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Children with post-surgical capillary leak syndrome can be distinguished by antigen expression on neutrophils and monocytes

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ABSTRACT

Our initial studies indicate that children who develop post-operative capillary leak syndrome (CLS) following cardiac surgery with cardiopulmonary bypass (CPB) can be distinguished based on their pre-operative level of circulating cytokines and adhesion molecules. We tested flow cytometric analysis of surface antigen expression as a potential assay for risk assessment of CLS. 24h preoperative blood samples were stained with monoclonal antibodies for the adhesion molecules ICAM-1, LFA1, MAC1, ß-integrin, activation markers CD25, CD54, CD69, HLA-DR, CD14 or CD4. Cells were measured on a dual-laser flow cytometer calibrated with microbeads. Antigen expression was detected as mean fluorescence intensity. The data indicate, that neutrophils of CLS patients express preoperatively higher levels of LFA1 and monocytes higher levels of HLA-DR and activation markers thus are in a state of activation. This could in combination with surgical trauma and CPB lead to their additional stimulation and migration into sites of inflammation and induce postoperative CLS. It is planned to set up a Flow-Classification program (CLASSIF1) for individual risk assessment. By discriminance analysis over 80% of the patients were correctly classified. Our preliminary study indicates that flow cytometry with its low sample requirement and rapid access of the results could be a powerful tool to perform risk assessment prior to pediatric open heart surgery.

Keywords: flow cytometry, prognosis, cardiac surgery, antigen expression, adhesion molecules, activation markers

1. INTRODUCTION

During cardiopulmonary bypass (CPB) there is extensive contact between blood anticoagulated with heparin and foreign surfaces of the extracorporal circuit. CPB has been associated with major qualitative and quantitative alterations of humoral pathways, cell types and inflammatory substances generating a systemic inflammatory response with generalized capillary leakage. [1] In adults some authors described a loss of activated T-lymphocytes and a drop in the CD4/CD8 T cell ratio [2] or increased expression of the adhesion molecule LFA-1 CD11a/CD18 on lymphocytes during CPB [3]. Heart surgery with CPB in children has a pronounced suppressive effect on the immune system compared to surgery without CPB [4, 5]. In addition, CPB induced decrease of activated lymphocytes in the peripheral blood probably due to increase of serum IL-10 levels [6] and complement activation [7, 8]

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The proposed mediators are able to reproduce the post-perfusion (post-pump) syndrome which may represent a wide spectrum of organ dysfunction ranging from subclinical to profound multiorgan failure. The sum of these clinical features which resemble very strongly after bone marrow transplantation [9], in severe burns or in septic shock, are titled capillary leak syndrome (CLS). The definition of CLS includes, independent from triggering agent, an endothelial damage with elevated microvascular permeability and generalized vasodilatation leading to a volume refractory, mostly vasopressor agents requiring arterial hypotension or blood pressure instability with generalized edema [1] including pleural effusions or ascites. [10]

Our initial studies indicate that children who develop post-operative complications including CLS after cardiac surgery with CPB can be distinguished based on their 24h pre-operative level of circulating cytokines and adhesion molecules.[11, 12] As an example, in risk patients C1-esterase inhibitor level was reduced whereas the serum level of soluble adhesion molecules (ICAM-1, E-selectin) were increased [11, 12]. However, the determination of these values is time consuming and requires a substantial volume of peripheral blood. In order to develop a faster and less sample consuming technique we tested the value of flow cytometric analysis of surface antigen expression as a potential assay for risk assessment of CLS.

Surgery of patients with/without post-operative complications following open-heart surgery with cardiopulmonary bypass in children		
	Number of patients without postoperative complications	Number of patients with postoperative complications
ASD	14	6
VSD	4	3
Ross/Homograft	4	3
Fontan	0	2
Glenn	0	4
Other	6	3

Table 1: Surgery of patients with/without postoperative complications following open-heart surgery with cardiopulmonary bypass in children (ASD – atrial septal defect, VSD – ventricular septal defect).

