Inflammation and Immune Suppression following Protein Losing Enteropathy after Fontan Surgery Detected by Cytomics

József Bocsi, Dominik Lenz, Ursula Sauer, Lena Wild, John Hess, Dietmar Schranz

Pediatric Cardiology, Heart Center Leipzig, University of Leipzig, Germany
Clinic of Anesthesiology, Children’s Hospital, University of Leipzig, Germany
Pediatric Cardiology, German Heart Center Munich, Germany
Pediatric Cardiology, Heart Center Leipzig, University of Leipzig, Germany

Summary

Background: Protein losing enteropathy (PLE), the enteric loss of proteins, is a potential late complication after total cavopulmonary connection (TCPC – Fontan circulation) surgery. PLE etiology is poorly understood, but immunological factors seem to play a role. This study aimed to gain insight into immune phenotype alterations following post-TCPC PLE.

Patients and Methods: Patients were studied over a period of up to 5 years after surgery. During routine follow-up, blood samples of TCPC patients without (n = 21) and with manifest PLE (n = 12) and of age-matched healthy children (control, n = 22) were collected. Routine laboratory, immune phenotype and serological parameters were determined.

Results: Following PLE the immune phenotype dramatically changed with signs of acute inflammation (increased neutrophil and monocyte count, C-reactive protein, serum IL-8 and complement activation). In contrast, the lymphocyte count (NK cells, αβ T cell receptor-positive (TCR+) CD4+ cells, αβ TCR+ CD8+ cells) decreased (60–80%, p < 0.001). The residual T cells had elevated CD25 (IL-2R) and CD69 expression. In PLE patients unique cell populations with CD3− αβγδ TCR− and αβ TCR+ CD4− CD8− double negative phenotype were present in increased frequencies.

Conclusions: Our studies show for the first time a dramatically altered leukocyte phenotype, the appearance of double negative cells and the alteration of serum compounds after PLE in TCPC patients. These alterations resemble to changes in autoimmune diseases such as systemic lupus erythematosus and celiac disease. We conclude that autoimmune processes may play a role in the etiology and pathophysiology of PLE.

Key Words
Protein losing enteropathy · SLE · Systemic lupus erythematosus · CD · Cluster of differentiation · Natural killer cells · NK cells · CRP · C-reactive protein

Zusammenfassung

Hintergrund: Das Eiweißverlustsyndrom (protein losing enteropathy; PLE) zeichnet sich durch einen massiven Verlust an Serum-Proteinen aus. Vor allem bei Patienten mit einer Fontan-Kreislauf-Operation (total cavopulmonary connection; TCPC) kann sie als mögliche späte Komplikation auftreten. Die genauen Ursachen der PLE sind unbekannt. Allerdings scheinen immunologische Faktoren eine Rolle zu spielen.

Patienten und Methoden: Die Patienten wurden über einen Zeitraum von bis zu 5 Jahren nach der Operation studiert. Im Rahmen der Nachsorgeuntersuchung wurde Blut von TCPC-Patienten (n = 21), von Patienten mit manifest PLE (n = 12) sowie von gesunden Kindern gleichen Alters (Kontrolle, n = 22) entnommen. Routine-Blutparameter und serologische Parameter wurden ermittelt und eine Immunphänotypisierung durchgeführt.


**Introduction**

Protein losing enteropathy (PLE) is a complication in diverse autoimmune diseases such as systemic lupus erythematosus (SLE) and celiac disease. PLE is also a feared late complication of total cavopulmonary connection (TCPC or Fontan circulation) surgery for congenital heart disease. Its etiology following Fontan surgery is still poorly understood. The TCPC procedure was developed to separate the systemic and pulmonary circulation in patients with tricuspid atresia and was later extended to single ventricles [1]. Some of these patients exhibited a substantial decrease of the serum protein level and an excessive fecal secretion of protein, sometimes associated with lymphangiectasia, years after the surgical procedure [2–6]. According to retrospective studies these alterations, referred to as PLE, have a 10-year morbidity of 3.7–13%. The mortality of patients with manifest PLE is 46–59% after 5 years and >80% after 10 years [5, 6]. Single case reports indicate that surgical interventions such as fenestration of the atrial septum or treatment with prednisone or heparin could lead to a relief from the PLE symptoms [7–10]. However, a detailed European study showed that in spite of surgical intervention or medication >60% of the patients still maintain PLE [5]. Understanding the etiology of PLE after TCPC surgery is of substantial importance for the development of treatment strategies.

