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Serum profile of transferrin isoforms in rheumatoid arthritis treated with biological drugs

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ABSTRACT

Background: In the chronic inflammation process in the course of rheumatoid arthritis (RA), many alterations in the expression of plasma proteins, as well as their posttranslational modifications (including glycosylation) can occur. Taking into account the disturbances in protein glycosylation and the emerging new treatment regimens, the aim of this study was to assess the serum profile of transferrin isoforms in RA patients treated with biological drugs.

Methods: The study included 20 patients (16 females and 4 males; mean age: 53.4 years; range: 24–67) with rheumatoid arthritis treated with rituximab. Serum samples were taken 3 times: before and 3 and 6 months during treatment. The isoforms of transferrin were separated by capillary electrophoresis (MINICAP electrophoretic system, Sebia, France) into five major fractions: asialo-, disialo-, trisialo-, tetrasialo- and pentasialotransferrin. The results were calculated as relative concentrations of each fraction.

Results: The median trisialotransferrin relative concentrations after 3 and 6 months treatment (4.40% and 4.10%, respectively) were significantly higher ($p = 0.013$, $p = 0.009$, respectively) than before treatment (3.50%). The levels of serum pentasialotransferrin were also increased 3 and 6 months following treatment (16.5% and 17.7%, $p = 0.005$ and $p = 0.006$, respectively) as compared to those before therapy (14.5%), while tetrasialotransferrin concentrations were lower (80.3% and 78.4%, $p = 0.009$ and $p = 0.008$, respectively) than before treatment (81.5%). Trisialotransferrin relative concentration correlated with Hb ($p = 0.019$), whereas pentasialotransferrin with PLT ($p = 0.036$) after treatment.

Conclusions: This study indicates that treatment with rituximab of RA patients alters the serum profile of transferrin isoforms. Tri-, tetra- and pentasialotransferrin relative concentrations measurements can be a useful tool to monitor therapy.

1. Introduction

Rheumatoid arthritis (RA) is a long-term autoimmune disease that is characterized by persistent inflammatory synovitis, predominantly affecting the peripheral joints. It is associated with pannus formation, cartilage destruction, bone erosion and joint destruction. The synovial membrane is characterized by hyperplasia, increased vascularity and greater permeability to the inflammatory cells, mainly $CD4^+$ T cells [1]. Since RA can also affect body systems, it is called a systemic disease. The pathogenesis of RA is still incompletely known.

RA is usually treated with one or more of many available disease-modifying antirheumatic drugs (DMARDs). Conventional DMARDs (such as methotrexate, sulfasalazine) and such drugs as steroids are effective, although they also have several limitations, including slow onset of action, adverse effects, modest remission, and retention rates. The steadily increasing knowledge regarding pathogenesis mechanism in autoimmune rheumatic disease has enabled scientists to develop different therapeutic approaches and drugs termed “biologicals”. Biological agents specifically inhibit the key inflammatory cytokines, such as tumor necrosis factor (TNF-α) or interleukin-1 (IL-1), cellular activation, and inflammatory gene transcription by means of monoclonal antibodies, soluble cytokine receptors and natural antagonists [2]. Biological therapies prove to be efficacious, highly specific and better tolerated than standard treatment. Rituximab (RTX) is among the

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wide spectrum of available biological treatments [3–5]. RTX is a chimeric mouse/human monoclonal antibody that targets the transmembrane protein CD20 molecule expressed on the surfaces of pre-B and mature B lymphocytes. It leads to the apoptosis of these cells with antibody- and complement-dependent cytotoxicity. This mechanism contributes to a selective peripheral B cell depletion for more than 24 weeks [5]. There is some evidence suggesting that B cells play an important role in the pathogenesis of RA [6,7].

In acute and chronic inflammatory conditions, increased synthesis of some positive acute phase glycoproteins has been observed to be accompanied by alterations in their microheterogeneity [8–10]. It seems that the regulation of synthesis and glycosylation of these glycoproteins is mediated by tumor necrosis factor (TNF-α), some interleukins and transforming growth factor (TGF) β1 [11–13]. In chronic inflammatory process in the course of RA, also many alterations in the expression of plasma proteins, as well as their posttranslational modifications (including glycosylation) can occur [14,15]. Changes in the relative proportions of the transferrin variants, reflecting alterations in glycosylation, have already been described in pregnancy and in several chronic diseases, including RA, hemochromatosis and cancer [16,17]. Over the last three years we have also examined the profile of transferrin isoforms in a few diseases, including RA [18–22]. Transferrin is a negative acute phase protein and due to its microheterogeneity has become a good model to investigate the shift in the relative proportions of its isoforms, reflecting changes in glycosylation. Its microheterogeneity is determined by different sialic acid content in N-linked oligosaccharide chains (nine isoforms are possible, from asialo- to octasialotransferrin).