2. PATIENTS AND METHODS

After informed written consent was obtained from the parents of the patients, 49 children undergoing cardiac surgery with CPB were analyzed. The age range of the patients was 3 to 16 years. Table 1 shows the type of congenital heart defects or surgery and the respective numbers of patients. Peripheral blood was obtained 24 h before cardiac surgery. All children received similar anesthesia, medication and intra- and post-operative care. Blood was collected in syringes containing ethylen-diamine-tetra-acetate (EDTA) to prevent clogging of the cells. From the same blood sample routine laboratory parameters were determined.

2.1. Cardiopulmonary bypass

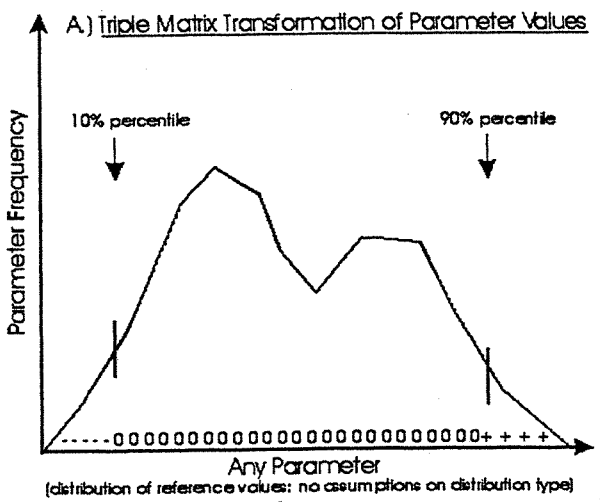
The extracorporeal circuit was realised in a standardised manner. A heparin-coated system was used. CPB was performed with a roller pump (Stoeckert-Shiley, Munich, Germany) and hollow-fibre oxygenator (DIDECO, Mirandola, Italy). Deep hypothermia was induced by cooling the priming solution (crystalloid solution, mannitol, Iono-lactat) in the extracorporeal circuit and the circulating blood with the heat exchanger. During the cooling period all patients received sodium nitroprussid for vasodilatation. Bretschneider's cardioplegic solution was used for myocardial perfusion. (For details see [8])

2.2. Flow cytometry

Phenotyping of blood cells was done by the whole-blood staining method.[13] 40µl of a blood sample was mixed with appropriate volume of a cocktail of directly fluorescence dye conjugated monoclonal antibodies. Optimal antib concentrations were determined for each antibody by titration. The cells were stained for 15 min at room temperatu the dark. Then 1ml of a lysing solution (Becton-Dickinson, San Jose, CA) was added, mixed and incubated for 20 m room temperature in the dark. The cells were spun down at 300 x g, the supernatant discarded, the cells were wa twice in 1 ml phosphate buffered saline (PBS, Sigma, Deisenhofen, Germany) and finally resuspended in 500µl containing 0.1% paraformaldehyde (Sigma). The samples were stained and analyzed by three or four color dual-l flow cytometry (FACSCalibur; Becton-Dickinson). The flow cytometer was calibrated using quantitative calibra beads (Spherotech Inc., Libertyville, IL) if necessary. Cells were stained with one of the following antibody cocktail:

1. CD45/CD14/HLA-DR/CD3,
2. CD25/CD54/CD3/CD19
3. CD11a/CD18/CD16+CD56
4. CD3/CD69/CD19/CD45

The antibodies were labeled with the fluorescent dyes fluorescein isothiocyanate (FITC), phycoerythrin (PE), PerC and allophycocyanine (APC), respectively. With CD45 (pan leukocyte antigen), HLA-DR (MHC-II), CD25 (interleu 2 receptor α), CD54 (ICAM-1), CD18 (ß-2 integrin), CD11a (LFA1), CD69 (early activation antigen), CD16 (Receptor III), CD56 (N-CAM). Antibodies were obtained from Becton-Dickinson, Beckmann-Coulter Corp. or Dak