Systematic investigations correlating the PLE risk after TCPC surgery with clinical parameters described increased systemic venous pressure as a risk factor for PLE [3, 5]. However, a substantial percentage of PLE patients (38%) had no obvious hemodynamic alterations compared to PLE-free patients [3]. Increased pressure as well as disadvantageous hemodynamics may stimulate cells. Neuronal cells, endothelial cells, platelets and leukocytes are sensitive to pressure and/or mechanical stress. This sensitivity can induce intracellular calcium signaling and activation [11]. Cell activation may then lead to elevated release of inflammatory mediators and to further stimulation of the immune system [12]. Concurrently, pressure alterations such as pulmonary hypertension can lead to immunological alterations [13]. Therefore, one inducer of PLE could be an altered activity of the immune system. One indication of an involvement of the immune system is that in some patients immune suppression can at least temporarily resolve PLE [7]. In addition, it was reported in a single case study that PLE after Fontan surgery coincides with decreased cell count of circulating CD4+ T cells but normal counts of CD8+ T lymphocytes [14]. Protein loss and/or lymphangiectasia have been described also for non-Fontan and non-cardiac patients – among others patients with celiac disease [15] or systemic lupus erythematosus (SLE) [16]. These autoimmune diseases are also associated with dramatic changes of the immunophenotype, including preferential loss of CD4+ cells [17, 18]. Furthermore, after PLE with non-cardiac etiology association with infections [19] and additional dramatic changes of the cellular immune system were reported [9]. In the recent years it became evident that for the understanding of complex diseases the understanding of the complex changes of cellular networks is of importance [20]. The systemic investigation of cellular systems is termed ‘cytomics’ [21, 22], and the technology best suited for its investigation is cytometry.

With respect to PLE in TCPC patients there is still a tremendous lack of knowledge in the understanding of the ongoing cellular processes in the disease. The present study reports of flow cytometric immune phenotype and serological changes of pediatric patients following PLE after TCPC. Data of the patients with manifest post-TCPC PLE, PLE-free post-TCPC subjects and healthy, age-matched controls were compared. This information may facilitate the development of treatment strategies for post-TCPC patients suffering from PLE.

**Patients and Methods**

**Patients**

The study was approved by the ethical committee of the University of Leipzig and started in autumn 1995. Written informed consent was obtained from the parents of all patients (inclusion criteria: age 3–18 years). Children < 3 years were excluded in order to reduce the influence of age-dependent changes due to the developing immune system [23]. The clinical data and sampling of the all TCPC patients are summarized in table 1. Ten patients who underwent TCPC surgery in our hospital (average age 6.8 ± 2.6 years at surgery; mean ± SD) were analyzed over a period of up to 5 years starting before surgery and thus yielding also pre-PLE phenotyping values. One of these children developed PLE during the time period of investigation. The PLE group (average age 12.8 ± 4.5 years at sampling) contained 11 patients with manifest PLE (7 patients from the Deutsches Herzzentrum München, 2 patients from the Herzzentrum Gießen and 2 patients who were operated in the Deutsche Herzzentrum Berlin but were followed up in the Herzzentrum Leipzig). For the PLE patients clinical laboratory data before and after the onset of PLE were available from the patients records, allowing to compare serological values before and after PLE onset. A control group of 22 age- and sex-matched, infection-free, healthy children (age 8.6 ± 2.5 years, range 3.0–15.8 years) was recruited at the Department of Anesthesiology of the Children’s Hospital of the University of Leipzig.

**Sampling**

Blood samples were obtained regularly (in 3- to 6-month intervals) during the pre- and postoperative outpatient follow-up. Samples up to 30 days after surgery were ignored to avoid influences of the surgical trauma. Routine laboratory and clinical chemistry parameters (differential blood count, C-reactive protein (CRP), creatinin, electrolytes, protein concentrations, hematocrit, blood coagulation parameters) were determined in all blood samples. In all collections EDTA anticoagulation or coagulation tubes were used.