Previously, we found alterations in the profile of serum transferrin isoforms in rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). In the current study, taking into consideration the changes in protein glycosylation, as well as, new treatment regimens, we wanted to assess this profile in RA patients treated with biological drugs. Thus, the purpose of our study was to determine the diagnostic usefulness of transferrin isoform measurements in RA patients in monitoring rituximab therapy.

2. Material and methods

2.1. Subjects

The study group consisted of 20 selected patients (16 females and 4 males), aged 24–67 years with rheumatoid arthritis treated at the Department of Rheumatology and Internal Diseases, Medical University of Bialystok. The diagnosis of RA was confirmed according to the ACR 2010 classification criteria [23]. The mean duration of the disease was 9 years (range 2–30 years). The patients were RF-positive. They had a past history of lack of response to conventional non-biological disease-modifying antirheumatic drugs (DMARDs) (including methotrexate) as a first-line treatment and an inadequate response to the first biological drug – anti-TNF-α throughout a 6-month course of treatment (second-line treatment). Therefore, they were qualified for further therapy with the second biological drug (third-line treatment). The decision of treatment dose and schedule was made by a treating physician on the basis of DAS28 ESR score. The patients underwent one 6-month course of rituximab (RTX, MabThera®, Roche Products Ltd) therapy. Repeated treatment could be administered after 24 weeks [24]. The study subjects received 1000 mg of RTX in combination with methotrexate (MTX), administered twice by intravenous infusion on days 1 and 15, two weeks apart. MTX was given orally in a dose of 20–25 mg/week. Approximately half of the patients received methylprednisolone. RA activity was evaluated by disease activity score DAS28 ESR. DAS28 is a key outcome measure to evaluate the response to therapies and is commonly used in clinical trials and treat-to-target strategies. The treatment was monitored and assessed with DAS28 ESR, every six months. All the patients qualified for biological therapy had a high RA activity (DAS28 > 5.1). The study group started with a DAS28 ESR average score of 6.185 (high activity) before treatment with a prompt decrease to an average of 3.125 (low activity) in the 6th month, after therapy. An adequate response is defined as an improvement in DAS of ≥1.2 points – moderate or good response. All RA patients achieved complete disease remission. The control group consisted of 20 healthy volunteers (10 males and 10 females), aged 21–54 years (mean age 30). They did not receive any treatment and did not exhibit any symptoms of rheumatic diseases.

Table 1
Characteristics of RA patients at start and after therapy.

Clinical and laboratory data	Before therapy	After therapy	P value
Number of patients (N)	20	20	–
Gender (F/M)	16/4	16/4	–
Disease duration (years)	9/2–30	9/2–30	–
Age (years)	53.4/24–67	53.4/24–67	–
RF positive (%)	100	100	–
ESR (mm/h)	42.5/30–96	12/8–20	< 0.001
CRP (mg/L)	15.1/8.3–26.1	2.75/1.2–5.40	< 0.001
Hb (g/dL)	13.0/11.0–16.40	13.55/ 10.60–16.80	0.023
RBC ($x10^{12}$/L)	4.29/3.04–5.57	4.34/3.66–5.67	0.020
WBC ($x10^{9}$/L)	6.55/3.36–10.9	6.67/3.86–11.0	0.642
PLT ($x10^{3}$/μL)	293.5/177–411	288.5/210–381	0.816
DAS28-ESR (median/range)	6.19/5.69–7.26	3.13/1.94–3.94	< 0.001
DAS28-ESR (N/%):			
- Remission (< 2.6)	0/0	8/40	–
- Low activity (2.6–3.1)	0/0	3/15	
- Moderate activity (3.2–5.1)	0/0	9/45	
- High activity (> 5.1)	20/100	0/0	

Data are medians and range. The differences between data before and after therapy (Wilcoxon signed rank test).
Significant differences at $P < 0.05$.