B.) Introduction into Triple Matrix Database

	numeric database parameters			triple matrix database parameters		
patient	1	2	3	1	2	3
1	40.1	4.02	18.38	0	+	0
2	39.5	4.20	5.25	-	+	-
3	41.2	3.46	35.35	0	-	+
4	53.2	3.78	30.72	+	0	+
5	41.3	3.95	27.46	0	0	0
6	79.3	3.80	16.29	+	0	0
7	48.0	3.98	28.33	0	0	0

Figure 1: Triple matrix transformation of numerical data by the CLASSIF 1 algorithm

postoper. clin.diag.	patients (n)	CLASSIF1 classification (%) N	CLS
N	9	100.0	0.0
CLS	9	0.0	100.0

Figure 2: Preoperative triple-matrix classificat patient data (34 provided parameters, 20/80% perc

2. 3. Data analysis

Flow cytometric data were analyzed with the CellQuest software package (Becton-Dickinson) and transferred and further calculated using a commercial spreadsheet program (SigmaPlot, Jandel Scientific GmbH, Erkrath, Germany). The cell populations of monocytes, neutrophils or eosinophils were characterized by forward and sideward angle light scatter [13]. In these subsets the percentage of the respective cell population and the fluorescence intensity of the different subsets was determined as a measure for antigen expression. From these values the cell numbers and, with the aid of the fluorescence microbeads as shown elsewhere[14], the number of antibodies bound per cell were calculated. This resulted in 178 data columns. Statistical analysis was done by unpaired Student's t-test or Wilcoxon's Mann Whitney U-test as appropriate (SPSS, Knowledge Dynamics, USA). Classification was performed by discriminance analysis with the SPSS classification program. Briefly, each parameter was tested by the classification programm for discrimination between good and bad outcome. The parameter with the highest discrimination was then combined with a second parameter and the combination with the highest discrimination was selected again and combined with a third parameter etc.. Typically there was an optimal number of parameters for discrimination, additional parameters did not increase discrimination but often reduced it. One typical discrimination procedure took more than one hour.

(A) patient nr	(B) clinical outcome	(C) CLASSIF1 preoperative prediction	(D) CLASSIF1 patient classification masks	(E) CLASSIF1 positional identity with reference classification mask
1	N	N	0-00++00++	0.60
2	N	N	00--0+0+00	0.70
3	N	N	++0x00+000	0.60
4	N	N	00+000-00+	0.80
etc.				
10	CLS	CLS	++0x0++00+	0.50
11	CLS	CLS	++-x+++++0	0.80
12	CLS	CLS	++-x+0000+	0.50
13	CLS	CLS	++-x++++++	0.90
14	CLS	CLS	0--+00++++	0.60
etc				

(B) clinical outcome	(F) CLASSIF1 reference classification masks
N(ormal)	0000000000
CLS	++-+++++++

0 = unchanged + = increased
- = decreased x = missing param.

parameters of reference classification masks:

1. patient age
2. patient weight
3. C1 inhib.
4. IL-10
5. soluble ICAM-1
6. soluble E-selectin
7. serum histamine
8. urine histamine
9. granulocyte count
10. % granulocytes

Figure 3: Partial triple matrix printout for N(ormal) and CLS risk patients of Fig.2. Patients are classified (C) according to the highest positional identity between patient (D) and reference (F) classification masks. The reference classification masks (F) are determined for each classification category during the learning phase by combining the most frequently observed triple matrix characters in each of the CLASSIF1 selected data columns. CLASSIF1 classifiers are inherently error tolerant. This is indicated by positional identity indicators (E) of <1.00. Patients are in many instances correctly classified although the positional identity indicator does not reach the identity value of 1.00 with the best fitting reference classification mask (F). Data in part from [11, 12].