**Serology**

EDTA blood or blood in coagulation tubes was centrifuged at 2,800 × g for 10 min at 4 °C with supernatant collection. Aliquots were stored at −80 °C within 1 h after sampling. The concentration of the complement components (C3, C4, C5, C1 inhibitor, C3d) and Ig subclasses was determined by radial immune diffusion (The Binding Site, Heidelberg, Germany) with serum or EDTA plasma (C3d) and total hemolytic complement CH100 by lysis of antibody-coated sheep erythrocytes (The Binding Site). All other

---

**Immune Suppression following Protein Losing Enteropathy after Fontan Surgery**

Transfus Med Hemother 2007;34:168–175
Phenotyping of leukocytes was done by the whole blood technique [24, 25]. Cells were spun down at 3000 × g, and the supernatant discarded. The cells were resuspended in 200 μl 0.5% paraformaldehyde (Sigma) in PBS. Antibodies were obtained from BD Biosciences (CD4 (helper/inducer cells), CD8 (cytotoxic-suppressor cells), CD14 (monocytes, granulocytes, LPS receptor), CD45 (pan leukocyte antigen), CD69 (early activation antigen), CD8 T cell receptor (TCR), γδ TCR, HLA-DR (MHC II)), from CalTag, Hamburg, Germany, CD16 (neutrophils, NK-subset, FcγReceptor III), CD19 (B cells), CD25 (IL-2 receptor-α), CD56 (NK subset, N-CAM)), from Beckman-Coulter Corp., Hialeah, FL, USA, (CD45RA (naïve T cell subset, B cells, granulocytes), CD54 (ICAM-1)), or from DAKO, Glostrup, Denmark, (CD3 (T cells), CD45Ro (thymocytes, activated/memory T cells)). Background fluorescence was quantified after staining with appropriate control antibodies (BD Biosciences). Antibodies were labeled with the fluorescent dyes fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP™) or allophycocyanin (APC) in cocktails of three- or four-color combinations. Cells were measured on a dual laser flow cytometer (FACSCalibur; BD Biosciences) calibrated in standardization as suggested [26, 27].

Statistics
Flow cytometric data were analyzed with the CellQuest software package (BD Biosciences). The percentage of leukocyte subsets was quantified, and their cell counts per volume were calculated based on the differential blood count. For each cell subset the mean fluorescence intensity as a measure of antigen expression was determined after subtraction of the background fluorescence of control antibody-stained cells [25]. Mean values were calculated and compared using Student’s t-test or Mann-Whitney U-test as appropriate.

Results

Clinical Laboratory Parameters after PLE
All available routine laboratory parameters from the records of the patients before (at least 2 samples/patient) and immediately at PLE onset (at least 2 samples/patient) were tested. Figure 1 shows only values that were significantly changed after PLE. Hematocrit, hemoglobin concentration and lymphocyte count decreased, but platelet count increased. Total serum protein albumin and γ-globulin concentration decreased; however, α1- and α2-globulin and β-globulin concentrations increased significantly. The serum albumin level remained constant in patients with albumin substitution but decreased without. For 7 PLE patients no pre-PLE values of neutrophil and monocyte counts were available. At PLE onset most of the affected patients suffered from viral, bacterial or fungal infections of heterogeneous origin [28].

Leukocyte Subsets after TCPC and after PLE
The changes of the count of circulating leukocytes and their major subsets are shown in figure 2. Total leukocyte and neutrophil counts were not significantly different in the TCPC groups with and without PLE, but they were significantly higher than in the control group. Monocyte counts changed in a similar fashion as neutrophil counts. These results may indicate an acute inflammatory status with TCPC circulation. The lymphocyte subset was increased in the TCPC patients without PLE, but in TCPC patients with PLE in contrast, they be-

