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Original Article

Complement Activation Profiles in Patients with Juvenile Idiopathic Arthritis

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Abstract

Objectives: Besides T- and B-cells also the innate immunity is playing an effective role in the pathogenesis of Juvenile idiopathic arthritis (JIA) reported in literature. The complement system (CS) is activated by the three different ways classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). This is a controlled prospective observational single center study focused on the three pathways of CS and its effector, the terminal complement complex (TCC), associated with disease activity in all clinical JIA subgroups.

Methods: Peripheral blood (PB) samples (n=159) of 60 JIA patients (partially also longitudinal visits) were analyzed for specific complement pathway activation (COMPL300 ELISA), complement factor H (CFH)-autoantibodies (CFHAb ELISA), and the soluble TCC (sC5b-9 ELISA) in serum (S) and EDTA plasma (P). TCC was also analyzed in synovial fluid (SF) of JIA patients (n=9) controlled with samples of healthy adults (n=12) obtained by arthroscopy (primarily gonarthrosis). As healthy controls (HC) adults (n=100) and children (n=18) without inflammatory diseases were tested. The JADAS10 defined the acute phase of the disease.

Results: We compared the elevated serum TCC levels of JIA patients in the acute phase of the disease (13.32 IQR 8.09-19.48; p<0,001) with the controls' levels (7.78 IQR 4.93-10.31). Subgroup analyses showed statistically significant (s.s.) elevation in PArf+, OAper, and OAext. COMPL300 analyses revealed that JIA patients with acute disease had lower CP (82% IQR 37-97% vs 105% IQR 98-115%; p<0.001), LP (31% IQR 2-86% vs 80% IQR 20-101%; p<0.001), and AP (25% IQR 2-88% vs 85% IQR 70-98%; p<0.001) capacity compared to the HC average due to chronic activation in all three pathways. Both TCC and COMPL300 analyses during acute phase and remission showed a clear tendency, especially in PArf+ but only s.s. in the individual course. Remarkable sJIA patients neither in TCC nor in COMPL300 analyses showed complement activation. No evidence of CFH-autoantibodies was found in our study group. The TCC levels in SF showed a median of 2.48 IQR 1.71-2.76 AU/ml in JIA patients and surprisingly high levels in HC with 6.7 IQR 3.9-9.24 AU/ml.

Conclusion: Specific subgroups of JIA patients (extOA, PARf+, ERA) showed increased CS activation in TCC in PB in the acute course of the disease. Furthermore, the decreased CS capacity, especially in the CP and AP, confirms the consumption of complement components as an additional contributor to the disease pathogenesis and acute course. Due to the CS activation throughout all disease activity states, we could not determine s.s. between the acute and remission phases in our cohort. However, COMPL300 in combination with TCC seems to be a helpful acute disease biomarker in the individual course.

The pharmacological modulation of CS components might be a valid alternative approach in treatment-resistant patients, although further investigations are needed.

Introduction

Juvenile Idiopathic Arthritis encompasses all forms of chronic inflammatory arthritis with an onset before 16 years of age [1,2 and symptoms persisting for at least six weeks [2], according to t the International League of Associations for Rheumatology (ILAR). JIA is the most common chronic rheumatic disorder in children [3].

The seven different clinical subgroups defined by the ILAR can be listed as follows: The largest subgroup, oligoarthritis (OA), accounts for approximately 65% of cases and is characterized by an early onset, before the age of six. Its diagnosis has to be further explored after six months to distinguish between persistent oligoarthritis (OAper) (four or fewer joints affected) or extended oligoarthritis (OAext) (more than four joints affected). Additionally, a large number of patients develop positive antinuclear antibodies (ANA), a high risk of chronic iridocyclitis, and often a specific HLAassociation (e.g., HLA DRB1*01, DRB*08 and DRB*11) [1,4,5]. Polyarticular JIA involves more than five joints in the first six months and is divided into rheumatoid factor positive polyarthritis

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(PARf+) with detectable anti-citrullinated protein antibodies (ACPA) and rheumatoid factor negative polyarthritis (PARf-). Both forms can share similar features with OAext or present symmetrical synovitis of large and small joints, school-age onset, and negative ANA [1,3,6-8]. Enthesitis related arthritis (ERA) is a subgroup with a clear, distinguishable cluster of symptoms predominantly involving the hip and including most forms of childhood spondyloarthritis [9]. The disease is commonly observed in children older than six, and most patients are HLA-B27 positive [10]. Juvenile psoriatic arthritis (PsA) is defined by the simultaneous presence of arthritis and a typical psoriatic rash [2,3]. Its clinical presentation, as early as two years of age, is more common among females with expression of ANAs, dactylitis, and small joint involvement. Later onset is associated with enthesitis, axial joint disease, and persistent oligoarthritis [11]. Systemic juvenile idiopathic arthritis (sJIA) also known as "Still's disease," is a very distinctive Interleukin (IL)-1- and IL-6-mediated condition potentially leading to macrophage activation syndrome (MAS) [2,12,13]. Periodic febrile states typically precede or accompany joint swelling, protract for at least three days, and occur along with one or more of the following symptoms: splenomegaly and/or hepatomegaly, erythematous rash, generalized lymph node enlargement, and serositis.

JIA is deemed a multifactorial disease, originating from genetic features (HLA-types), mutations in cytokine genes regulation like TNF-Alpha [14-16], inappropriate activation of T- and B-cells with deregulation of the adaptive immune system [16,17], and especially in sJIA an overproduction of the proinflammatory cytokine IL-6, Il-1, and IL-18 [6,13,18] and strong involvement of macrophages linked to a fatal complication the macrophage activation syndrome (MAS) [19,20].

Research has focused on TNF-alpha as a potent inflammatory functions contributor and target of therapy [21-23], and the innate immune system, with its major component, the complement system. Previous studies on adult patients with RA showed complement activation in the blood [24,25] and a high turnover of C3, C4, and C5 in inflamed joints [26,27]. Further investigations on the complement activation in peripheral blood [28,29] and synovial fluid (SF) [30] of patients with JIA revealed evidence of activation, primarily through the classical (CP) and alternative pathway (AP) of the complement system [31].

The complement system in RA and JIA

The complement system is a well-orchestrated mechanism that bridges the innate and adaptive immune systems and consists of more than 50 fluid- and membrane-associated proteins [32,33]. There are three pathways of complement activation: the classical pathway (CP) initiated when the C1q subcomponent binds to IgG or IgM-bearing immune complexes [34], the mannose-binding-lectin pathway (MBLP), activated by interaction of mannan-binding lectin-associated serine proteases (MASP) with mannose groups on the surface of microbes, and the alternative pathway (AP) which needs neither an antibody nor another binding ligand, but is induced by a spontaneous cleavage of C3 into C3a and C3b, and a subsequent circuit amplification step of C3 convertase to cleave more C3 molecules. The regulation of this pathway is crucial to prevent over-activation through an active complement system . The C3b element can bind to all plasma-exposed surfaces and therefore initiate autoimmune responses. This regulation comes through cofactors (factor I, factor H, CD46) on the surface of host cells that bind and dismantle the active cleavage components. (33) All three pathways lead to the formation of the MAC and production of anaphylatoxins by the cleaved proteins (C3a, C4a and C5a) [33].