The Flow-Classification program CLASSIF1 for individual risk assessment is used as an alternative way for data classification[15]. The CLASSIF1 program permits the establishment of standardized, instrument and laboratory independent cytometric and other multiparameter classifiers e.g. on clinical chemistry or other clinical data [16]. The

CLASSIF1 classification algorithm shortly works as follows: Pairs of percentiles e.g. 5/95%, 10/90%, 15/95% etc. are calculated for the value distribution curve of the reference samples of each database column of the learning set (Fig. 1.A). All values i.e. of reference and abnormal samples of each database column are then transformed into triple matrix characters by assigning: 0 to values between the respective percentiles, + to values above and - to values below the respective upper and lower percentiles (Fig. 1.B). A confusion matrix is subsequently established between the known e.g. the clinical classification of reference and abnormal samples on the ordinate and the same classification states for the CLASSIF1 triple matrix classifier on the abscissa (Fig. 2). The triple matrix printout (Fig. 3) permits to identify the most discriminative parameters and to check the matrix for systematic errors in samples or assays with time.

The classification result is ideal when a 100 % recognition value is obtained in each diagonal box of the confusion matrix. This indicates correct classification of all samples by the CLASSIF1 classifier which usually does not occur when all database columns are considered for the classification i.e. prior to the optimization process. Triple matrix classifiers are inherently standardized onto the group of reference samples during the classification process. Classifiers from different flow cytometers or laboratories can thus be compared in an instrument and laboratory independent way provided no differences between the respective reference groups are detected by the CLASSIF1 algorithm.

The advantage of this classification strategy is that triple matrix classifiers can be numerically compared during interlaboratory consensus trials e.g. on leukemia, HIV and thrombocyte classifications by immunophenotyping because they are portable [16, 17, 18, 19]. In view of immunophenotyping, the performance of triple matrix classifiers depends preferentially on individual laboratory precision rather than on interlaboratory accuracy. Instrument accuracy cancels out by percentile normalization on the mean values of the reference samples. Reference samples can be obtained in many instances from age and sex matched blood donors who represent a comparatively homogeneous group of persons.

Post-operative complications following open-heart surgery with cardiopulmonary bypass in children		
	Number of Patients	Post-operative outcome
With complications	10	liver swelling, edema, pericardial effusion
	6	liver swelling >1cm, edema
	3	pericardial effusion
	2	pleural effusion
Without complications	19	none
	6	edema
	3	liver swelling <1cm

Table 2: Postoperative complications following open-heart surgery with cardiopulmonary bypass in children.

3. RESULTS AND EXAMPLES

3.1. Patients data

Patients were grouped according to the clinical outcome as shown in Table 2. Patients grouped into the complication group had one or more of the shown post-operative complications such as pericardial or pleural effusion, liver swelling and edema. Complication free patients had only minor edema or a liver swelling of < 1cm above pre-operative values. All patients were discharged well about one week after surgery.

As can be seen in Table 3, patients with bad outcome were significantly older, had higher body weight and had increased duration of CPB (not significant) and total duration of surgery. This result is in agreement with own earlier findings [11, 12] and results from other authors [20, 21, 22].

Clinical and surgical data			
	Patients without post-operative complications	Patients with post-operative complications	t-test (p value)
Age (yrs.)	8.12 ± 2,93	10.17 ± 3.31	0.031
Weight (kg)	25.93 ± 10.49	35.09 ± 12.97	0.011
Stay on ICU (d)	1.93 ± 1.09	3.37 ± 2.81	0.018
Aortic crossclamping - duration (min)	35.1 ± 30.2	40.0 ± 28.7	0.577
CPB - duration (min)	66.2 ± 40.8	91.6 ± 48.7	0.053
Surgery + Anesthesia duration (min)	181.3 ± 65.8	236.6 ± 118.4	0.043

Table 3: Clinical and surgical data of patients with and without post-operative complications. Data show mean ± 1 standard deviation.

