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Serum IL-10 and IL-12 levels reflect the response to chemotherapy but are influenced by G-CSF therapy and sepsis in children with soft tissue sarcomas*

Surowicze stężenia IL-10 i IL-12 odzwierciedlają uzyskiwaną odpowiedź na chemioterapię u dzieci z mięsakami tkanek miękkich, jednak ulegają modyfikacji podczas terapii czynnikiem wzrostu kolonii granulocytów (G-CSF) oraz w przebiegu sepsy

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- B Data Collection
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Summary

Introduction:

Pre-treatment serum IL-10/IL-12 balance has been recently found deregulated in childhood soft tissue sarcomas (STS). Its role in STS monitoring and assessment of response to therapy is unknown.

Objective:

To establish whether serum IL-10 and IL-12 levels and their reciprocal ratios reflect childhood STS course and actual activity and whether G-CSF therapy and central vein catheter (CVC)-related sepsis influence the interleukins levels.

Materials and methods:

ELISA determinations of serum interleukins were performed before treatment, in remission without complications (CR), at relapse and after treatment in 59 STS patients and during G-CSF administration and CVC-related sepsis (in 18) and also in 30 healthy controls.

Results:

In CR IL-10 declined and IL-12 increased as compared to pretreatment levels; in relapse IL-10 rose and IL-12 decreased significantly as compared to levels in CR. Also rates of IL-10, IL-12, and IL-10/IL-12 ratios recently estimated by us as of prognostic significance reflected well the STS course. During G-CSF therapy and CVC-related sepsis, IL-10 increased and IL-12 decreased significantly from levels in CR without complications. IL-10 levels and rates of IL-10 ≥ 11 pg/ml in sepsis could falsely suggest relapse. However, IL-12 levels, rates of IL-12 ≤ 60 pg/ml and/or simultaneous determination of both interleukins differed significantly from levels at relapse.

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|-----------------------|--|
| Conclusion: | Serial determinations of serum IL-10 and IL-12 reflected well the course of STS in children and enabled remission and relapse phases to be distinguished. To avoid G-CSF and sepsis influence, IL-12 and IL-10/IL-12 ratio and not IL-10 alone should be analysed. |
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INTRODUCTION

Childhood soft tissue sarcomas (STS) are a highly heterogeneous group of malignant tumours, constituting the fifth most common paediatric malignancy (after leukaemias, brain tumours, lymphomas and neuroblastoma). The prognosis in children with STS has improved markedly over the last decades, yet still a proportion of patients develop relapse and die of cancer progression, despite aggressive, multimodal treatment, including chemotherapy (CHT), radiotherapy (RTX) and surgery.

Therapy assignment and prognosis in childhood STS depends on a set of established clinical and pathological factors, including histological subtype, age, stage of disease, tumour size, invasiveness, and localization and the feasibility of its complete resection [7, 8, 66]. Whether the response to neo-adjuvant CHT, as judged by anatomic imaging, influences the outcome of paediatric STS is still a matter of debate [60, 61]. In contrast to most studies concerning STS of adults [20, 71], good response to induction CHT has been reported to be associated with better EFS and OS in paediatric STS [30, 55, 56]. Accordingly, the German Cooperative Soft Tissue Sarcoma Study (CWS) has been using response to three initial cycles of CHT to tailor subsequent therapeutic options [15, 31]. Furthermore, the correlation between initial response and outcome has been observed also in adults with rhabdomyosarcoma in the United States [35].

In clinical oncological practice, the response of STS to induction CHT is defined as shrinkage based on the results of radiological examinations, including: chest X-ray, ultrasonography (USG), computed tomography (CT) and/or magnetic resonance (MR). Those methods, however, do not show the activity of the residual tumour and are not able to define undoubtedly whether

the patient has achieved complete remission (CR) of cancer. In this aspect, promising data have been reported recently for clinical utility of fluorodeoxyglucose positron emission tomography (FDG PET) /CT imaging in adult STS patients [3, 13]. However, in children with STS this diagnostic method has not been validated and recommended yet. That is why it is of utmost importance to seek new reliable and non-invasive markers of childhood STS that could complement the standard radiological workup to facilitate proper assessment of the actual disease activity and monitor the response to therapy.

It has been reported that malignant solid tumours, including sarcomas, are frequently associated with marked alterations of pro- and anti-inflammatory cytokine patterns. Selected interleukins, chemokines, growth factors, and interleukin receptors have been found elevated in sera of cancer patients and associated with biological aggressiveness, clinical stage and prognosis [16, 25, 26, 52, 57, 64]. Among them, serum IL-10 and IL-12 levels and their reciprocal ratio have gained much interest recently. Sarcoma cell lines have been found to express IL-10 mRNA and IL-10 protein [54] and significantly elevated serum levels of IL-10 compared to healthy controls were detected in adult sarcomas of soft tissue [47, 48] and bones [49]. In children with cancer, increased IL-10 and decreased IL-12 were found before treatment in poor-prognosis and high-risk STS, acute lymphoblastic leukaemia (ALL) and Hodgkin's lymphoma (HL). To the contrary, children with less advanced cancers presented with higher serum IL-12 and lower IL-10 levels [5, 8]. Moreover, initial serum IL-10 and IL-12 were shown to predict EFS and OS in paediatric STS, especially when both interleukins were analysed simultaneously [8].

IL-10 and IL-12 act in an antagonistic fashion, but their definite sources and roles in carcinogenesis and immune responses towards cancer are unclear. It is speculated that elevated IL-10 and decreased IL-12 found in advanced and aggressive cancer are likely to reflect deregulated Th1/Th2 immune responses with Th2 predominance and Th1 suppression. Increased pre-treatment concentrations of serum IL-10 might also reflect tumour burden [28, 36, 45, 68]. Cancer patients with more advanced disease and worse prognosis were found to present with decreased IL-12, most likely as a result of its suppression mediated by IL-10 and a sign of ineffective anti-tumour Th1 immune response.