A growing body of research on the role of the complement system in the pathogenesis of rheumatic diseases converges on its involvement in the pathogenesis of arthritis in general. The inappropriate or uncontrolled activation of the complement system especially influences the pathogenesis and clinical course of rheumatoid arthritis [26,35]. C5a, a potent chemoattractant, plays an important role in the inflammatory process within an affected joint. The second cleavage product of C5, C5b and its induction of the TCC also affect pathogenesis. In clear contrast to JIA, RA patients often have RFs, a clear hint to the autoimmune origin of the disease. Okroj and Heinegård [27] described the pathogenesis of RA as a reaction between antigens on the cartilage and different antibodies. This reaction can lead to the formation of immune complexes that can react with complement receptors and, therefore, activate the system [36]. Another complement system activation trigger in RA is the cartilage molecule fibromodulin that binds directly to C1q and activates the classical pathway [27]. This mechanism becomes important after initial cartilage destruction.

The central role of the complement system in Juvenile Idiopathic Arthritis is still subject to research. Several papers showed clear evidence of the involvement of the complement system in the pathogenesis of JIA. On the one side, researchers proved that different subtypes of JIA have a different activation patterns in specific complement pathways, and on the other side, they determined the general activation of parts like C3 or CRP [28,37]. Additionally, their efforts showed that JIA patients, especially those diagnosed with polyarthritis, had higher levels of IgM antibodies than the control groups. Polyarthritis patients also had an increased expression of complement receptor 1 (CR1 on activated monocytes and B-lymphocytes) [28]. The role of CR1 in the pathogenesis of JIA remains unclear, as it is typically a negative regulator of the complement system. Prokopec et al. [28] interpreted the increased level of CR1 as a regulation attempt that the complement system starts to minimize the damage caused by activation. Elevation of CR1 expression on B-cells is among the major differences between rheumatoid arthritis patients and JIA patients. Besides CR1 activation, these studies suggested the critical role of C3a as a chemoattractant in the pathogenesis of polyarticular JIA [28,38]. Aggarwal and Bhardwaj [39] postulated that the elevated sC5b-9 levels in most JIA patients indicate the activation of the complement system. The involvement of the classical pathway in JIA remains to be elucidated. A poster presentation at the Pediatric Rheumatology Symposium by Gilliam and Reed [40] suggested the involvement of the classical pathway in the pathogenesis of JIA. A recently published review hypothesized the involvement of several pathways and parts of the complement system [36]. Ultimately, Petri et al. theorized a connection between the lectin pathway and oligoarticular and systemic JIA [41].

This controlled prospective observational study focused on the signs of activation/deficiency of the three pathways of CS and its effector, the terminal complement complex (TCC). TCC levels in the blood and synovial fluid of JIA patients correlated with disease activity and inflammatory markers differentiated among the JIA subgroups.

Patients and Methods

Collection of blood samples of JIA patients

The pediatric rheumatologists used the ILAR classification [2] to sort 60 JIA patients into subgroups. Our study cohort consists of seven patients with sJIA (20 samples), four PArf-

positive (22 samples) and 12 PArf-negative (38 samples) patients, eight patients with OAext (20 samples), 20 patients with OAper (43 samples), five with ERA (10 samples) and four patients with PsA (6 samples).

Sample treatment: Blood samples (n=159) from 60 patients (18 male and 42 female) with a median age of 13 years (range: 1 to 17 years) were collected at the Department of Pediatric Rheumatology, Innsbruck Medical University Hospital, Austria. Native serum and EDTA blood were centrifuged at 2000 rpm for 10 minutes to remove cells, separated in aliquots, and stored at -20°C (or after six months at -80°C) immediately after blood draws to minimize in vitro activation of the CS.

Samples of synovial fluid (preparation described below) from 10 patients (two male and eight female) were collected during routine ultrasound-guided injections of swollen knees during the active phase of the disease and treated as described below.

Patients inclusion criteria: Written informed consent (parents and patients, if possible), diagnosis of Juvenile Idiopathic Arthritis by the treating pediatric rheumatologist, according to the ILAR criteria.

Exclusion criteria: Acute viral or bacterial infection, active medical treatment for chronic diseases other than JIA.

TCC was measured in bough blood samples, centrifuged plasma, and serum if separated immediately after blood draw. COMPL300 could only be analyzed in serum. We excluded some samples from the statistical analysis to ensure the best clinical plausibility and minimize pre-analytic failure.

Treatment regimens: During clinical remission, 13 patients received no further treatment, 13 patients received NSAIDs, 21 patients received DMARDs, five patients were treated with oral corticosteroids, seven patients received DMARDs and oral corticosteroids, and 22 patients were treated with biologicals (with or without DMARDs).

Besides the TCC ELISA assays [42] of adult controls (n=100) aged 19-64 (56 female and 46 male), we tested those of healthy children (n=18) aged 2-18 (10 female and eight male) for sC5b-9 levels. Samples from children controls were collected during routine diagnostic procedures on admitted patients without inflammatory or complement-activating diseases. The blood draws for diagnostic evaluation were performed after obtaining parents' and young patients' informed consents: written informed consent no diagnosed acute or chronic diseases no regular use of anti-inflammatory medicine, not pregnant.

Collection and preparation of synovial fluids

Human synovial fluid (SF) was obtained through joint aspiration in nine (n=9) JIA patients in the acute phase of the disease, along with twelve samples of SF obtained through arthroscopy from healthy adults without acute inflammatory disease or RA. SF was first centrifuged at 5000 RPM and 4°C for 25 minutes. The supernatant was pipetted in an Eppendorf tube and frozen at -20°C. Because of the special biological matrix and its non-Newtonian nature, the preparation protocol required SF treatment with hyaluronidase to improve ELISA assay quality. SF was again centrifuged at 1000 RPM for 10 min. The projection was pipetted in an Eppendorf tube and hyaluronidase (HAse) was added 1:1. The HAse concentration was 4mg/ml, diluted in a phosphate buffer (0,02M, PH7 + 77mM NaCL and 0,01% BSA) as recommended by Jayadev and Rout [43]. The solution was incubated at room temperature for one hour in a shaker and again centrifuged at 3000 RPM for 5 minutes. The samples were tested by sC5b-9 ELISA kits to detect TCC.

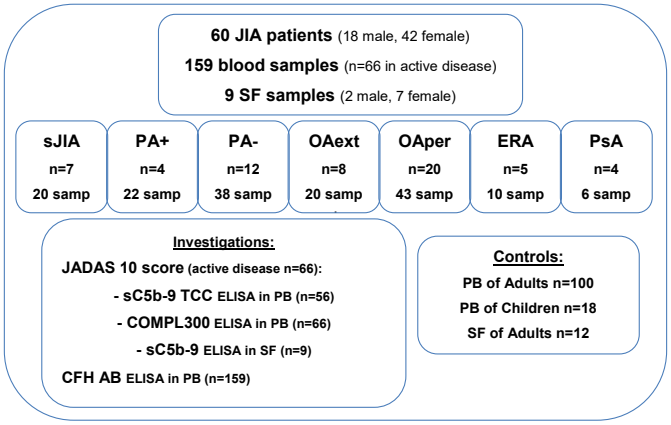


Table 1. *Overview of analyzed samples.*

Nearly all blood samples in the acute phase of the disease could be tested with the sC5b-9 ELISA, complement pathway activity Wieslab© Complement System Screen kit (COMPL300) (Figure 1).

Definition of inactive and active disease

We applied the American College of Rheumatology provisional criteria for defining clinical inactive disease in selected categories of Juvenile Idiopathic Arthritis to ascertain active and inactive states at the time of blood draws [44]. The criteria for clinical remission were: no joints with active arthritis, no fever, rash, serositis, splenomegaly or generalized lymphadenopathy, no active uveitis, ESR or CRP level within normal limits or not attributable to JIA; a physician's global assessment of disease activity, with the best possible score on the adopted scale,and duration of morning stiffness of less than 15 minutes. Pediatric rheumatologists evaluated disease activity using the JADAS10 Score to detect the acute phase [45].