3.2. Immunological data and classification

Children who developed post-surgical complication had a higher expression of surface adhesion molecules (e.g. HLA-DR, CD69, LFA-1) on monocytes, neutrophils and eosinophilic granulocytes (Table 4). On the other hand, we found a lower expression of CD 25 and CD54 on monocytes and CD16 on neutrophils in samples of children with post-surgical complications.

24 h preoperative differences in surface antigen expression				
	Patients without postoperative complications	Patients with postoperative complications	t-test (p-value)	U-test (p-value)
Monocytes HLA-DR	189.2 ± 239.4	571.9 ± 614.0	0.0072	0.0095
Monocytes CD69	5.2 ± 5.0	8.6 ± 6.9	0.0488	0.0191
Monocytes CD25	7.3 ± 5.5	3.9 ± 1.7	0.0886	0.0206
Monocytes CD54	73.6 ± 64.5	40.3 ± 15.4	0.1428	0.0156
Monocytes CD11a	437.5 ± 380.8	703.1 ± 428.2	0.0285	0.0627
Neutrophils CD18	86.4 ± 73.6	139.9 ± 105.4	0.0419	0.1297
Neutrophils CD11a	162.9 ± 136.0	245.5 ± 126.4	0.0352	0.0269
Neutrophils CD16	180.3 ± 94.7	116.1 ± 38.3	0.0488	0.0253
Eosinophils % leuko	2.3 ± 1.7	4.0 ± 3.1	0.0191	0.0199
Eosinophils count	168.5 ± 142.5	278.8 ± 233.1	0.0489	0.0546
Eosinophils CD11a	267.5 ± 225.2	364.8 ± 199.5	0.1290	0.0447

Table 4: 24h preoperative surface adhesion molecule expression on monocytes, neutrophils or eosinophilic granulocytes in patients with or without postoperative complications. Data show antigen expression (median fluorescence intensities; mean of all measurements ± 1 standard deviation). Only values with significant or near significant differences are shown.

A typical example of altered antigene expression is shown for LFA-1 in figure 3. Leukocytes from both patients were analyzed on the same week However the patient with post-operative complications had increased CD18 and CD11a expression (Fig. 4). This increase was calculated to be approximately 8,000 fluorescein or 11,000 PE equivalents per neutrophil for CD18 and CD11a.