Only a few studies have reported on the association between tumour resection or its shrinkage after systemic therapy and change of the serum levels of IL-10 and/or IL-12 [1, 44, 63, 73]. There have been no data on this issue in children treated for STS. Therefore in the present study we aimed to investigate whether the serum IL-10 and IL-12 and their reciprocal ratios might reflect disease course and actual activity of cancer in children with STS. Bearing in mind that many components of oncological treatment may interfere with the host immunological responses [9, 12, 27, 34, 62, 67], we aimed also to establish whether G-CSF therapy of severe post-CHT neutropenia and central vein catheter (CVC)-related sepsis influence the interleukin levels. Previously, G-CSF administration in neutropenic children as well as infectious complications have been found to increase the levels of soluble IL-2 receptor α (sIL-2R α) and thus falsely suggest a relapse in children who are in remission of STS [6]. We have recently reported that sIL-2R α correlates negatively with IL-12 and positively with IL-10 serum levels [8, 29], so it is likely that also IL-12 and IL-10 serum levels in children treated for STS might be influenced by G-CSF and sepsis.

To our best knowledge this is the first study on this issue in children with STS and the first ever to prospectively monitor the possible changes of serum IL-10, IL-12 and their reciprocal ratios during chemotherapy for STS in children.

MATERIAL AND METHODS

Patients

The study consisted of 59 children with STS (F/M: 33/26; aged 23 to 183 months, mean age 103, median 119 months), registered in the Polish STS database and treated in the Department of Paediatrics, Haematology and Oncology, Medical University of Gdansk, Poland between 1996 and 2008. The histopathological diagnoses of all patients were confirmed before treatment by open biopsies and verified in two distinct pathological institutions. The diagnostics and therapy were performed according to the CWS-91, -96, -2002 and 2006 protocols approved for use in Polish centres of paediatric oncology. In addition to a complete medical history and physical examination, diagnostic procedures included

radiological examinations: chest X-ray, USG, CT and/or MR of the sites involved by the neoplasm or sites of possible metastases. Bone scintigraphy and bilateral bone marrow aspiration were obligatory to exclude or confirm metastases. The therapeutic strategies did not change substantially over the years and comprised surgical excision of the primary tumour, involved lymph nodes (LN) and/or metastatic foci (primary or delayed), RTX of the disease site(s) and systemic CHT given on a neo-adjuvant and/or adjuvant basis. Full details on all relevant clinical data were available for all patients, including treatment modalities, response to CHT, relapse occurrence and outcome.

Control group

The control group consisted of 30 healthy children (8 females and 22 males; aged from 45 to 213 months, mean age 134.7, median 135.0 months) without any history or signs of inflammatory, autoimmune or infectious diseases within the previous three months. None of them was receiving medications or suffered from any organ insufficiency.

Sample collection

Patients with STS were sampled prospectively at four main phases of disease:

I - at diagnosis (before any oncological treatment) - in all 59 children,

II - in CR during oncological therapy (CR without complications) - in 34 children,

III - during relapse and poor response to therapy - in 31 children,

IV - in CR at least 3 months after oncological treatment discontinuation - in 34 children (in 19 patients it was the first CR after first line therapy, and in 15 the second CR after therapy of relapse).

CR was defined as the complete disappearance of measurable disease while a relapse was defined as the detection of a new lesion at the primary or other site(s) in a patient who was previously in CR. Poor response to therapy comprised patients not responding to therapy (NR; tumour reduction <25% of initial volume) and patients with disease progression (PD; increase in tumour size or new lesions detected). All time points of sample collections were carefully planned to avoid the possible influence of immunological activation caused by factors other than malignancy at the levels of analysed markers [6]. The blood samples were collected from patients with no clinical signs of actual or previous (at least 14 days before) infectious or other inflammatory disorders, with no renal impairment, at least 30 days after general anaesthesia, surgery and termination of radiotherapy and at least 14 days after stopping the G-CSF administration.

Among the 59 study patients there were 18 children (10 girls and 8 boys aged 23.2 – 147.5 months at diagnosis, mean age 83; median 71 months), who were examined in phases I-IV (termed phases 1-4) and additionally:

- in CR of disease during G-CSF therapy for WHO grade IV neutropenia (phase 2a, 18 patients),
- in CR of disease during CVC-related sepsis (phase 2b, 18 patients),
- in relapse or with poor response to therapy during CVC-related sepsis (phase 3a, 13 patients of 16 relapsed).

At stage 2a the samples were collected in children at days 5-9, median day 7 of therapy with G-CSF. The G-CSF therapy was introduced because of post-CHT neutropenia of WHO grade IV (absolute neutrophil count <0.5 G/l) and was given at a dose of 5 micrograms per kilogram once a day subcutaneously.

The diagnosis of CVC-related sepsis was based on two positive cultures with Gram positive bacteria of blood taken from a Hickman-Broviac catheter implanted in the superior caval vein through the subclavian vein at the beginning of oncological treatment. The patients displayed fever over 38°C with or without chills, and tachycardia, with no other specific infectious foci detected in physical and radiological examinations. The patients with CVC-related sepsis were not given G-CSF before or at the time of the sample collection; in those with grade IV neutropenia, G-CSF was introduced after the samples had been collected. None of the patients developed septic shock or organ failure and all recovered due to early institution of wide spectrum empirical intravenous antibiotic therapy and careful monitoring of vital signs, including blood pressure, heart rate, breathing rate, oxygen saturation, blood gases and values of acute phase reactants. No laboratory or clinical signs of renal failure or haemolysis were found in the patients at the time of sample collection. The samples in phases 2b and 3a were taken during the first two days of sepsis since its first symptoms.