Table 1. Cardiac diagnosis and sampling of patients undergoing TCPC

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pre PLE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RL</td>
</tr>
<tr>
<td>1</td>
<td>DILV</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>Cor univentriculare, PA, ASD</td>
<td>*</td>
</tr>
<tr>
<td>3</td>
<td>TA, VSD, ASD</td>
<td>*</td>
</tr>
<tr>
<td>4</td>
<td>TI, ASD</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>TA</td>
<td>*</td>
</tr>
<tr>
<td>6</td>
<td>DILV, MAG, AVSD, PS</td>
<td>*</td>
</tr>
<tr>
<td>7</td>
<td>PA, TS, HRV</td>
<td>*</td>
</tr>
<tr>
<td>8</td>
<td>TA, MAG</td>
<td>*</td>
</tr>
<tr>
<td>9</td>
<td>TA, MAG, PFO, MI (1°)</td>
<td>*</td>
</tr>
<tr>
<td>10</td>
<td>DORV, Situs inversus</td>
<td>*</td>
</tr>
<tr>
<td>11</td>
<td>HLS</td>
<td>*</td>
</tr>
<tr>
<td>12</td>
<td>TA, MAG</td>
<td>*</td>
</tr>
<tr>
<td>13</td>
<td>MA, VSD</td>
<td>*</td>
</tr>
<tr>
<td>14</td>
<td>Cor univentriculare, DILV</td>
<td>*</td>
</tr>
<tr>
<td>15</td>
<td>MAG, VSD</td>
<td>*</td>
</tr>
<tr>
<td>16</td>
<td>DILV</td>
<td>*</td>
</tr>
<tr>
<td>17</td>
<td>VSD, ASD</td>
<td>*</td>
</tr>
<tr>
<td>18</td>
<td>TA, WPW syndrome</td>
<td>*</td>
</tr>
<tr>
<td>19</td>
<td>DILV, AV Valve atresia</td>
<td>*</td>
</tr>
<tr>
<td>20</td>
<td>HLS</td>
<td>*</td>
</tr>
<tr>
<td>21</td>
<td>TOF, VSD</td>
<td>*</td>
</tr>
</tbody>
</table>

ASD = Atrial septal defect; AVSD = atrial and ventricular septum defect; BAS = balloon atrial septostomy; BTS = Blalock-Taussig shunt; DILV = double inlet left ventricle; DORV = double outlet right ventricle; HLS = hypoplastic left heart syndrome; HRV = hypoplastic right ventricle; MAG = malposition of the great arteries; TCPC = total cavopulmonary connection; MI = mitral insufficiency; PA = pulmonary valve atresia; PFO = persistent foramen ovale; Pheno = phenotyping; RL = routine laboratory; TA = tricuspid valve atresia; TI = tricuspidal valve insufficiency; TOF = tetralogy of Fallot; TS = tricuspidal stenosis; VSD = ventricular septum defect; WPW = Wolff-Parkinson-White.

Enabled.
haved oppositely (fig. 2) and decreased to values 40% lower than those of the controls or 60% lower than those of the PLE-free patients (p = 0.005).

**PLE Patients Lose High Quantities of Cells, Preferentially T Lymphocytes**

The most striking finding of the analysis of the main lymphocyte subsets was that during PLE T cell counts were dramatically reduced by >70% compared to those of TCPC patients without PLE or controls. Moreover, also, the NK cell count decreased by 63% during PLE (fig. 3). B cell counts, in contrast, were significantly increased in TCPC patients, but were not significantly different in the PLE and in the control group.

**Changes of T Cell Subsets after the Onset of PLE**

The most striking finding was that following PLE the cell count of CD3+ αβ TCR+ cells and of CD3+ γδ TCR+ T cells was reduced by 75 and 60%, respectively, compared to the control (fig. 4). We found a discrepancy between the count of T cells (CD3+) and that of αβ TCR+ plus γδ TCR+ cells in PLE and TCPC patients (fig. 3). This finding indicates the existence of an unusual lymphocyte population with a CD3+ αβ/γδ TCR– phenotype. This subset was also present in the controls, but their frequency and their cell count (not shown) in TCPC and PLE patients was increased (p < 0.0005). In 2 PLE patients this population even exceeded 40% of all circulating T cells. The percentage of this double negative population of T cells did not correlate with that of CD3+ cells co-expressing the NK cell markers CD16/CD56 (not shown) [24]. In addition to these unusual cells, a second unusual cell type that was αβ TCR+ but double negative for CD4 and CD8 (αβ TCR+ CD4− CD8−) was present in PLE patients. Its proportion among αβ TCR+ cells was significantly higher in PLE pa-
The decrease of αβ TCR+ T cell counts affected both the CD4+ and the CD8+ subsets (fig. 4). However, after PLE, the cell count of αβ TCR+ CD4+ cells dropped by >78% but that of αβ TCR+ CD8+ cells only by >56%, indicating the preferential loss of T helper cells. This effect is emphasized by the decrease of the CD4/CD8 T cell ratio from 1.8 (control) to 0.9 in PLE patients (fig. 4). In 3 PLE patients T helper cell counts were <100/µl, and in 3 patients the CD4/CD8 ratios were even below 0.8. The data indicate that the increased CD4/CD8 ratios in TCPC patients without PLE were due to high αβ TCR+ CD4+ and low αβ TCR+ CD8+ cell counts (fig. 4).