Measurement of TCC ELISA (sC5b-9)

The levels of soluble terminal complement complex (TCC), also called sC5b-9, were determined by ELISA in EDTA plasma and serum (46), as previously described by Würzner [46], Prüfer, and Scheiring [47]. Additionally to the blood testing, we established a protocol to measure the levels of TCC in synovial fluid (SF), and adopted the preparation protocol with hyaluronidase by Jayadev et al. [43] to reach a better test quality in ELISA [43]. The antibody named "WU 13-15", Prof. Würzner from the Institute of Hygiene, Microbiology and Social Medicine of the Medical University of Innsbruck, developed against the complement factor 9 was used to rout the sC5b-9 complex to the microtiter plate. The TCC bound and assembled on the cell surface is not measured with this test. The second antibody was directed against C6 or C7, both part of the sC5b-9 complex. The enzyme label added to the third antibody was avidin-phosphatase (aP). The chemical reaction of aP and the used detection substrate p-Nitrophenyl Phosphate resulted in yellow color. The intensity of the color, which directly correlates with the concentration of the soluble TCC, was measured with an ELISA-reader ("bio-rad," microplate reader 680) at 415 nm wavelength with a 490 nm reference filter. The results were converted to concentration values using Microplate Manager and a standard curve of maximal activated normal human serum (concentration 500 AU/ml) by treatment with zymosan [46-50].

Measurement of Wieslab© complement system screen kit (COMPL300)

This commercially available ELISA complement screening kit allows the determination of the three different complement pathways and their activation patterns. Its microtiter plates consist of three wells, each coated with specific activators of the classical (IgM), the MBL (Mannan), and the alternative pathway (Lipopolysaccharides). The diluents contain specific blocking agents for each pathway to prevent cross-reactions. The coatings on the respective wells activate complement by means of sC5b-9 formation. After washing steps, the concentration of sC5b-9 is detected with the same principle of the TCC ELISA. The avidin phosphatase (aP) enzyme is attached to an antibody to detect the antigen-antibody complex bound to the microtiter plate. The chemical reaction between aP and the added substrate results in a specific intensity of yellow color. The intensity of the color is measured with a spectrophotometer ("bio rad," microplate reader 680) with a wavelength of 415 nm and a reference filter of 490 nm. The complement activation for each pathway was expressed as a percentage of the activity of a positive normal human standard serum included in the test kit.

The positive control equals 100%, and the negative control equals 0%. All samples were tested at least twice, and the mean value was used for calculation. The calculated values for all samples directly correlate to the value reached by the positive control and are presented as percentage values. The diagnostic reference levels for this ELISA were determined in a study with 120 healthy adult blood donors [51]. The standard values were defined as the mean percentage of activity minus two times the standard deviation. This calculation sets the standard values for the CP at 74% and for the AP at 39%. The cut-off in the LP was set arbitrarily at 10% because 30% of all healthy blood donor samples were below that threshold [51].

Anti-CFH ELISA

The CFH-ELISA is a sandwich kit for the quantitative analysis of human anti-CFH in serum and EDTA plasma, according to the procedure established by Dragon-Durey, Loirat [52]. The microtiter plate is coated with human Factor H, and anti-factor H antibodies are fixated to the wall of each well.The IgG antibody linked to avidin phosphatase (aP) binds to the fixated antibody-antigen complex. As shown in the TCC ELISA, the aP is an enzyme that converts the substrate (p-nitrophenyl phosphate) through a chemical reaction resulting in yellow color. The intensity of the color directly corresponds to the concentration of antibodies against CFH in the specimen and is measured with an ELISA-reader (bio-rad, microplate reader 680) at 415 nm wavelength with a 490 nm reference filter. The extinction of each sample is converted to concentration values generating a standard curve using a defined amount of the CFH antibody positive sample and using MS Excel for calculation. The standard used in the Anti-CFH ELISA contains positive tested samples of serum, courtesy of Dragon-Durey, Loirat [52] and retested by Watson and Lindner [53]. We applied the standard curve in dilution from 1:100 to 1:6400 in duplicates. The absorbance of the positive plasma reference was used to calculate the arbitrary titer of CFH Autoantibodies.

Statistical analysis

Data and graphs were evaluated with SPSS version 22.0 for MAC and Windows. The statistically significant differences were controlled with the Mann-Whitney-U test. P-values below 0.05 were considered statistically significant.

This single centre study was performed according to the declaration of Helsinki 2000 and approved by the local Ethics Committee (UN 3731).

Results

TCC ELISA (sC5b-9) in controls

As described above, two samples were excluded from the calculation step of TCC evaluation as the related results were clinically invalid due to pre-analytical errors (n=116/118). The 116 healthy controls median TCC level was 7.78 AU/ml in serum (IQR 4.83 – 10.32 AU/ml) and 1.22 AU/ml in plasma (IQR 0.78 – 1.82 AU/ml) without normal distribution (Kolmogorov-Smirnov test $p<0.001$). There were no significant gender differences in serum and plasma Biomarker levels (Mann-Whitney U test $p<0.27$ and $p<0.45$) (Figure 2). Because of the large age distribution of the healthy blood donors compared to our pediatric patients, we created six subgroups with a 10-year cut-off to analyze TCC levels by age intervals. The oldest group

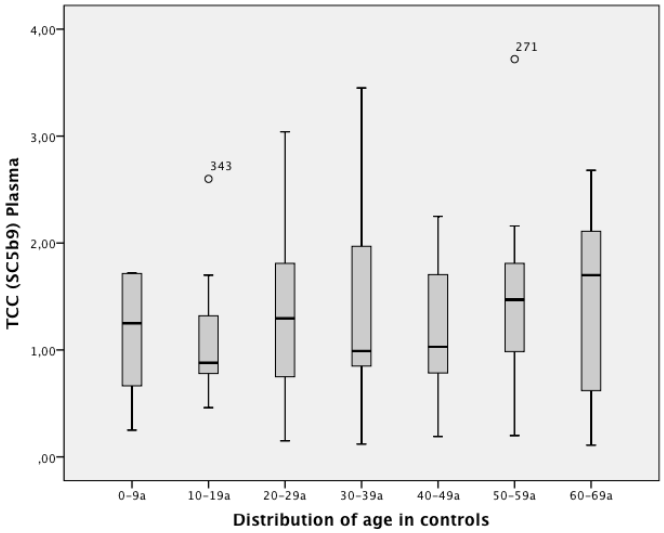


Figure 2. *TCC distribution by age in the control group including children*

Table 1. *TCC concentration in the control group. The table shows the values and distributions of the TCC in the blood samples in male and female control samples.*

	TCC serum AU/ml	TCC plasma AU/ml
Controls tested (n)	116	116
Median	7.78	1.22
Interquartile range	4.90 – 10.32	0.78 – 1.81
Minimal	0.16	0.11
Maximal	59.1	10.4
Female tested	63	63
Median in female	8.84	1.23
Male tested	53	53
Median in male	7.72	1.07
gender difference	n.s.	n.s.