The children of the control group were sampled once. The blood count, renal and liver function tests, erythrocyte sedimentation rate and C-reactive protein (CRP) values were within the normal range for age in all controls (for results see [5]).

All samples were stored at -70°C until assayed.

Informed consent was obtained from all patients and controls or their guardians according to institutional guidelines applied over the years. The study was approved by the Local Ethical Committee of the Medical University of Gdansk (decisions no. NKEBN/367/95, NKEBN/678/2002 and NKBBN/60/2013).

Determination of serum IL-10 and IL-12 levels

Determinations of serum levels of IL-10 and IL-12 (IL-12p70) were performed in duplicate in the Department of Immunopathology and Transplantology, Medical University of Gdansk, Poland. Commercially available sandwich enzyme immunoassay kits (Endogen, Cambridge, MA) were used. The assays were specific for human IL-10 and human IL-12p70 and did not cross-react with other known cytokines. The lower limit of detection for the ELISA assays for both IL-10 and for IL-12p70 was 3 pg/ml, with an intra-assay and an inter-assay variation less than 10%, as provided by the manufacturer. The procedures of determination of interleukins were performed according to the manufacturer's recommendations and have been described in detail previously [5]. The results of IL-10 and IL-12 were found to be independent of age and are expressed in pg/ml. In 18 patients sampled serially in all crucial phases of the disease course (i.e. before therapy, at CR during treatment, at relapse and in CR after therapy) and also during G-CSF administration and CVC-related sepsis, the serum levels of sIL-2R α , lactate dehydrogenase (LDH), erythrocyte sedimentation rate, CRP and beta2 microglobulin (B2-M) were available, obtained according to the methods described previously [6]. In all phases of disease, the rates of patients with IL-10 ≥ 11 pg/ml, IL-12 ≤ 60 pg/ml, IL-10/IL-12 ≥ 0.15 , IL-10 <9.5 and IL-12 >65 pg/ml and IL-10 ≥ 9.5 and IL-12 ≤ 65 pg/ml were assessed. These values have been previously found significant for prognosis assignment in childhood STS [8]. The patients' clinical status and the phase of disease at the time of sample collection were not known by the persons running cytokine assays. Repeated analyses and regular validation of results were aimed at higher precision of each measurement series.

STATISTICAL ANALYSIS

The clinical data and laboratory findings were collected in a medical database constructed in the software Microsoft Excel for Windows XP (Microsoft). The results of the study were submitted to statistical analysis using the statistical package EPIINFO Ver. 3.4.3 (08-11-2007), Centers for Disease Control and Prevention, Atlanta, GA. In all analyses, $p < 0.05$ was considered statistically significant. For all groups mean values (X), median values (M), standard deviations (SD), interquartiles and range of all analysed continuous variables were calculated.

The hypothesis of equality in mean values among different groups versus the control group was verified using the non-parametric Kruskal-Wallis one-way analysis of variance by ranks. To verify the hypothesis of equality in mean values among the groups of cancer patients at different phases of disease, the non-parametric Wilcoxon signed-rank test was used. Both tests were chosen because of the small number of cases in analysed groups. The correlation between the analysed variables was tested with the Spearman or Pearson's rank correlation coefficient.

Results

Monitoring of STS course with serial IL-10, IL-12 and IL-10/IL-12 determinations

As shown in Table 1, the serum levels of IL-10 and IL-12 and values of their ratios changed depending on the phase of disease and response to CHT. Achievement of CR was associated with a significant decline of elevated median pre-treatment level of IL-10 and increase of IL-12. Serum IL-10 level obtained in CR during therapy was similar to those seen in patients after therapy and in controls while IL-12 levels significantly exceeded the levels found in healthy children. In fact, IL-12 levels were significantly higher than in controls in all examined phases of cancer except for the phase of relapse, when the IL-12 level was similar to that observed in healthy children. The hallmarks of relapse/progression were rise of IL-10 and decrease of IL-12 as compared to the levels obtained in CR. The differences

were significant; thus it was possible to discriminate between CR and relapse phases based on IL-10 and IL-12 levels and their ratio.

It was previously found that pre-treatment IL-10 correlated positively and IL-12 correlated negatively with sIL-2R α and LDH levels [8]. In the present study we observed that in CR without complications IL-12 correlated negatively with sIL-2R α ($r=-0.34$, $p=0.047$) and IL-10/IL-12 correlated positively with sIL-2R α ($r=0.35$, $p=0.041$) and LDH ($r=0.34$, $p=0.049$). In relapse/progression of cancer IL-12 correlated negatively and IL-10/IL-12 correlated positively with LDH ($r=-0.60$, $p=0.000$ and $r=0.43$, $p=0.017$; respectively).

As shown in Table 2, also the rates of patients with the values of IL-10, IL-12 and IL-10/IL-12 ratio that were previously found important for prognosis prediction in childhood STS reflected well the course of disease.

Table 1. The results of serum IL-10, IL-12 and IL-10/IL-12 ratio in children with STS at different phases of disease and in controls