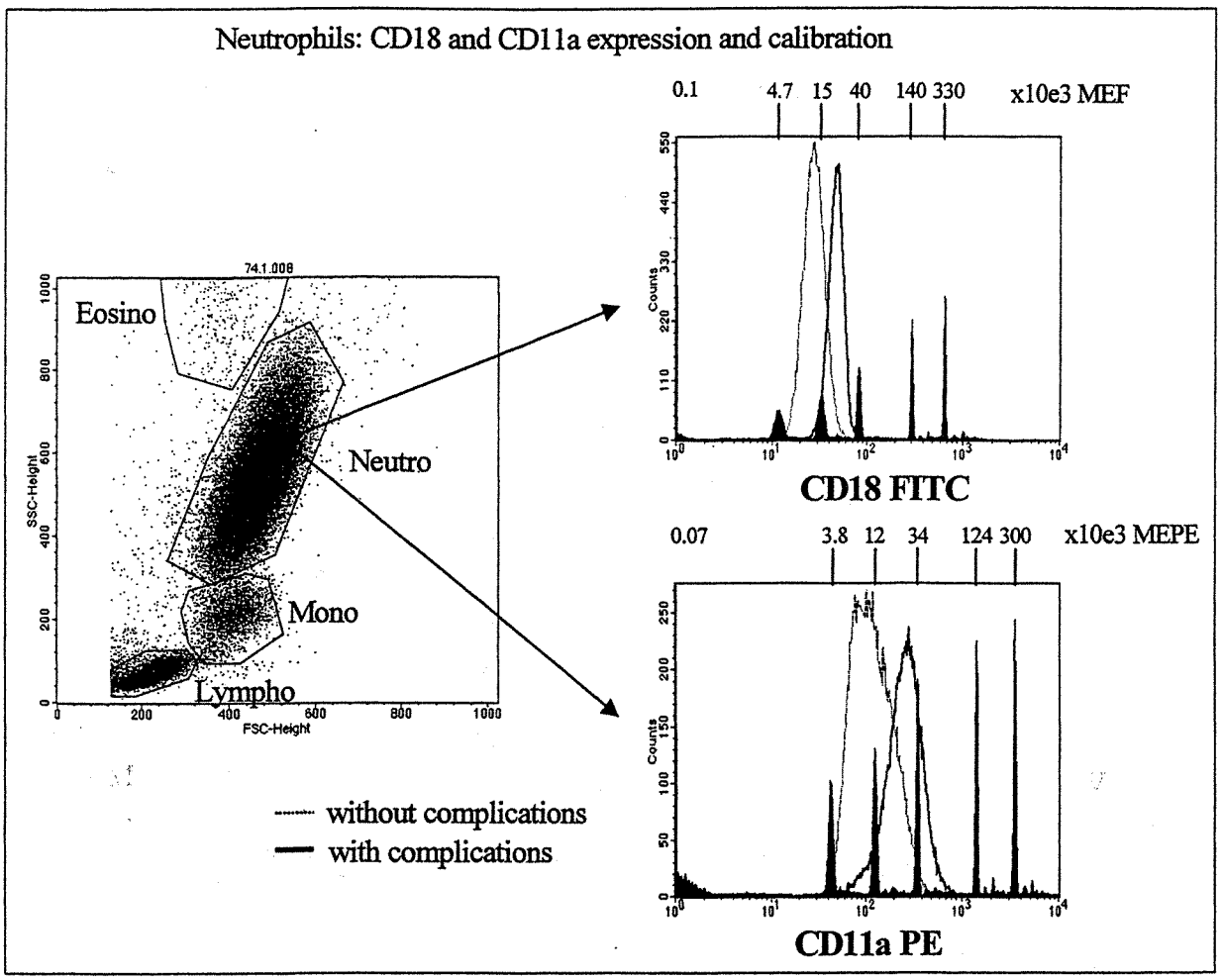


Figure 4: Expression of LFA-1 peptides on the surface of neutrophilic granulocytes of children with or without complications. Data in the histograms are gated for neutrophils only (left image shows gate for neutrophilic granulocytes) and display cell count (y-axis) versus CD18 or CD11a expression (fluorescence intensity) and corresponding molecules of equivalent fluorescein (MEF) and PE (MEPE) using beads (black histograms). The figures above each histogram indicate the numbers of fluorescence molecule equivalents per peak. Data were provided by the manufacturer.

Increased state of activation of the immune system can be deduced from elevated surface level of HLA-DR (MHC II), CD69, CD11a on monocytes and LFA-1 on neutrophils. In addition percentage and count of eosinophilic granulocytes is higher. As shown in figure 4, only one parameter (fluorescence intensity or percentage of cell subpopulations) is insufficient for individual risk assessment. Although some of the patients could be grouped correctly based e.g. on elevated HLA-DR expression on monocytes (above mean of complication free values plus two standard deviations, Fig. 5) most of the patients (> 80 %) had similar values to the complication free group. Therefore we performed risk assessment based on discriminance analysis. For this analysis we used all 178 directly measured or calculated

parameters. As shown in Table 5 by SPSS classification the patients can be classified by surface antigen expression on monocytes and neutrophils, allowing a pre-operative risk assessment. The retrospectively prospective classification shows that over 84 % patients with postoperatively complications can be preoperatively identified using just three of all analysed parameters. The patients could be discriminated by the increased surface expression of HLA-DR and CD11a on monocytes and CD11a on neutrophils. (Table 5).