Table 2. *Median TCC levels for each subtype of the JIA in clinical remission*

Classification JIA	TCC in serum (AU/ml)		TCC in plasma (AU/ml)	
sJIA (in median)	7.67 (IQR 4.35-11.23)	n.s.	0.42 (IQR 0.28-1.34)	p<0.007*
n=(remission/total)	n=11/20		n=11/20	*sJIA<HC
PArf + (in median)	10.94 (IQR 8.9-13.3)	p<0.027	0.71 (IQR 0.50-1.46)	n.s.
	n=11/22		n=11/22	
PArf - (in median)	11.06 (IQR 8.18-11.91)	p<0.011	0.63 (IQR 0.41-0.83)	n.s.
	n=14/38		n=14/38	
OAext (in median)	14.76 (IQR 10.67-19.96)	p<0.001	2.67 (IQR 0.77-4.46)	n.s.
	n=12/20		n=12/20	
OAper (in median)	14.71 (IQR 8,98-17.85)	p<0.001	2.08 (IQR 0.95-3.62)	n.s.
	n=15/43		n=15/43	
ERA (in median)	11.15 (IQR 6.74-13.76)	n.s.	0.63(IQR 0.51-0.93)	n.s.
	n=4/10		n=4/10	
PsA (in median)	14.15 (IQR 10.44-18.98)	n.s.	3.52 (IQR 2.06-3.93)	n.s.
	n=3/6		n=3/6	
All subgroups (median)	11.35 (IQR 8.18-14.84)	p<0.001	0.83 (IQR 0,74-2.94)	n.s.
	n=70/159		n=70/159	
Controls (in median)	7.78 (IQR 4.93-10.31)		1.22 (IQR 0.79-1.81)	
	n=116		n=116	

exhibited a slight increase in TCC levels t with no significant difference among the age groups (Kruskal-Wallis test p<0.35, 0.79, 0.89, 0.86) (Table 1).

We performed a sensitivity analysis excluding the oldest group (>50 years) and repeated the statistical analysis on our patient cohort. All results were reproducible and statistically significant, although the higher levels of complement activation in the oldest subgroup would have reduced the differences within the patient cohort.

TCC ELISA (sC5b-9) in JIA patients

JIA patients in clinical remission

We excluded some samples due to errors during the above-mentioned pre-analytical phase to ensure the maximum clinical validity of the results. Subsequently, we could analyze 148 blood samples from 55 patients (40 female and 15 male) during the regular clinical visits in all stages of the disease. The data of the study population in clinical remission showed no statistically significant differences in TCC levels between female (S 11.21; IQR 8,5-14.81 AU/ml and P 0.81; IQR 0.91-1.91 AU/ml) and male (S 12.32; IQR 7.18-15.67 AU/ml and P 1.68; IQR 0.4-3.48 AU/ml) JIA patients' serum and plasma.

The serum TCC levels of JIA patients in clinical remission/without active disease were significantly higher (11.35 IQR 8.18-14.84 AU/ml) compared to the controls' (7.78 IQR 4.9-10.3 AU/ml, median (p<0.001)). As seen in the control group, the TCC plasma concentrations were inferior in all tested patients with a lower mean variation resulting from a smaller IQR. There was no statistically significant difference between the JIA patients (0.83 AU/ml; IQR 0.74-2.94) and the control group (1.22 AU/ml, IQR 0.78-1.81) (Tab 2). The Kruskal-Wallis test showed a significant difference among the subgroups (p<0.001 in serum and p<0.001 in plasma). The highest median serum and plasma TCC levels were observed in both OA subgroups and the second clinical group of PA. Statistical significance to HC could only be confirmed in serum samples. Surprisingly, no elevated TCC could be detected in sJIA patients' blood, and TCC levels in plasma were significantly lower than the HC's.

TCC concentration in clinically active and inactive disease

JIA patients during clinically active disease showed statistically higher TCC levels in serum (13.32; IQR 8.09-19.48 AU/ml) than the control group (7.78, IQR 4.9-10.3 AU/ml). However, this could not be statistically confirmed in plasma despite a clear tendency.

As listed in Table 3 and illustrated in Figure 4 and 5 the OAext, OAper, and PArf+ subgroups registered the highest, statistically significant TCC levels in serum during the clinical remission phase. However, only the OAper category manifested significantly elevated levels in plasma compared to the HC. Similar to the patients in remission, the sJIA subgroup did not exhibit elevated TCC in serum and plasma.

The critical issue was the role of TCC during active and inactive phases of a disease involving complement activation. Collective TCC measurements in all subgroups yielded no statistically significant difference in disease state between JIA active (S 13.49 IQR 8.93-19.55 / P 1.41 IQR 0.63-3.09 AU/ml in median) and inactive (S 11.32 IQR 8.18-14.78 / P 0.82 IQR 0.47-2.22 AU/ml in median) patients. Individual subgroup analyses could not confirm a statistically significant difference between active and inactive JIA patients despite a clear tendency in PArf+ patients' serum, as listed in Table 3.

Complement screening kit - COMPL 300

The test process included aliquots of patient serum samples collected during the TCC ELISA preparation step to exclude augmented in-vitro activation. We compared JIA patients with HC and subsequently considered cut-off values for complement

Table 3. *TCC concentrations in active disease of the subgroups in JIA study group.*
** TCC levels of the clinical subgroups compared to the healthy controls (Mann-Whitney-U test)*

Classification JIA	TCC serum (AU/ml)	vs HC vs inactive*	TCC plasma (AU/ml)	vs HC vs inactive*
sJIA (in median)	7.35 (IQR 5.76-9.12)	n.s.	0.42 (IQR 0.39-0.53)	n.s.
n=(active/total)	n=3/20		n=3/20	
PArf + (in median)	17.43 (IQR 12.07-19.55)	p<0.001	1.75 (IQR 0.63-2.93)	n.s.
	n=9/22	n.s.*	n=9/22	n.s.*
PArf - (in median)	10.08 (IQR 5.2-13.15)	n.s.	0.86 (IQR 0.36-1.19)	n.s.
	n=15/38	n.s.*	n=15/38	n.s.*
OAext (in median)	14.09 (IQR 9.11-20.23)	p<0.005	1.75 (IQR 1.28-3.6)	n.s.
	n=7/20	n.s.*	n=7/20	n.s.*
OAper (in median)	15.1 (IQR 13.27-21.38)	p<0.001	1.99 (IQR 0.87-4.16)	p<0.017
	n=18/43	n.s.*	n=18/43	n.s.*
ERA (in median)	11.53 (IQR 5.77-17.3)	n.s.	1.61 (IQR 0.67-2.55)	n.s.
	n=2/10		n=2/10	
PsA (in median)	12.58 (IQR 7.36-17.82)	n.s.	1.17 (IQR 0.71-1.64)	n.s.
	n=2/6		n=2/6	
All subgroups (median)	13.32 (IQR 8.09-19.48)	p<0.001	1.32 (IQR 0.63-3.00)	n.s.
	n=56/159	n.s.*	n=56/159	n.s.*
Controls (in median)	7.78 (IQR 4.93-10.31)		1.22 (IQR 0.79-1.81)	
	n=116/118		n=116/118	