| Analyzed markers | I | II | III | IV | controls | |
|------------------|------------|-----------------------|----------------------|-----------------------|-----------------------|----------------------|
| | N=59 | N=34 | N=31 | N=34 | N=30 | |
| IL-10 | M | 11.8 ^{bdef} | 5.63 ^{acg} | 13.4 ^{bdeh} | 4.90 ^{aci} | 6.27 ^{ac} |
| | Min-max | 3.1-32.8 | 3.01-17.35 | 6.1-29.3 | 3.07-18.01 | 1.85-11.52 |
| | X \pm SD | 12.7 \pm 8.7 | 6.96-3.48 | 14.2 \pm 5.7 | 5.94 \pm 2.88 | 6.33 \pm 2.60 |
| | 25Q-75Q | 4.9-15.8 | 4.36-9.22 | 9.94 – 18.8 | 4.53 – 6.85 | 5.16 – 7.31 |
| IL-12 | M | 68.9 ^{bde} | 64.8 ^{ace} | 56.2 ^{bd} | 94.1 ^{ace} | 55.8 ^{bdh} |
| | Min-max | 4.0-120.3 | 93.8-121.1 | 12.1 - 79.3 | 62.3 - 115.3 | 13.7 - 86.6 |
| | X \pm SD | 65.2 \pm 30.2 | 93.0 - 13.3 | 52.7 \pm 17.5 | 93.8 \pm 11.2 | 54.7 \pm 20.3 |
| | 25Q-75Q | 53.4 – 89.3 | 84.7 – 100.6 | 39.5 – 64.3 | 89.2 – 100.0 | 40.0 – 72.2 |
| IL-10/IL-12 | M | 0.148 ^{bdef} | 0.059 ^{age} | 0.256 ^{bdeh} | 0.051 ^{acei} | 0.127 ^{bcd} |
| | Min-max | 0.037 - 7.399 | 0.031 - 0.268 | 0.091 - 2.040 | 0.033 - 0.289 | 0.029 - 0.539 |
| | X \pm SD | 0.699 \pm 1.651 | 0.080 - 0.052 | 0.365 \pm 0.380 | 0.067 \pm 0.047 | 0.148 \pm 0.114 |
| | 25Q-75Q | 0.063 – 0.350 | 0.047 – 0.115 | 0.155 – 0.427 | 0.045 – 0.071 | 0.076 – 0.163 |

Legend: phases of STS course: I – before treatment, II – in complete remission (CR) without complications, III – at relapse and in poor responders, IV – in CR after treatment discontinuation.

^a $p < 0.01$ as compared to the level of the marker at phase I. • ^b $p < 0.01$ as compared to the level of the marker at phase II. • ^c $p < 0.01$ as compared to the level of the marker at phase III. • ^d $p < 0.01$ as compared to the level of the marker at phase IV. • ^e $p < 0.01$ as compared to the level of the marker in healthy controls • ^f $p < 0.05$ as compared to the level of the marker at phase III • ^g $p < 0.5$ as compared to the level of the marker at phase IV • ^h $p < 0.5$ as compared to the level of the marker at phase I • ⁱ $p < 0.5$ as compared to the level of the marker at phase II

Table 2. The rates of selected values of IL-10, IL-12 and IL-10/IL-12 ratio in children with STS at different phases of disease and in controls.

| Analyzed markers | I | II | III | IV | controls |
|--|----------|----------|----------|----------|----------|
| | N=59 | N=34 | N=31 | N=34 | N=30 |
| IL-10 ≥ 11 pg/ml | 33 (56%) | 6 (18%) | 22 (71%) | 1 (3%) | 1 (3%) |
| IL-12 ≤ 60 pg/ml | 22 (37%) | 0 (0%) | 18 (58%) | 0 (0%) | 15 (50%) |
| IL-10/IL-12 ≥ 0.15 | 30 (51%) | 3 (9%) | 25 (81%) | 1 (3%) | 10 (33%) |
| IL-10 < 9.5 and IL-12 > 65 pg/ml | 22 (37%) | 27 (79%) | 3 (10%) | 29 (85%) | 8 (27%) |
| IL-10 ≥ 9.5 and IL-12 ≤ 65 pg/ml | 27 (46%) | 1 (3%) | 22 (71%) | 1 (3%) | 1 (3%) |

Legend: phases of STS course: I – before treatment, II – in complete remission (CR) without complications, III – at relapse and in poor responders, IV – in CR after treatment discontinuation.

Serum levels of IL-10 and IL-12, and IL-10/IL-12 ratio during therapeutic complications

It was found that median concentrations and the rates of selected values of IL-10, IL-12 and IL-10/IL-12 ratio changed significantly during oncological therapy and its complications (grade IV neutropenia treated with G-CSF and CVC-related sepsis) in 18 children with STS (Figs. 1ABC and 2ABC).

A) Influence of G-CSF therapy on the serum levels of IL-10 and IL-12 and IL-10/IL-12 ratio

The median serum levels of IL-10 and the rates of IL-10 ≥ 11 pg/ml in children in CR with concomitant neutropenia treated with G-CSF were significantly higher than in patients in CR without complications ($p=0.0025$) but lower than in relapse ($p=0.0494$). The median levels of IL-12 obtained during G-CSF administration were significantly lower than in CR without complications but not as low as in relapse ($p=0.0033$ and $p=0.004$, respectively). Rates of IL-12 values ≤ 60 pg/ml were found in none of the patients in CR without complications or in CR with concomitant G-CSF administration while in relapse they were found in 50% of children. Also the median IL-10/IL-12 ratios and the rates of IL-10/IL-12 ≥ 0.15 differed significantly between CR without complications, CR with G-CSF therapy and relapse, making it easier to distinguish between the phases ($p=0.0043$ and $p=0.0019$, respectively).

B) Influence of CVC-related sepsis on the serum levels of IL-10 and IL-12 and IL-10/IL-12 ratio

The median levels of IL-10 increased and of IL-12 decreased in patients in CR with CVC-related sepsis in comparison to values obtained in CR without complications ($p=0.0010$ and $p=0.0002$, respectively). During sepsis median IL-10 level and the rates of IL-10 ≥ 11 pg/ml were

similar to those found at relapse ($p=0.134$). To the contrary, median level of IL-12 was still higher than at relapse and no patients had IL-12 ≤ 60 pg/ml in comparison to 50% at relapse.