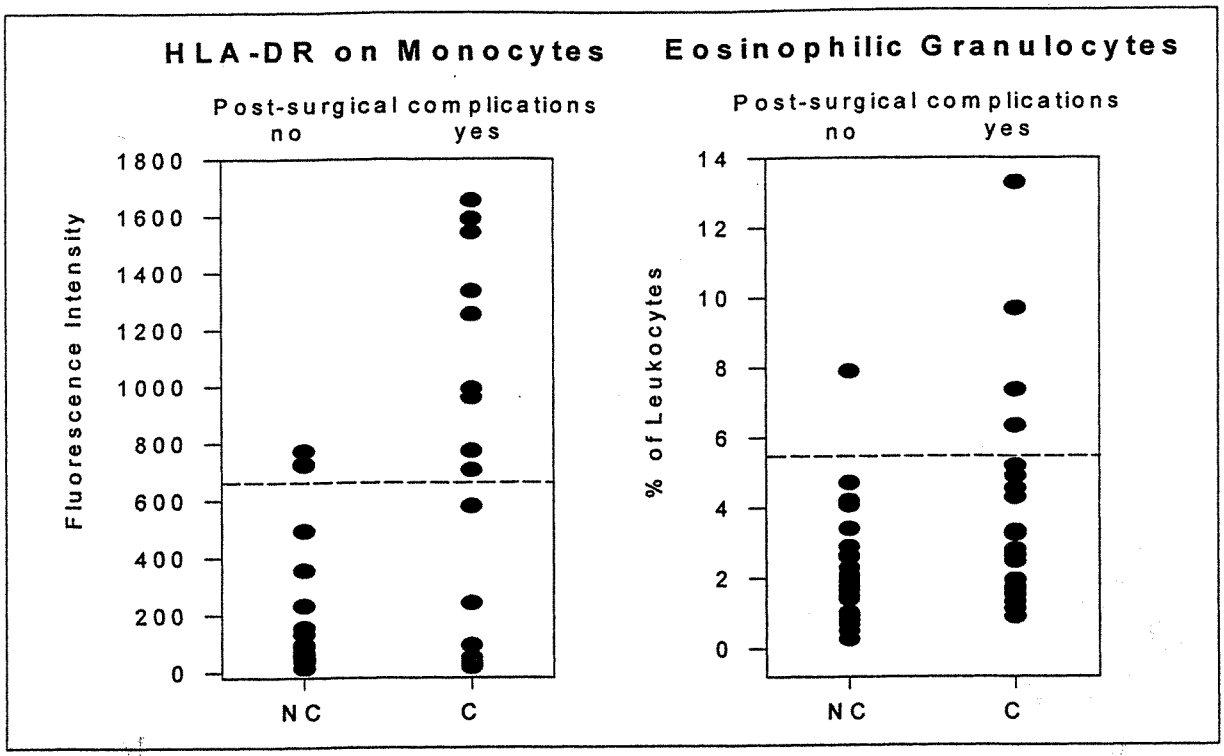


Figure 5: Expression of HLA-DR on monocytes (fluorescence intensity) and percentage of eosinophilic granulocytes of individual patients without (NC) or with (C) complications. The dashed lines indicates mean + 2 standard deviations of NC patients.

		Prospective SPSS Classification	
Clinical outcome (complications)	**Number of Patients**	**no Complications**	**Complications**
no	**24**	**79 %**	21 %
yes	**20**	10 %	**90 %**

Table 5: Classification of patients with and without postoperative complications based on 24h preoperative data. An overall classification of 84 % was obtained using monocyte HLA-DR and CD11a and neutrophil CD11a expression. Due to missing data only 44 patients could be analysed by the SPSS classifier.