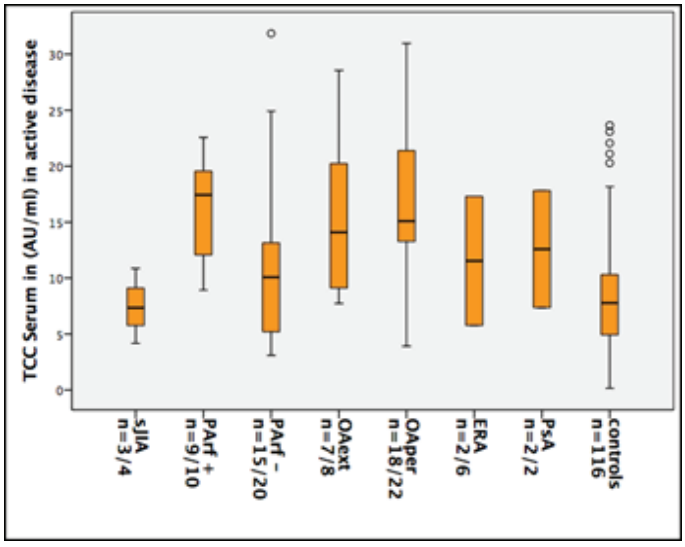


Figure 3. *TCC serum concentrations in active disease of the subgroups in JIA study group.*

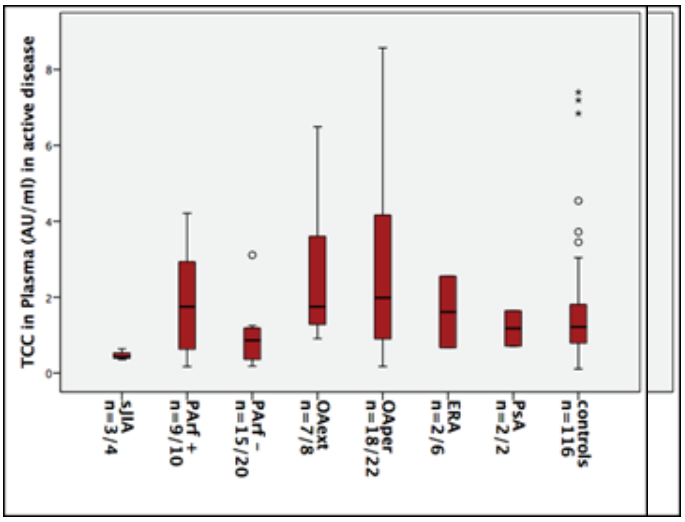


Figure 4. *TCC plasma concentrations in active disease of the subgroups in JIA study group.*

deficiency reported by Seelen and Roos [51]. Reduced activation ability (expressed in percentage from the standard control) can prove, on the one hand, general deficiency of complement factors or, on the other hand, ongoing complement activation with consumption of complement factors for each specific complement pathway (CP, LP, AP) [37]. The Kolmogorov-Smirnov test (p<0.001) showed no normal distribution in the classical and lectin pathways, therefore we used median and IQR for statistical analyses (Mann-Whitney-U-test for calculation of p-value) on all three pathways. The levels showed no statistically significant differences in the CP and LP between male (n=43/91) and female (n=48/91) subjects but significantly lower AP levels in female (p<0.003) controls (82%, IQR 68-93% female vs. 94%, IQR 74-109% male).

Also the levels in JIA patients (n=142) showed no normal distribution (Kolmogorov-Smirnov test p<0.001) and no statistically significant difference between males and females in all three pathways. Our study groups' complement activity

median levels for each pathway are listed in Table 5 (remission) and Table 6 and shown in Figure 6 (active disease).

Classical Pathway (CP) – 74% cut-off value

JIA patients in the acute phase of disease had a noticeably ($p<0.001$) lower capacity with a median of 82% IQR 37-97 compared to the control group with 105% IQR 98-115. Additionally, 30 of the 66 tested samples in the acute phase of disease (46%) were under the 74% cut-off value. PArf-positive, OAext, OAper, and ERA patients had the lowest mean capacity levels, in contrast with the control group. No significant difference emerged despite the apparent lower capacity

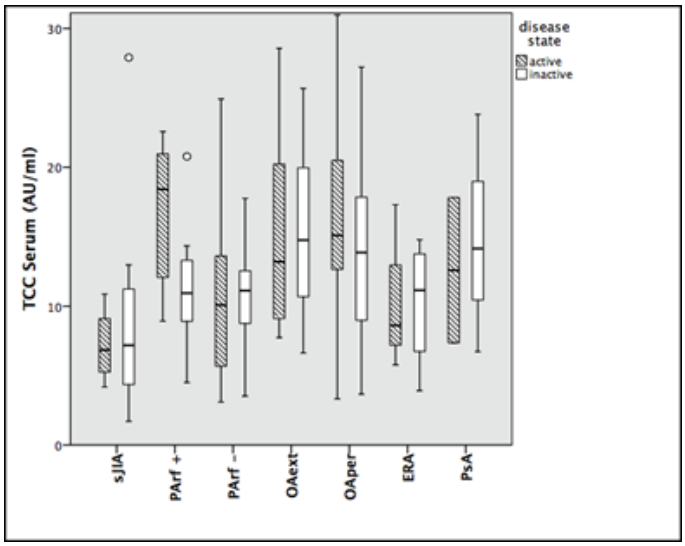


Figure 5. *TCC levels of JIA subgroups in clinical active and inactive disease*

Lectin Pathway (LP) – depending on MBL plasma concentration

Selen and Roos [57] arbitrarily set the cut-off value of the LP at 10% because one-third of their controls were below that limit. We noticed corresponding levels in our control group, as 25 out of 66 samples (38%) were below said value. In the JIA patients in the acute phase of the disease, the median capacity was 31% IQR 2-86, as opposed to the HC with 80% IQR 20-101, implying statistical significance ($p<0.042$). OAext and ERA patients showed the lowest capacity levels in the LP, substantially inferior to the HC. Similarly, PArf+ patients showed appreciably lower lectin pathway capacity levels in the acute phase compared to subjects in clinical remission ($p<0.043$).

Alternative Pathway (AP) – 39% cut-off value

As for the AP, JIA patients' capacity in the acute phase was 25%, IQR 2-88 ($p<0.001$), against 85%, IQR 70-98 of the HC. Thirty-five out of 66 tested samples (53%) were under the 39% cut-off value. As registered in the classical pathway, the lowest median capacity levels found in PArf-positive, OAext, OAper, and ERA patients, significantly differed from the control group. Despite this clear tendency, especially in PArf+ patients in acute disease compared to individuals in clinical remission, we found no significant difference.

The differences between active disease and remission are shown in Figure 7.

Table 4. *TCC concentrations in active disease of the subgroups in JIA study group.*
** TCC levels of the clinical subgroups compared to the healthy controls (Mann-Whitney-U test)*

Classification JIA	TCC serum (AU/ml)	vs HC vs inactive*	TCC plasma (AU/ml)	vs HC vs inactive*
sJIA (in median)	7.35 (IQR 5.76-9.12)	n.s.	0.42 (IQR 0.39-0.53)	n.s.
n=(active/total)	n=3/20		n=3/20	
PArf + (in median)	17.43 (IQR 12.07-19.55)	p<0.001	1.75 (IQR 0.63-2.93)	n.s.
	n=9/22	n.s.*	n=9/22	n.s.*
PArf - (in median)	10.08 (IQR 5.2-13.15)	n.s.	0.86 (IQR 0.36-1.19)	n.s.
	n=15/38	n.s.*	n=15/38	n.s.*
OAext (in median)	14.09 (IQR 9.11-20.23)	p<0.005	1.75 (IQR 1.28-3.6)	n.s.
	n=7/20	n.s.*	n=7/20	n.s.*
OAper (in median)	15.1 (IQR 13.27-21.38)	p<0.001	1.99 (IQR 0.87-4.16)	p<0.017
	n=18/43	n.s.*	n=18/43	n.s.*
ERA (in median)	11.53 (IQR 5.77-17.3)	n.s.	1.61 (IQR 0.67-2.55)	n.s.
	n=2/10		n=2/10	
PsA (in median)	12.58 (IQR 7.36-17.82)	n.s.	1.17 (IQR 0.71-1.64)	n.s.
	n=2/6		n=2/6	
All subgroups (median)	13.32 (IQR 8.09-19.48)	p<0.001	1.32 (IQR 0.63-3.00)	n.s.
	n=56/159	n.s.*	n=56/159	n.s.*
Controls (in median)	7.78 (IQR 4.93-10.31)		1.22 (IQR 0.79-1.81)	
	n=116/118		n=116/118	