Patients were interviewed regarding their use of alcohol as some isoforms of transferrin may change during alcohol abuse. Detailed characteristics of RA patients are presented in Table 1. Written informed consent was obtained from patients after explanation of the nature of the study. The study was approved by the local research ethics committee for Medical University of Bialystok (R-I-002/400/2017).

2.2. Sample collection

Blood samples were taken immediately before drug administration and after 3 and 6 months therapy. The sera were separated by centrifugation at 1500 × *g* for 10 min, frozen and stored at −86 °C until analysis. Besides serum, a portion of each blood sample was collected into 2 tubes containing liquid sodium citrate (for determination of erythrocyte sedimentation rate – ESR) and EDTA-2 (for haematological analysis). Biochemical findings such as CRP, RF and anti-CCP were measured on the Architect ci8200 analyser (Abbott, USA) by immunochemical method. Blood cell counts was determined on the Sysmex XS-800i analyser (Sysmex, Singapore).

2.3. Determination of transferrin isoforms

The isoforms of transferrin were separated by capillary electrophoresis on a MINICAP electrophoretic system used the MINICAP CDT reagent kits (Sebia, Evry, France) into five major fractions according to their sialylation level: asialotransferrin, disialotransferrin, trisialotransferrin, tetrasialotransferrin and pentasialotransferrin (Fig. 1). The system calculates relative concentrations (%) of each fraction automatically. The within-run precision of transferrin isoforms measurement by this method was as follows – for disialotransferrin: CV = 8.90% at the mean value of 0.97%, for trisialotransferrin: CV = 3.10% at the mean value of 3.17%, for tetrasialotransferrin:

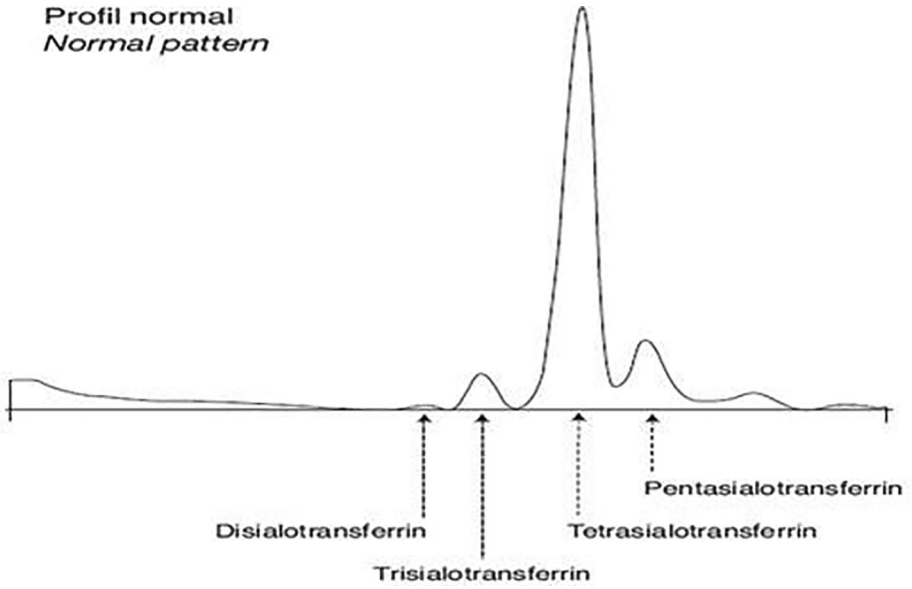

Fig. 1. Normal pattern of transferrin isoforms.

CV = 1.40% at the mean value of 81.1% and for pentasialotransferrin: CV = 5.98% at the mean value of 14.88%.

2.4. Statistical analysis

Statistical analysis was performed using Statistica 12 PL (StatSoft, Poland). The results were given as medians and range. To compare differences between clinical and laboratory data before and after treatment in the same patient, the Wilcoxon signed rank test was used. The differences between healthy control subjects and RA patients after treatment and between RA moderate and high activity groups were evaluated using the Mann-Whitney *U* test. The correlation between variables was assessed by Spearman's rank correlation coefficient. The results were considered to be statistically significant when p-values were less than 0.05.