It was possible to discriminate between CR with sepsis and relapse phases based on the median IL-12 levels and/or based on IL-10/IL-12 ratios (72.9 vs. 63.7 pg/ml, $p=0.0019$, and 0.140 vs. 0.181, $p=0.0084$, respectively). Also when the levels of both interleukins were analysed simultaneously (IL-10 < 9.5 and IL-12 > 65 pg/ml or IL-10 ≥ 9.5 and IL-12 ≤ 65 pg/ml) the results found in patients in CR with sepsis were different from the results found in relapse.

In children sampled while in relapse and during CVC-related sepsis the IL-10 level was not different from the level at relapse without complications ($p=0.0869$) but IL-12 was significantly lower ($p=0.0277$) and was similar to the level observed in healthy controls. The median IL-10/IL-12 ratio in patients in relapse with sepsis was the highest ($p=0.201$), indicating mostly decreased IL-12.

DISCUSSION

To our best knowledge this is the first report on the clinical significance of determination of the balance between serum IL-10 and IL-12 levels in patients with STS measured prospectively during anti-tumour therapy – with respect to the influence of G-CFS and sepsis on the levels of analysed interleukins.

MONITORING OF THE DISEASE COURSE

the study has shown that serum levels of IL-10 and IL-12 change during oncological therapy and are able to reflect well the course of disease. Elevated serum IL-10 and IL-10/IL-12 ratios were found in active phases of cancer (at diagnosis and at relapse), while the opposite results with

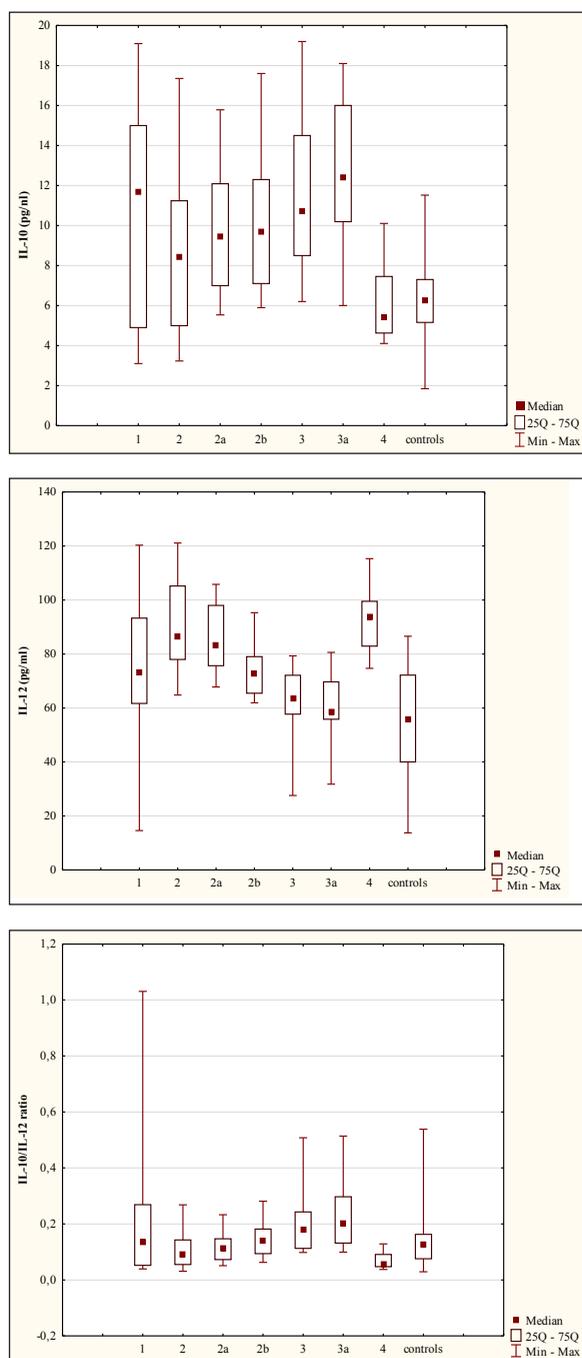


Fig. 1. Median serum levels of IL-10 (A), IL-12 (B) and IL-10/IL-12 in 18 children with STS sampled during the course of disease in six phases (1 – before treatment, 2- in CR during therapy, without complications, 2a – in CR during G-CSF therapy for neutropenia, 2b – in CR, during CVC-related sepsis, 3 – at relapse, 3a – at relapse during sepsis, 4 – in CR after treatment discontinuation) and in healthy controls.

Fig 1A: $p < 0.005$ for: 1 vs. 2; 1 vs. 4; 1 vs. controls, 2 vs. 2a; 2 vs. 2b; 2 vs. 3; 2 vs. 3a, 2a vs. 3; 2a vs. 3a; 2a vs. 4; 2a vs. controls, 2b vs. 3a; 2b vs. 4; 2b vs. controls, 3 vs. 4; 3 vs. controls, 3a vs. 4; 3a vs. controls.

Fig 1 B: $p < 0.005$ for: 1 vs. 2; 1 vs. 2a; 1 vs. 4; 1 vs. controls, 2 vs. 2a; 2 vs. 2b; 2 vs. 3; 2 vs. 3a; 2 vs. controls, 2a vs. 2b; 2a vs. 3; 2a vs. 3a; 2a vs. controls, 2b vs. 3; 2b vs. 3a; 2b vs. 4; 2b vs. controls, 3 vs. 3a; 3 vs. 4, 3a vs. 4, 4 vs. controls.

Fig 1 C: $p < 0.005$ for: 1 vs. 2; 1 vs. 2a; 1 vs. 4, 2 vs. 2a; 2 vs. 2b; 2 vs. 3; 2 vs. 3a, 2a vs. 2b; 2a vs. 3; 2a vs. 3a; 2a vs. 4, 2b vs. 3; 2b vs. 3a; 2b vs. 4, 3 vs. 3a; 3 vs. 4; 3 vs. controls, 3a vs. 4; 3a vs. controls; 4 vs. controls

decreased IL-10 and IL-10/IL-12 ratios were seen in the phases of cancer CR: during and after treatment. Also the rates of selected values of analysed markers assessed at different disease phases reflected well the course of STS. What is important, it was possible to differentiate between the phase of CR and the phase of suspected relapse based on the levels of serum IL-10 and IL-12 and their ratios. These findings suggest that serial determinations of serum IL-10 and IL-12 in children with STS could help to assess more precisely the phase of disease and response to therapy in particular patients.