Table 5. *COMPL300 - complement pathways median activity expressed in percentage for the JIA subgroups (in clinical remission), with the calculation of significant difference to controls.*

JIA subgroup	CP in %	Vs HC	LP in %	Vs HC	AP in %	Vs HC
sJIA (median)	80% IQR 43-92	p<0.001	63% 43-118	n.s.	29% IQR 2-75	p<0.001
n=(remission/ total)	n=14/20		n=14/20		n=14/20	
PArf + (median)	86% IQR 41-108	n.s.	101% IQR 62-121	n.s.	85% IQR 8-93	n.s.
	n=11/22		n=11/22		n=11/22	
PArf - (median)	85% IQR 73-113	p<0.006	61% IQR 33-118	n.s.	67% IQR 28-127	n.s.
	n=16/38		n=16/38		n=16/38	
OAext (median)	56% IQR 52-79	p<0.001	7% IQR 1-29	p<0.001	7% IQR 1-50	p<0.001
	n=12/20		n=12/20		n=12/20	
OAper (median)	49% IQR 16-76	p<0.001	14% IQR 2-69	n.s.	7% IQR 1-57	p<0.001
	n=15/43		n=15/43		n=15/43	
ERA (median)	41% IQR 1-93	p<0.014	33% IQR 2-78	n.s.	3% IQR 1-27	p<0.001
	n=4/10		n=4/10		n=4/10	
PsA (median)	96% IQR 58-110	n.s.	1% IQR 1-14	p<0.018	40% IQR 20-89	n.s.
	n=3/6		n=3/6		n=3/6	
JIA pts (median)	74% IQR 39-92	p<0.001	43% IQR 4-97	p<0.042	31% IQR 1-80	p<0.001
	n=75/159		n=75/159		n=75/159	
Controls (median)	105% IQR 98-115		80% IQR 20-101		85% IQR 70-98	
	n=91		n=91		n=91	

Anti-CFH ELISA

This ELISA was designed to detect antibodies against the complement factor F (CFH), the most important regulator of the alternative pathway. We tested all samples at least twice and analyzed the concentration of CFH-antibodies using the ELISA screening kit. Two patients of our study group initially showed borderline detecTable CFH autoantibodies. In an additional testing we performed serial dilutions of the serum sample with negative results, consequently no CFH autoantibodies in our cohort could be found.

Synovial fluid

We tested nine samples from seven female and two male JIA patients, listed in Table 7. Due to the lack of normal TCC values in SF and the impossibility of recruiting healthy pediatric controls, we investigated samples from adults with gonarthritis without inflammatory joint disease during planned arthroscopies The results showed a median TCC of 2.48 IQR 1.71-2.76 AU/ml in JIA patients and surprisingly higher median levels in HC with 6.7 IQR 3.9-9.24 AU/ml. The majority of samples (7/9) from patients with OAper showed a median TCC of 1.9 IQR 1.35-2.48 AU/ml. One patient with PsA had a TCC of 3.7 AU/ml in SF, and one sample from a patient with OAext showed the highest TCC level with 8.2 AU/ml.

Discussion

This is the first study dealing with detailed analysis of the CS in JIA. Due to the long pre-analytical steps, COMPL300 analyses of SF samples yielded invalid results. All samples showed low capacity in all three pathways. We assume in the100 minutes preparation interval, the samples underwent in vitro activation of the complement system and showed a non-existing complement activation capacity. Complement activation products are currently under investigation in the SF.

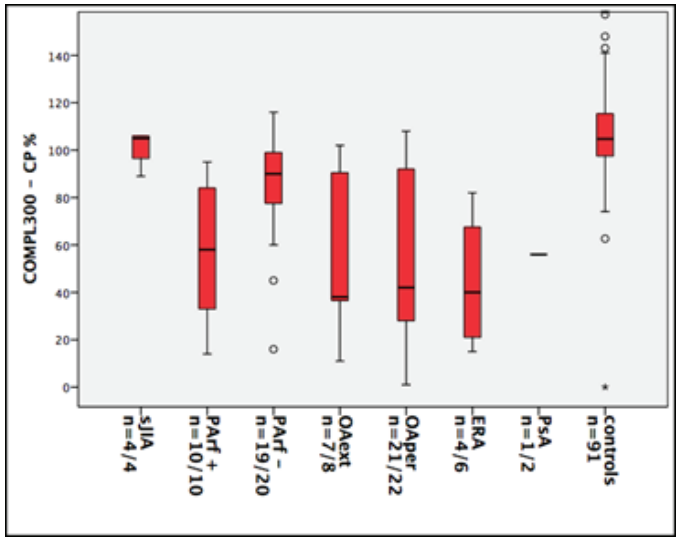
Rapid pre-analytical handling of hemolytic samples is crucial in most complement analyses for a correct interpretation of TCC results in PB. We excluded some HC samples (n=2/118) and some of the patients (n=11/159) to generate clinically valid results. As for most reliable specimen for in vivo representative measurements, Mollnes (55) proposed the use of Ethylenediaminetetraacetic acid (EDTA) plasma in the field of in vitro diagnostics as EDTA inhibits complement activation. We used immediately separated and frozen native serum to investigate the three different pathways using the COMPL300 ELISA. The consequent in vitro activation in all three pathways led to the formation of TCC and indicated the CS capacity in each pathway. The serum samples (stored at -20°C) assayed for TCC concentrations revealed six-fold higher levels than in EDTA plasma in both HC and JIA patients. Ergo, TCC levels in native serum confirmed the statistically significant complement activation in our JIA patients' EDTA plasma and consequently seems a more sensitive specimen. However, the literature does not contain comparable findings. Due to the insufficient number of pediatric HC, we integrated adult blood donors. The age-subgroup analysis showed no statistically significant difference in TCC levels among the age groups (0-18y, 20-29y, 30-39y, 40-49y, 50-59y), and the TCC levels can be considered representative of our study population. Former studies [31,37] measured different complement components in synovial fluid and showed intra-articular complement activation. Therefore, we also tested the nine synovial fluid samples for TCC-levels to infer the complement system activity within the affected joints.