3. Results

The distribution of transferrin isoforms is given in Table 2. There were significant differences in the relative concentration of transferrin isoforms in patients before and after treatment. We found a significant increase in trisialotransferrin and pentasialotransferrin concentrations after the 3-month (4.40% versus 3.50%, p = 0.012 and 16.5% versus 14.5%, p = 0.005, respectively) and 6-month (4.10% versus 3.50%, p = 0.009 and 17.70% versus 14.5%, p = 0.006, respectively) therapy with rituximab in comparison to these before treatment. In contrast, the relative concentrations of tetrasialotransferrin after the 3- and 6-month therapy were significantly lower than these before treatment (80.3% versus 81.5%, p = 0.009 and 78.4% versus 81.5%, p = 0.008, respectively. No significant differences were noted in the concentrations of transferrin isoforms between RA patients after 6-month therapy and healthy control subjects (disialotransferrin: 0.50% versus 0.60%, P = 0.140, respectively; trisialotransferrin: 4.10% versus 3.65%, P = 0.605, respectively; tetrasialotransferrin: 78.4% versus 78.1%, P = 0.790, respectively and pentasialotransferrin: 17.7% versus 17.2%, P = 0.958, respectively). Since this paper is a continuation of our previous work on the profile of transferrin isoforms in rheumatic diseases, we decided to present our earlier results that are opposite to the current ones. Previously we observed a significant decrease in the relative concentrations of trisialotransferrin and pentasialotransferrin, and a significant increase in tetrasialotransferrin when compared to the control group (2.13% versus 3.61%, p < 0.001; 13.56% versus 18.61%, p < 0.001 and 83.64% versus 76.84%, p < 0.001, respectively).

Table 2
The results of transferrin isoforms in healthy control and RA patients before and after 3- and 6-month therapy.

Healthy control/RA patients	Disialo-Tf (%)	Trisialo-Tf (%)	Tetrasialo-Tf (%)	Pentasialo-Tf (%)
Healthy control	0.60	3.65	78.1	17.20
	0.30–5.40	1.60–5.60	65–84.7	11.1–32.8
Before therapy	0.50	3.50*	81.5*	14.5*
(n = 20)	0.20–2.10	0.90–5.00	73.6–85.0	10.0–22.1
	P = 0.916	P < 0.001[c]	P < 0.001[c]	P < 0.001[c]
After 3-month therapy	0.60	4.40*	80.3*	16.5*
(n = 20)	0.30–3.50	1.30–7.70	72.0–84.6	12.0–23.8
	P = 0.284[a]	P = 0.012[a]	P = 0.009[a]	P = 0.005[a]
After 6-month therapy	0.50	4.10*	78.4*	17.7*
(n = 20)	0.20–1.30	1.50–5.90	71.1–83.0	11.8–24.3
	P = 0.834[a]	P = 0.009[a]	P = 0.008[a]	P = 0.006[a]
	P = 0.059[b]	P = 0.726[b]	P = 0.779[b]	P = 0.499[b]
	P = 0.140[d]	P = 0.605[d]	P = 0.790[d]	P = 0.958[d]

Data are medians and range. The differences between transferrin isoforms before and after 3- and 6-month therapy (Wilcoxon signed rank test).
Significant differences at P < 0.05:
[a] when comparing RA patients before and after 3- or 6-month therapy.
[b] when comparing RA patients after 3- and 6-month therapy.
[c] when comparing healthy control and RA patients before therapy.
[d] when comparing healthy control and RA patients after 6-month therapy.

There were no significant differences in transferrin isoforms levels between the 3- and 6-month follow-up. Before the treatment, among the transferrin isoforms, only the concentration of tetrasialotransferrin correlated with DAS28 ESR (R = 0.507, p = 0.038), but none of them correlated after therapy. At baseline, median DAS28 ESR was high and significantly decreased from 6.19 to 3.13 after the start of treatment. It was a statistically significant difference (p < 0.001). It means that all patients achieved complete disease remission. Due to the fact that after the 6-month therapy we found RA patients with a low (DAS28 ESR < 3.2, median: 2.59, range: 1.94–2.97, n = 10) and moderate (DAS28 ESR 3.2–5.1, median: 3.54, range: 3.28–3.94, n = 10) disease activity, we wanted to find out if there were any relative differences in sialylated transferrin between these groups. We found no significant differences in transferrin isoforms between RA patients with low and moderate disease activity after the 6-month-therapy (disialotransferrin:

0.55% versus 0.50%, P = 0.736, respectively; trisialotransferrin: 4.00% versus 4.10%, P = 0.908; tetrasialotransferrin: 78.35% versus 78.4%, P = 0.487, respectively; pentasialotransferrin: 15.7% versus 18.0%, P = 0.693, respectively). Trisialotransferrin relative concentration negatively correlated with Hb (R = −0.790, p = 0.019), while pentasialotransferrin with PLT (R = −0.762, p = 0.028) after treatment.