The reasons and nature of marked differences in serum IL-10 and IL-12 concentrations observed between the phase of active cancer growth and the phase of cancer remission are complex. From one point of view, the increase of serum IL-10 before initiation of therapy and in patients who relapsed can reflect the tumour bulk and disease progression. It has been reported previously that IL-10 is released by the cells of several cancer types, including sarcomas [4,46,51,54]. Accordingly, the serum levels of IL-10 have been found to correlate with the disease stage in children with STS [8] and in patients with several other malignancies [28,36,45,68]. The studies on changes of serum IL-10 levels during anti-neoplastic therapy are scarce; however, it was reported that in adult STS, the serum IL-10 level reflected tumour burden and response to therapy, decreasing significantly after complete tumour resection [48]. Similarly, the surgical excision of the colorectal and gastrointestinal cancers resulted in a significant reduction of plasma IL-10 [18,57].

Due to the fact that IL-10 can be secreted by the tumour infiltrating cells and many other types of immune cells, the elevation of IL-10 in phases of active and aggressive cancer and its decrease in CR of disease may also reflect the specific immune responses towards cancer and the interrelationship between the cancer and the host. The role of IL-10 in carcinogenesis has recently been found to be complex and pleiotropic [40, 41], but there is still a strong body of evidence that IL-10 plays an important immunosuppressive role [51], allowing malignant cells to evade immune surveillance [37], and promote cancer growth and dissemination [17, 50].

Among many immunosuppressive actions, IL-10 is a potent inhibitor of IL-12 and in many cancers the two cytokines act in an antagonistic fashion. A strong negative correlation between IL-10 and IL-12 in children with STS, but not in healthy controls, was reported recently [8]. Elevated IL-10 and decreased IL-12 levels in patients with more advanced cancer, poor response to therapy and worse prognosis are thought to result from an imbalanced Th1/Th2 axis, with the latter predominating over the former. In our present study good response to CHT and achievement of CR were associated with a significant decrease of IL-10 and rise of IL-12, which can be explained as reversal of Th2-dominant to Th1-dominant immune responses. Similarly, initially elevated IL-10 values (to-

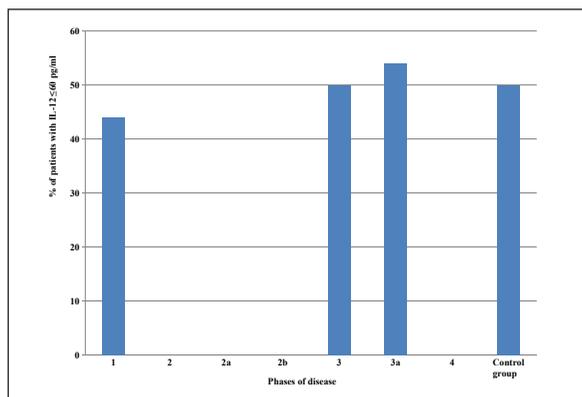
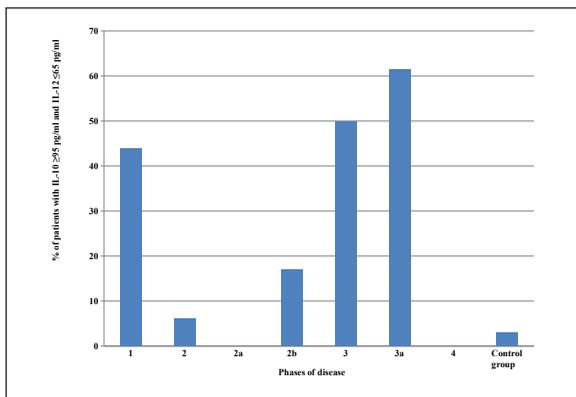
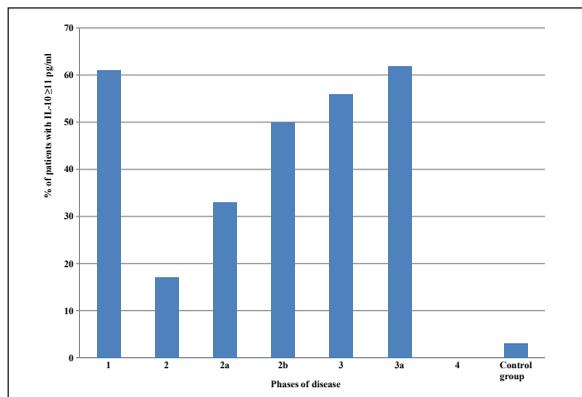
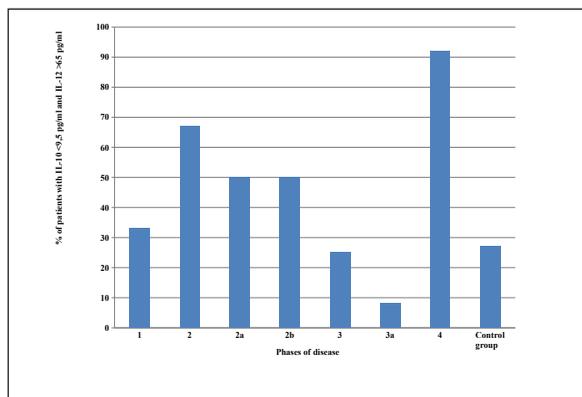
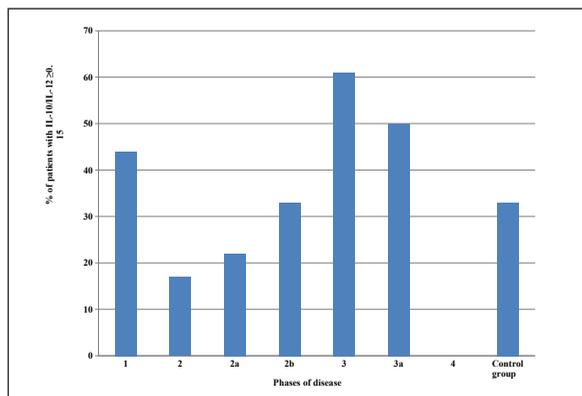