**Changes of T Cell Activation after PLE**

PLE was associated with changes in the degree of T cell activation as demonstrated by an altered surface activation antigen expression (fig. 5). After PLE CD25 (IL-2R) expression was 7-fold higher than in the controls. This increase was associated with an elevated expression of the early activation antigen CD69, MHC class II (HLA-DR) expression, in contrast, was not increased (not shown). PLE was also associated with a selective loss of naïve (CD45RA−) T lymphocytes during PLE (not shown).

**Serum Compounds**

The serum levels of all IgG subgroups as well as of IgM, IgE and IgA were reduced in PLE patients when compared to the controls (all p < 0.001). Beside the massive protein loss, the concentration of several serum components changed characteristically, showing that a pro-inflammatory response is present in PLE patients. Indicators for a pro-inflammatory response were elevated C3d as well as neopterin and IL-8 concentrations (PLE vs. control group: 6.2±2.6 mg/l, 7.4±3.3 nmol/l, 10.4±6.5 pg/ml, vs. 2.6±0.7 mg/l, 2.0±0.7 nmol/l, 4.8±3.0 pg/ml), (all p < 0.008) and reduced anti-inflammatory IL-10 concentrations (PLE vs. control group: 0.5±0.6 vs. 4.0±1.8 pg/ml; p < 0.0005). The TNF-α concentration and the sE-selectin level were lower in the PLE (9.5±7.6 pg/ml, 25±20 pg/ml) than in the control group (24.2±60.9 pg/ml (not significant), 65±18 pg/ml (p = 0.0001)). The concentration of soluble (s)IL-2 receptor (sCD25) increased in PLE compared to control (863±306 U/ml vs. 488±318 U/ml; p = 0.01), corroborating the finding of elevated CD25 expression on T cells (fig. 5). There were no significant differences with respect to the other analyzed serum compounds and between both groups of patients.
Discussion

Our study shows for the first time that PLE after TCPC surgery is accompanied by signs of an acute inflammatory response and the dramatic loss of T cells, in particular of the $\alpha\beta$ TCR+ CD4+ subtype. Some laboratory parameters of PLE-free TCPC patients showed differences from the control. Because all of these patients have a TCPC circulation, it cannot be excluded that some of them are at risk to develop PLE in the future. As pre-PLE serological data do in general not differ from that of age-matched healthy controls, it can be demonstrated that post-PLE observations of serological and immunological changes, which were observed also by others [14], are in fact PLE-associated and not due to an immune modulation already present before PLE. Although the number of PLE patients analyzed in our study is quite low, with approximately 100 TCPC surgeries per year in Germany [29], a 10-year morbidity for PLE of about 10% and a mortality of >80% [3, 5], our 12 patients represent a substantial number of PLE survivors.

The mechanism of action of PLE is poorly understood. The current theory is that chronic venous congestion causes the lymphatics to decompress into low-pressure cavities (pleural cavity or abdomen). Loss of proteins and lymphocytes and an inflammatory response leads to chronic malnutrition. PLE results in ascites and global immune dysfunction [30]. Some authors suggested that mesenteric hyperperfusion, because of the increased mesenteric vascular resistance, would be a possible trigger for the development of the PLE [31]. The results of Chaloupecky et al. [32] support the hypothesis that the abnormalities in the coagulation profile observed in patients after the Fontan operation are related to coagulation factor production in the liver.