Mean ESR and CRP levels at treatment start were 42.5 mm/h and 15.1 mg/L, respectively (Table 1). These levels decreased from baseline over time (12 mm/h and 2.75 mg/L, respectively, p < 0.001 for both comparisons). There was a significant increase in the following hematological parameters from baseline to the 6-month therapy: Hb: 13.0 g/dL versus 13.55 g/dL, p = 0.023 and RBC: 4.29×10^{12}/L versus 4.34×10^{12}/L, p = 0.020.

4. Discussion

Alterations in glycosylation of serum glycoproteins have been observed in several pathological conditions [14,15,18–22]. Disturbances in this process play a key role in the pathogenesis, progression and diagnosis of many disorders. This paper is a continuation of our scientific interests focused on the profile of transferrin isoforms in rheumatic diseases [19,22,25,26]. Previously, we examined the transferrin fractions in RA adult patients and in children with juvenile idiopathic arthritis (JIA) [19,22]. In the current study we investigated the profile of transferrin isoforms in RA patients treated with a biological drug – rituximab. Thus, we wanted to determine the diagnostic usefulness of transferrin isoform measurements in these patients for monitoring rituximab therapy. To our knowledge this is the first study investigating the effects of rituximab therapy on the profile of serum transferrin isoforms.

The present study revealed significantly higher relative concentrations of trisialo- and pentasialo- and lower levels of tetrasialotransferrin in patients with rheumatoid arthritis after 3- and 6-month rituximab treatment in comparison to those before therapy. Our earlier research on RA patients showed opposite results, i.e. lower concentrations of trisialo- and pentasialo- and a higher level of tetrasialotransferrin. We postulate that rituximab treatment leads to the normalization of the transferrin isoform profile. Thus, we can say that the present results are in line with our previous findings. The level of disialotransferrin was normal in the previous study and did not change after therapy. We also found a significant reduction in inflammatory markers and disease activity, indicating that a single course of treatment with rituximab was effective in reducing the inflammation of rheumatoid arthritis in RA patients. Surprisingly, the glycosylation pattern (only tetrasialotransferrin) correlated with disease severity prior to treatment, but not afterwards. The lack of this correlation may be associated with complete disease remission and reduced inflammation connected with decreased ESR level and return of acute-phase protein to baseline. Transferrin as a negative protein of acute-phase reaction, that has been reduced in RA patients, now returns to normal. Another explanation may be also the shift in the glycosylation profile of transferrin (tetrasialotransferrin back to baseline). It would be very interesting to see the relative differences in the sialylated transferrin isoforms in RA patients with different disease activity (different DAS28-ESR categories) prior to treatment. However, our subjects showed high RA activity and it was only them who qualified for further therapy with the second biological drug – rituximab. After the 6-month therapy, however, we separated 2 categories of RA patients with low and moderate activity. We found no differences in the profile of transferrin isoforms between these disease categories.

As we mentioned earlier, RA patients before biological therapy with rituximab received also other medicines, such as methotrexate alone or combined with methylprednisolone as a conventional non-biological disease-modifying antirheumatic drug (DMARD). Therefore, we could not divide this study group into subgroups according to the drug received to check its potential effect on transferrin sialylation. However, this seems to be irrelevant as biological therapies are applied in combination with a conventional DMARD, e.g. methotrexate. As methotrexate was given before and after RTX therapy, its potential impact on changes in the sialylated transferrin profile could be neglected. Therefore, we postulate that the significant alterations in the sialylated transferrin profile were caused by rituximab.

We also included a group of healthy subjects, not receiving RTX, in our study, to compare their results with the findings obtained from RA patients biologically treated with RTX. Lack of differences in the profile of transferrin isoforms confirmed its normalization after treatment, thus indicating that the drug affects transferrin sialylation.