Fig. 2. The rates of IL-10 ≥ 11 pg/ml (A), IL-12 ≤ 60 pg/ml (B), IL-10/IL-12 ≥ 0.15 pg/ml (C), IL-10 < 9.5 pg/ml and IL-12 > 65 pg/ml (D) and IL-10 ≥ 9.5 pg/ml and IL-12 ≤ 65 pg/ml (E) in children with STS at different phases of disease (1 - before treatment, 2 - in CR during therapy, without complications, 2a - in CR during G-CSF therapy for neutropenia, 2b - in CR, during CVC-related sepsis, 3 - at relapse, 3a - at relapse during sepsis, 4 - in CR after treatment discontinuation) and in healthy controls



gether with CA-125) decreased in women with ovarian cancer responding to CHT, while they remained elevated in non-responders [73]. Patients with bladder cancer were also reported to develop Th2 dominant status (inter alia reflected by elevated serum IL-10 level) and deficient Th1 immune response, that tended to reversal following combination therapy with intravesical bacillus Calmette-Guerin plus interferon-α2b [1].

Similarly to the results of our present study, in patients with head and neck cancer good response to therapy was accompanied by the increase of IL-12 serum levels [44]. However, the levels of IL-12 at diagnosis of these patients were low, which is in contrast to our results, where the median pre-treatment serum level of IL-12 was found to be higher than in healthy controls. IL-12 is regarded as an anti-neoplastic cytokine, so its elevated serum levels can obviously be expected in remission phases of cancer, but it appears that they can also coexist with active tumour mass – probably as a sign of the enhancement of Th1 cytokines production in response to cancer development, which has been discussed elsewhere [8]. In this context, determination of IL-12 alone cannot serve for diagnostic purposes; however, serial changes of IL-12 during therapy combined with simultaneous determination of IL-10 have been found to be of potential clinical utility. In active disease before therapy and at relapse, the IL-10/IL-12 ratio was higher than in controls, reflecting elevation of IL-10 with simultaneous elevation of IL-12 (before therapy) or decrease of IL-12 (at relapse). To the contrary, very low values of IL-10/IL-12 ratios were characteristic for remission phases, resulting from lowered IL-10 and highly increased IL-12 levels. Decrease of IL-10 under anti-tumour therapy can also be responsible for de-suppression of IL-12 production in patients with remission of cancer. Accordingly, in the report of Tsavaris, administration of edrecolomab in patients with resected Dukes' stage C

colorectal cancer resulted in a significant increase of pre-treatment low IL-12 levels, while pre-treatment high IL-10 levels decreased. Interestingly, this phenomenon was not observed in patients who received standard 5-fluorouracil-based CHT. The authors concluded that patients with resected Dukes' C colorectal cancer display *in vivo* deficient immune responses which can be restored with postoperative adjuvant therapy with edrecolomab [63]. It was also reported that serial determination of selected cytokines of the Th1/Th2 axis might be used as an early signal of disease recurrence/progression and for evaluation of the response to immunotherapy. In the study of Lauerova a significant decrease of IL-2 and IL-12 and rise of IL-6 and IL-10 were found to precede relapse by at least one month in patients with melanoma treated with rIFN-alpha [33]. In our study we determined the interleukin levels once the relapse was confirmed in radiological and/or histopathological examinations, not before.

The results of our study show promising clinical utility of IL-10 and IL-12 as markers of response to CHT. However, it should be underlined that the patients were not sampled randomly. The time points for sample collection were chosen very carefully to avoid the possible influence of concomitant infections, RTX, general anaesthesia, surgical procedures and medications known to interfere with immune properties of the host on the interleukin levels. Such a situation is rather idealized, which is why the interpretation of data obtained during anti-tumour therapy in children with STS should be cautious. It has been proven that many factors, other than malignancy, may influence the release of interleukins [6,9,12,27,34, 62,67].

INFLUENCE OF G-CSF AND INFECTION ON SERUM IL-10 AND IL-12 LEVELS

It was reported previously that administration of G-CSF and presence of CVC-related sepsis in neutropenic cancer patients significantly increased the level of serum sIL-2R α , so that patients in CR of cancer might display levels of the receptor similar to those found at relapse [6]. Recently, a positive correlation between sIL-2R α and IL-10 and negative with IL-12 was observed in children with STS [8]. In the present study we have found that G-CSF and infection also influence the serum IL-10 and IL-12 levels. Median IL-10 was found significantly higher and IL-12 lower than in the respective phases of cancer without G-CSF or the infectious complication. Levels of IL-10 obtained from patients during CVC-related sepsis could falsely suggest that they develop relapse. Also the number of patients with IL-10 ≥ 11 pg/ml was similar in the phase of CR with septic complications and in the phase of relapse. However, median levels of IL-12 and IL-10/IL-12 ratios assessed during CVC-related sepsis differed significantly from levels observed at relapse, making it easier to distinguish between the phases.