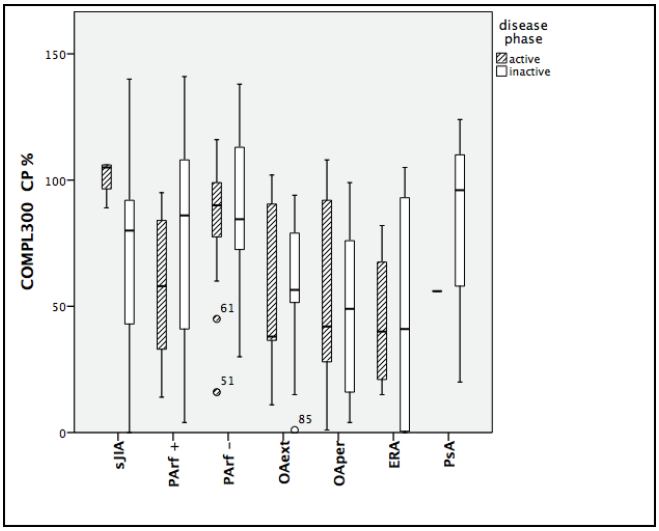
Although the etiology of JIA is not fully understood, the complement system is described as the major contributor to the disease pathogenesis [28,37,39,56]. Our study corroborates this hypothesis as markedly elevated TCC concentrations were

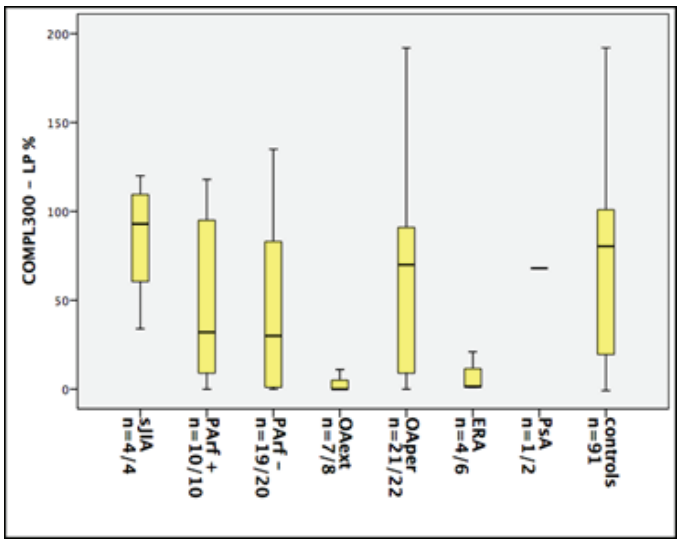
Figure 6. *COMPL300 - complement pathways median activity expressed in percentage for the JIA subgroups in the acute phase of disease and HC*

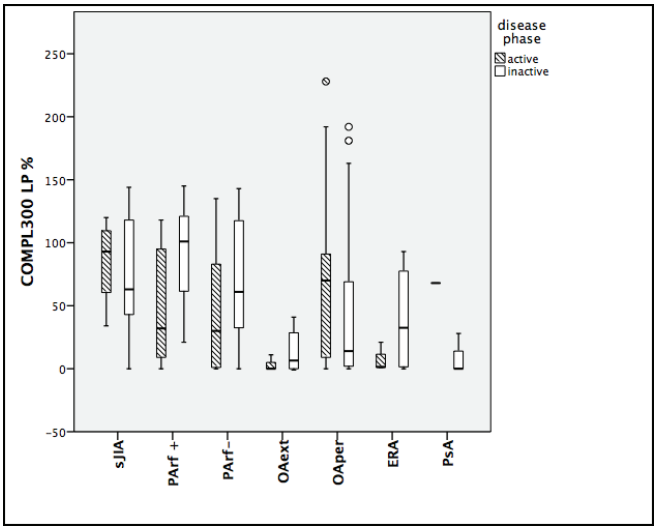
Figure 7. *COMPL300 - complement pathways median activity expressed in percentage for the JIA subgroups in the acute phase of disease and HC*

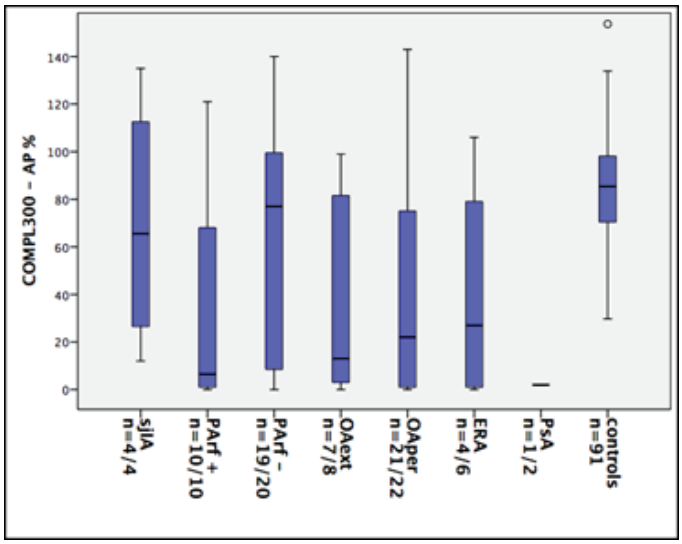
COMPL300 in JIA in active disease











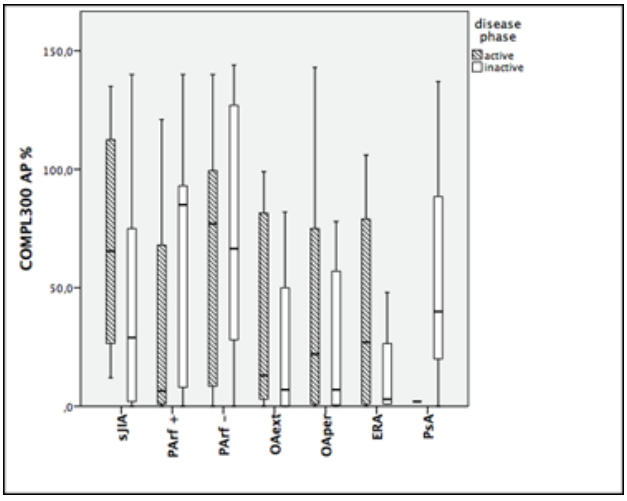


Table 6. *COMPL300 - complement pathways median activity expressed in percentage for the JIA subgroups (in active disease) with the calculation of significant difference to controls*

JIA subgroup	CP in %	vs HC vs inactive*	LP in %	vs HC vs inactive*	AP in %	vs HC vs inactive*
sJIA (median)	105% IQR 97-106	n.s.	93% IQR 61-110	n.s.	66% IQR 27-113	n.s.
n=(active/total)	n=4/20	n=4/20	n=4/20		n=4/20	
PArf + (median)	58% IQR 33-84	p<0.001	32% IQR 9-95	n.s.	7% IQR 1-68	p<0.001
	n=10/22	n.s.*	n=10/22	p<0.043*	n=10/22	n.s.*
PArf - (median)	90% IQR 78-99	p<0.001	30% IQR 1-83	n.s.	77% IQR 9-100	n.s
	n=19/38		n=19/38		n=19/38	
OAext (median)	38% IQR 37-91	p<0.001	1% IQR 0-5%	p<0.001	13% IQR 3-82	p<0.001
	n=7/20		n=7/20		n=7/20	
OAper (median)	42% IQR 28-92	p<0.001	70% IQR 9-91	n.s.	22% IQR 1-75	p<0.001
	n=21/43		n=21/43		n=21/43	
ERA (median)	40% IQR 21-68	p<0.001	2% IQR 1-12	p<0.015	27% IQR 1-79	n.s.
	n=4/10		n=4/10		n=4/10	
PsA (median)	56%	p<0.043	68%	n.s.	2%	p<0.022
	n=1/6		n=1/6		n=1/6	
JIA pts (median)	82% IQR 37-97	p<0.001	31% IQR 2-86	p<0.005	25% IQR 2-88	p<0.001
	n=66/159		n=66/159		n=66/159	
Controls (median)	105% IQR 98-115		80% IQR 20-101		85% IQR 70-98	
	n=91		n=91		n=91	

Table 7. *TCC Levels in synovial fluid in healthy controls (HC) and JIA patients during the acute phase of the disease.*

	SF of patients	SF of HC	OAper	OAext	PsA
samples tested (n)	9	12	7	1	1
Median TCC AU/ml	2.48	6.7	1.9	8.2	3.7
interquartile range	1.71 – 2.76	3.9 – 9.24	1.35 – 2.48		
female tested (n/N)	7 / 9 (78%)				
Age in years	11.4y (6-20y)				

found in serum, especially in the acute phase of the disease. Thus, TCC remains a highly sensitive activity marker. We could not confirm that the acute phase of the disease entails a significantly increased complement activation and consumption compared to clinical remission. This shortcoming could result from the remaining or ongoing CS activation in both clinical stages considered in our study. Nevertheless, despite abnormal differences in TCC levels among subjects, the disease course in one of our PArf+ patients showed significant differences in TCC in serum between the acute phase, especially during the initial manifestation, and remission (p<0.048). A similar circumstance was already noted by Jarvis et al. [57].