These results show that the normalization of the transferrin isoform profile is associated with disease remission after rituximab therapy and resolution of inflammation. It is known that chronic inflammatory conditions, including RA, are characterized by increased or decreased synthesis of several positive or negative acute phase glycoproteins and are accompanied by changes in glycosylation. Therefore, the regression of chronic inflammation restores tissue homeostasis and normalizes acute phase proteins [8–10,27]. Transferrin is considered to be a negative acute phase response and is a good subject to present the phenomenon of microheterogeneity due to its structural variation in the two N-linked oligosaccharide chains (nine isoforms) [28]. Changes in the relative proportions of the transferrin isoforms reflect alterations in glycosylation and can be accompanied by changes in their functional properties [16,17,24,27,29]. In our previous study, some of the transferrin isoforms were altered in RA patients treated with conventional therapy, in contrast to this study. Interestingly, the same fractions showed opposite directions of changes and returned to normal values. Therefore, we can speculate that the main cause of transferrin profile normalization would be complete resolution of inflammation (confirmed by reduced inflammatory markers). It is known that the regulation of both synthesis and glycosylation is mediated by tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6) and transforming growth factor β1 [11–13,30]. Cytokines are key modulators of inflammation, taking part in acute and chronic inflammation *via* a complex network of interactions [31]. In general, an excess of some cytokines is important in the pathogenesis of many diseases, especially those with an inflammatory background, such as rheumatoid arthritis that is an autoimmune condition characterized by chronic inflammation leading to tissue destruction [31]. Therefore, it can be expected that reduced cytokine production or neutralization of the already existing cytokines may have a beneficial effect on the course of these disorders [32]. Currently, there are a number of biological therapies that target the pro-inflammatory cytokines such as tumour necrosis factor alpha (anti-TNF-α agents), interleukin 1 (IL-1 receptor antagonist agents), interleukin 6 (anti-IL-6 receptor antibody agents), or are B cell depleting or T cell co-stimulant inhibitors [33]. Rituximab targets the transmembrane protein CD20 molecule expressed on the surface of pre-B and mature B lymphocytes [4]. It leads to the apoptosis of these cells with antibody- and complement-dependent cytotoxicity and to a selective peripheral B cell depletion for more than 24 months. B-cell repopulation after depletion therapy occurs after 6–9 months. Promotion of rapid and long-term depletion of B lymphocytes results in reduced recruitment of these effector cells at sites of immune complex deposition, thus reducing inflammation and tissue damage [4].

It is known that serum profile of transferrin isoforms is clearly associated with iron status in the body, because transferrin is an iron-carrying glycoprotein. In healthy people this pattern is normal, but in pathological conditions it may be altered [34–37]. Some authors observed changes in transferrin isoforms in RA patients [38,39]. Feelders et al. [39] noticed an evident shift in transferrin isoforms in the sera of non-anaemic RA patients, iron deficient RA patients and RA patients with anaemia of chronic disease involving increased synthesis of transferrin with highly branched glycan chains. These alterations were characterized by a significant increase in the percentage of highly sialylated transferrin isoforms, a significant decrease in

tetrasialotransferrin, and a reduction in the level of low sialylated fractions [39]. In our study, all RA patients had no anaemia before treatment, as shown by normal haemoglobin (Hb) levels. Nevertheless, Hb concentration and RBC (red blood cell) count were significantly increased after treatment. We observed a significant increase in relative concentrations of trisialo- and pentasialo- and a decrease in tetrasialotransferrin in RA patients after 3- and 6-month therapy. These changes in the transferrin isoform profile were contradictory to those observed in our previous research and resulted from the disease remission. It seems that the increased synthesis of pentasialotransferrin is involved in the compensatory mechanism attempting to facilitate iron transport to erythroblasts and leads to increased levels of RBC and Hb in RA patients after therapy. Highly sialylated transferrin isoforms have a higher affinity for transferrin receptors on erythroblasts surface, improving iron transport into the cell. We can say that the rituximab therapy is beneficial for improving iron stores, as evidenced by an increase in haemoglobin. Since no literature data are available in this field, further studies are needed in a group of patients with both RA and anaemia. We observed a negative correlation between pentasialotransferrin and platelet (PLT) number and also a negative relationship between trisialotransferrin and Hb after treatment.

In conclusion, the present study showed that treatment with rituximab of RA patients alters the serum profile of transferrin isoforms. Measurements of tri-, tetra- and pentasialotransferrin relative concentrations can be a useful tool for monitoring the therapy. Monitoring of transferrin glycosylation after therapy initiation allows evaluation of treatment efficacy and helps answer the question whether the cause of the disease has dissolved. In our opinion it is a better marker of the effectiveness of biological therapy, especially in the early stage of RA, and seems to be a more sensitive parameter than DAS28 as alterations in the glycosylation pattern occur earlier than in ESR.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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