLARATIONS:**ETHICS APPROVAL AND CONSENT TO PARTICIPATE:**

This study was conducted with the approval of the local Medical University of Lodz Ethics Committee [No RNN/58/13/KB].

At enrollment, all the patients and their parents gave their written informed consent to participate in the study.

CONSENT FOR PUBLICATION:

Not applicable.

AVAILABILITY OF DATA AND MATERIALS:

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS:

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS:

JL conceived of the study, designed and coordinated the study, participated in the ultrasound examination and draft the manuscript; was the main responsible for the sequence of alignment and drafted the manuscript. MK performed the statistical analysis and helped to revise the manuscript. JSJ participated in the collection of study group and ultrasound examination. ES participated in the design of the study, and revised the manuscript.

All authors read and approved the final manuscript.

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