The JIA subtypes analysis displayed complement activation in OAper, OAext, and PArf+ in the acute phase and clinical remission. Research has predominantly focused on complement activation in OA and PA, the largest JIA subgroups. We assumed that as systemic JIA affects multiple organs and joints, it would trigger the most aggressive inflammatory response. Our efforts could not confirm this hypothesis as we found relatively similar levels of TCC and COMPL300 outcomes both in our patients and controls. Petri et al. [41] examined specific lectin pathway markers in sJIA and discovered elevated M-ficolin, MASP-1, and MASP-3 in plasma and SF with localized traces in the joints. Despite this finding, the LP capacity was not different from our controls' in the active and remission phases. Our cohort did not show any statistically significant correlation between TCC concentration and disease activity, which could be explained by the consistently elevated TCC levels during clinically active and inactive disease, especially in the OAper and OAext subgroups. Only the PArf+ subjects exhibited higher TCC in the active phase than in remission, although not statistically significant due to the low number of patients (nine out of 22 with PArf+ in active disease). According to Vakeva and Meri [58], C5b-9 deposition is an activation marker for in situ in tissues, They defined the sC5b-9 complexes as usually formed in circulation and as an inactive form of the TCC on the cell membrane. Therefore, complement staining of synovial tissue could give further information about the in situ activation of the complement system. Nonetheless, Høgåsen and Mollnes [59] state that as result of complement activation in the fluid phase,

sC5b-9 is formed in the absence of the target cell surface and can therefore be seen as a complement activation marker. Although the sC5b-9 is classified as cytolytically inactive, a study has shown that it may trigger a pro-inflammatory milieu through osteoprotegerin expression and release by endothelial cells in patients with RA [60]. Besides its function, all previous studies suggest that measurable TCC levels reflect ongoing complement activation. The missing difference in TCC concentrations between clinically active and inactive disease in our study group may be interpreted as ongoing complement activation, regardless of the clinical state. Therefore, complement activation might represent a subclinical inflammation marker.

The result of our COMPL300 complement screening kit for specific complement pathway deregulation showed a debasement of functional activity in the alternative and the classical pathway. Thirty-one out of 49 blood samples (63%) showed levels below the 74% cut-off value in the classical pathway, and 37/49 blood samples (76%) showed levels below the 39% cut-off value in the alternative pathway. An abnormal pathway activation detected via COMPL300 is interpreted as a deficiency or chronic activation in the corresponding pathway [51]. The elevated TCC levels are evidence of systemic complement activation, and the COMPL300 screening indicates the alternative and classical pathway as the origin of activation. Brunner and Prelog [37] described the alternative pathway as the main contributor to the pathogenesis of oligoarticular JIA. Our findings confirm this postulation for all subtypes of JIA. The main activator of the classical pathway is C1q, the recognition subunit of the C1 complex. C1q normally triggers the classical pathway after opsonization through IgG- or IgM-bearing immune complexes, but additionally, newer studies have shown that a wide range of structures such as CRP, apoptotic cells, or extracellular membrane-associated receptors are recognized by C1q [34,35,61]. Comparably to the alternative pathway, the decreased complement capacity for the classical pathway can be interpreted as a high expenditure of complement components.

The constant turnover rate of the complement system confirmed by elevated TCC-levels suggests the involvement of the complement system within the pathogenesis or the course of JIA. The fundamental question is the cause of the complement system activation. Former studies have shown that all three pathways can be activated through apoptotic and necrotic cells [62,63]. As Happonen and Heinegard mentioned [61], necrotic and apoptotic cells regulate the complement activation through binding of complement inhibitors, but the high amount of dying cells or a disturbance in regulator uptake may cause an overstimulation of the complement system. Additionally, the authors showed that in RA patients, various extracellular matrix proteins released by cleavage during joint damage could also trigger the complement system. Although their results relate to RA patients, the pathophysiological process of cartilage damage and its relevance in complement activation is equivalent in JIA patients. As mentioned before, the sC5b-9 formation through complement activation can trigger pro-inflammatory responses and enhance complement activation [60]. While there is complement activation through cartilage damage within the joint, the COMPL 300 shows decreased complement activation capacity in the peripheral blood. The decreased activity within the mentioned pathways most likely is a result of high consumption. Gilliam and Reed's [40] analysis of 100 blood samples revealed activation of the classical pathway through circulating immune complexes and therefore proposed said pathway as the main contributor in the pathogenesis of JIA. The decreased activity we noticed in the classical pathway supports this argument. In summary, the AP and CP seem to be the activated pathways of the complement system in JIA

The first measurements of synovial fluid (SF) samples showed no valid results in simple dilution of SF with PBS-BSA. As mentioned before, we adapted the pre-analytical protocol established by Jayadev and Rout [43] to optimize measurements and serial dilution curves. The tests we conducted on the same serum with and without hyaluronidase to assess the effect of the enzyme on the complement factors, including the sC5b-9 complex, yielded no significant differences. Additionally, the comparison between the amino acid sequence cleaved by the hyaluronidase and the sequences of the complement proteins forming the TCC, produced no results, and no interference with our TCC test kit could be shown. The interpretation of our results remains challenging. All samples were collected during intra-articular corticosteroid injection and therefore present high local disease activity with clinical symptoms such as swelling, pain, and impaired movement. Four samples from patients with persistent oligoarticular JIA had a median TCC level of 1.62AU/ml, which was not significantly elevated compared with the blood specimen we simultaneously analyzed. The SF of the patient with Lyme arthritis and that of the single patient with extended oligoarticular JIA showed TCC levels of 9.8AU/ml and 8.89AU/ml, respectively Additional tests on more SF specimens are needed to draw a clinically relevant conclusion on intra-articular activation of the complement system. In 1991 Brodeur and Ruddy [64] showed that the mean synovial fluid levels of sC5b-9 were about eight times higher in RA than in crystal-induced arthritis and about sixteen times higher than in degenerative joint disease (DJD). Although they used a different analysis method, their cohorts could be potential comparison groups. Our future research endeavors will focus on arthroscopy and joint puncture to test more SF samples and explore TCC concentrations, regardless of underlying conditions.

Conclusion

Specific subgroups of JIA patients (extOA, PARf+, ERA) showed increased CS activation in TCC in PB in the acute course of the disease. Furthermore, the decreased CS capacity, especially in the CP and AP, confirms the consumption of complement components as an additional contributor to the disease pathogenesis and acute course. Due to the CS activation throughout all disease activity states, we could not determine s.s. between the acute and remission phases in our cohort. However, COMPL300 in combination with TCC seems to be a helpful acute disease biomarker in the individual course.

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Conflict of Interest

The authors do not have any conflicts of interest

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