4. COMMENTS

Our study indicates that flow cytometry with its low sample requirement and rapid access of the results could be a powerful tool to perform risk assessment one day prior to cardiac surgery. Our data shows for this group of patients that there is preoperative difference in the patients immune system which might influence the surgical outcome. The finding indicates that patients with increased risk have an activated immune system which might result in combination with the surgical trauma in an elevated immune response after surgery. The retrospectively prospective classification shows that over 84% patients with postoperatively complications can be preoperatively identified based on surface antigen expression. The increased activation of leukocytes is in agreement with earlier findings from our group showing that, based on serological data, elevated pre-operative activation of the immune system is correlated with impaired outcome [11, 12]. In these studies on a small group of patients increased levels of soluble adhesion molecules (e.g. ICAM-1, PECAM) [11] , histamine, neopterine and interleukin-10 levels as well as reduced C1-esterase inhibitor levels were measured [12]. See also Figure 3. Based on these data we were able to perform risk assessment with CLASSIF1 (and SPSS) yielding an overall classification > 95 % based on 7 data from a data set of over 50 parameters. However, the higher demand of serological analysis for time (hours to days) is a problem for immediate availability of the results. The high requirement for blood (50 µl to 300 µl per parameter) makes this approach not feasible for neonates and pre-term neonates with an overall blood volume of sometimes less than 100 ml. With the present paper we could show that risk assessment is also possible using flow cytometric data of leukocyte antigen expression and percentages. With flow-cytometry the lag time between drawing of the sample and the final results can be reduced possibly to 90 min. After defining the parameters essential for risk assessment the required blood volume can be reduced to 120 µl (three antibody cocktails). Thus, flow cytometry and data classification are an ideal combination for risk assessment in pediatrics and pediatric cardiology. The preoperative parameter changes in the risk patients are compatible with the existence of a latent preoperative infection. Individual risk assessment would allow for an individual prophylaxis of post-surgical complications (e.g. C1-Inhibitor substitution [1], immunosuppression) for the benefit of the patient as well as cost reduction.

Based on only three of 178 parameters we could classify over 80 % of our patients correctly using the SPSS classifier. Classification by the CLASSIF1 analysis should provide even an improvement over this classification. As our initial studies show it is much faster than the (manual step-by-step) classification with SPSS and therefore a more objective tool for analysis. In addition, it has the unique opportunity to analyze the original flow cytometric (ListMode) data-files without the (subjective) interference of an operator. Furthermore, SPSS and other classifiers fail to classify datasets when a single value is missing. In real life it will often happen that data are missing. This problem is outruled by the CLASSIF1 approach that allows classification even if the data set for one patient is incomplete. The CLASSIF1 triple matrix analysis provides a preoperative risk indicator, a hypothesis how CLS is generated and a means for therapy control. Provided the above hypothesis is correct, preoperative antibiotic treatment and recontrol of the data pattern at several days interval will permit to follow the normalization of the above parameter pattern. Once the parameter pattern is normalized, the risk for the postoperative occurrence of CLS should be minimal.

The development of disease classifiers is of high interest for the standardized and automated information extraction from multiparameter flow cytometric list mode and other (e.g. clinical chemistry) data. It is planned to set up an on-line classifier for CLS risk assessment on the internet. This classifier should allow for individual risk assessment also for small hospitals who would need due to low patient numbers years to set up a working database. This is in particularly a problem in pediatrics and pediatric cardiology.

The relative independence of the classification results of interlaboratory accuracy represents an advantageous feature of triple matrix classifiers. It is a frequent experience in flow cytometric ring trials that good accuracy is more difficult to achieve than good intralaboratory precision especially when different instrument types are used by the participants. The reason for this consists in the high technical complexity of flow cytometers and cell sorters in view of e.g. optical signal perception, amplification, thresholding, filter characteristics and data processing. The recent development of various standard bead preparations improves but does not generally solve this problem. One of the practical consequence of this is e.g. that diseases can be classified at institutions where no sufficient learning sets can be generated in reasonable times or where costly investigations are necessary to establish appropriate learning sets.

In addition, the biochemical properties of many body cell systems during disease can be immediately compared when the classifiers are standardized on generally accessible cells like e.g. peripheral blood cells. This standardization permits also to evaluate changes of blood cells in tissues or effusions during disease. A new way for a general and standardized biochemical diagnosis and prognosis system in medicine will be opened by the elaboration of consensus classifiers opens.

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