Several immunological alterations of our patients after PLE are similar to those found in patients with PLE of other etiologies. Muller et al. [33] reported the preferential loss of CD3+ and CD4+ cells into the gastrointestinal tract in patients with constrictive pericarditis. Dramatic T cell loss was also reported for intestinal lymphangiectasia by Yamamoto et al. [34]. As in lymphangiectasia [34], PLE after TCPC is associated with reduced IgG production as shown by us and by others [7]. This decrease is possibly an effect of reduced B cell differentiation or a lack of costimulatory T helper cells. However, B cells of PLE patients may still be stimulated to produce IgG as evidenced by in vivo corticoid administration [7]. The passive lymph loss secondary to high central venous pressure does not explain the selective loss of CD4+ lymphocytes, suggesting that the disturbance of the immune system can initiate changes in the local cytokine network of the gut, perturbing its homeostasis and immune defense. This could affect the structural integrity and patency of the intestinal wall, thus triggering PLE [14]. Patients with autoimmune diseases such as SLE and celiac disease may also develop PLE [15, 16]. In PLE of non-TCPC origin autoantibodies were reported [35], and viral infections were discussed as a trigger or even as an inducer of autoimmunity [36]. In non-cardiac patients some studies indicate that PLE is infection-associated and may even be induced by different pathogens [19, 37]. Importantly, TCPC patients also had infections coinciding with PLE onset [38]. Recent observations support the multiple insults hypothesis of PLE induction. It was suggested that viral or bacterial infection, mostly of the gastric tract, can serve as trigger of PLE onset [39]. Jejunal biopsies, taken during episodes of PLE, revealed an increased IFN-γ concentration [40], most likely as a response to viral infection, and elevated levels of the pro-inflammatory cytokine TNF-α [41]. Both cytokines are known to impair the integrity of the intestinal epithelial barrier [42]. Similar to other primary diseases associated with PLE, episodes of post-Fontan PLE are characterized by a loss of HSPG (heparan sulfate proteoglycan), particularly from the basolateral surface of intestinal epithelial cells. HSPG expression in the lamina propria is normal. The overall intestinal architecture remains intact, and the expression of other matrix components is also normal. The reasons why HSPG is lost during episodes of PLE is still unknown [43].

The significantly reduced CD4/CD8 T cell ratio after PLE found by us is due to preferential CD4+ cell loss. This was also reported in a single case report [14] as well as for SLE patients [18] and patients with celiac disease [17]. However, the mechanical removal of the lymphocytes via afferent lymphatic vessels is questionable because lymphocytes are able to actively migrate and escape simple excretion. Their loss could be due to apoptosis possibly induced by intraepithelial lymphocytes (IEL) [44] or may be accompanied by altered migration and chemokine patterns [45]. In addition, reduced generation of new (thymus-derived) T cells might be possible. Both, SLE and celiac disease are associated with signs of acute inflammation, such as elevated serum levels of neopterin, CRP, IL-6 [46], and increased T cell activation [47].

An intriguing finding of our study was the increased activation of circulating T cells and their elevated $\gamma\delta$ TCR phenotype after the onset of PLE. The residual phenotype resembles that of IEL [48] of the gut. In humans up to 40% of the IEL are $\gamma\delta$ TCR+, <10% CD4+ and >60% CD8+ T cells [48]. Most of these T cells are CD25+ and CD45RO+. These findings may suggest that in PLE after TCPC circulating thymus-derived $\alpha\beta$ TCR+ T cells vanish and the residual circulating T cell population is dominated to a higher extent by (possibly gut-derived) $\gamma\delta$ TCR+ T cells. Several studies have documented and suggested that $\gamma\delta$ TCR+ T cells may play a role in the regulation of the immune function [49]. Their number in the peripheral blood is significantly decreased in sepsis patients [50] being probably responsible for mediating the immune dysfunction after sepsis [49]. These alterations in T cells could lead to impaired generation of specific immune response to new (gut-derived) infections and to increased susceptibility of PLE patients to (opportunistic) infections.
We flow cytometrically detected interesting residual T cell populations in PLE patients with strong CD3+/CD4–/CD8– double negative phenotype. Similar populations have been described in the intestine in refractori-