Neutropenia is one of the most common complications of CHT regimens in childhood STS. Both the duration and severity of neutropenia increase the risk of infections,

including CVC-related sepsis [21]. To restore neutrophil counts and prime neutrophil functions for enhanced immune defence, G-CSF has been available in children in recombinant form as filgrastim or lenograstim [65]. Apart from increasing the production of mature, functional neutrophils, monocytes and lymphocytes [70], G-CSF also reduces release of several proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-12, in response to an infectious stimuli [62], increases the release of anti-inflammatory mediators such as IL-1ra, sTNF-receptors and IL-10 [10,11,23,39], and promotes mobilization of Th2-inducing dendritic cells [2] and Th2 immune deviation [53]. This unique combination of haematopoietic, anti-inflammatory and anti-infectious effects on the innate immune system has been confirmed as beneficial in cancer patients and thus G-CSF has been widely used in clinical practice for over two decades now [14]. Our findings confirm a shift from inflammatory to anti-inflammatory cytokine pattern during G-CSF therapy in children with STS.

Similarly, we have found a predominance of Th2 over Th1 immune responses in children diagnosed with CVC-related sepsis. It has been reported previously that this predominance of anti-inflammatory Th2 cytokine pattern with increased serum IL-10 levels in patients with sepsis may reflect immune suppression and be related to higher mortality rates [42]. Accordingly, Wu et al. reported that serum IL-10 levels in patients with severe sepsis were significantly elevated in comparison to healthy controls and the levels found in non-survivors were significantly higher than in survivors both on day 1 and on day 6 of sepsis [72]. To the contrary, the serum levels of IL-12 were much lower in septic patients than in healthy controls, with no evident difference between the levels in survivors and non-survivors. However, the survivors were found to produce more IL-12 from LPS-stimulated PBMCs than non-survivors [58]. This ability was increasing with time of sepsis duration, which might exert a protective effect by increased cellular immunity and phagocytic functions [72]. The role of IL-12 in sepsis is controversial [22]. Most experiments support the concept that impaired IL-12 production may severely limit the host's defence against infections and result in a higher mortality rate [19, 43, 69]. In an experimental model the production of IL-12 induced by surgery or sepsis was found to be protective against a lethal outcome, while neutralization of IL-12 resulted in higher mortality rates [59]. To the contrary, in infected mice, the neutralization of *E. coli*-induced release of biologically active IL-12 provided an evident survival benefit [74]. In our present study, the levels of IL-12 in children with sepsis were lower than in CR without complications, but none of the patients demonstrated IL-12 ≤ 60 pg/ml in contrast to 50% of patients in relapse who did. These findings may correspond with the fact that none of our patients developed septic shock and all recovered from infection. However, we did not monitor the IL-12 level during the sepsis course, so it is unclear whether the level of the IL rose with time. There are only a few studies investigating the clinical role of IL-12 determination in childhood sepsis [24, 38]. In the latter study of Martin,

IL-12 levels failed to differentiate between septic conditions in children with cancer, and were not related to disease severity at admission [38]. Kallio et al. have reported that adult cancer patients with infection had significantly higher serum levels of IL-10 (and also of CRP, procalcitonin, neopterin and IL-8) and lower IL-12 compared to patients without infection [29]. It was particularly visible in more advanced cancer. Similar findings were found in our present study, in which patients with relapse complicated by sepsis demonstrated an evident predominance of anti-inflammatory Th2 over the pro-inflammatory Th1 cytokine pattern as compared to patients with relapse but without sepsis. These findings reflect Th1/Th2 imbalance resulting from both infection and underlying malignancy. Thus it has been recommended that diagnosis of infection in adult cancer patients with solid tumours requires combinations of markers and analysis of the underlying malignancy [29]. These observations support our thesis that the results of interleukin levels during oncological therapy can be obscured by the infectious process, so careful interpretation of data is mandatory in the context of analysing the phase of underlying cancer and response to CHT. Also the cause of sepsis may be an important factor since fungal infection was found to decrease IL-12 to a lesser extent than bacteraemia of Gram positive origin [32]. In our group of patients all septic conditions were caused by Gram positive bacteria, the most common cause of CVC-related sepsis in cancer patients. In our opinion it is clinically important that also several other biochemical markers, including sIL-2R α , B2-M, LDH and CRP, are determined simultaneously with IL-10 and IL-12, which may help to distinguish the "real" relapse of STS from the "false" one, in which the interleukin levels are influenced by G-CSF therapy or sepsis [6].

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CONCLUSIONS

1. Serum levels of IL-10 and IL-12 and IL-10/IL-12 ratio reflect well the course of disease and response to CHT and enable differentiation between CR and relapse phases in children with STS.
2. Achievement of CR was associated with a significant decline of elevated pre-treatment median level of IL-10 and increase of IL-12. The hallmarks of relapse/progression were rise of IL-10 and decrease of IL-12 as compared to the levels obtained in CR.
3. Interpretation of the levels of IL-10 and IL-12 measured during oncological treatment must be cautious because during G-CSF therapy and CVC-related sepsis the levels of IL-10 increase and of IL-12 decrease significantly.
4. Levels of IL-10 and the rates of IL-10 ≥ 11 pg/ml obtained from patients during CVC-related sepsis could falsely suggest that they develop relapse. However, levels of IL-12, the rates of IL-12 ≤ 60 pg/ml and/or simultaneous determination of both interleukins differed significantly from levels observed at relapse, making it easier to distinguish between the phases.
5. The study confirms that the values of analysed interleukins, which have recently been found to be prognostically significant in childhood STS (IL-10 ≥ 11 pg/ml, IL-12 ≤ 60 pg/ml, IL-10/IL-12 ≥ 0.15 , IL-10 < 9.5 and IL-12 > 65 pg/ml and IL-10 ≥ 9.5 and IL-12 ≤ 65 pg/ml), reflect well the course of disease and facilitate differentiation between the real relapse and the "false" one caused by CVC-related sepsis.

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