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In this issue

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Clinical flow cytometry in 2019

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EDITORIAL

Flow cytometry is a relatively new scientific field and many of the people who contributed to its evolution are still active all over the world. Flow Cytometry has evolved into an important diagnostic and research tool in several areas of medicine and biology. The flow Cytometry laboratory therefore is a part of the Hematology or Immunology departments in the hospital and a core laboratory in a research institute.

We are envisioning clinical laboratories with new preanalytical instruments (most of them of large volume), small modern compact cytometers of 15+ colors, a small number of technicians preparing samples and acquiring events, modern “R” software uploaded on computers for the analysis. The dry reagent-tubes are kept in room temperature cupboards and the rest of the monoclonal antibodies in the preanalytical machines. The colors are not the “old friends” well known organic dyes. New not spilling bright dyes are replacing them.

The samples, peripheral blood, bone marrow and lots of biological fluids, cerebrospinal fluid, ascites, pleural effusions, are derived from hematological patients or cases receiving new “precision individualized immunological drugs” in large quantities.

The results are "flowing" quickly to the GDPR checked recipients and the files of the patients are packed with valuable information, while new software produces exact diagnosis from the patient's data.

Parts of this "brave new world" are presented in this issue of the eJIFCC, which is dedicated to flow cytometry. Practical advice about the diagnosis of Paroxysmal Nocturnal Hemoglobulinuria (PNH), where flow cytometry constitutes the golden standard method is presented by experts in the field (B. Brando et al). Primary immunodeficiency diagnosis, where flow cytometry plays a pivotal role through the study of all the immune cells immunophenotype and function is presented in two papers by experienced researchers (U. Saltzer et al and J. Wolf et al).

Flow cytometry is one of the first diagnostic tools concerning childhood acute lymphoblastic leukemia and the importance of the evaluation of sample quality is paramount (E. Szánthó et al). DNA analysis by flow, an old application of flow cytometry with completely new perspective is presented in the review about flow cytometry in breast cancer by the team, who renewed the interest in perioperative use of flow (M. Andreou et al). Finally two more papers on the importance of flow in Immunology, a study of monocytes polarization in sepsis (M.Greco et al) and a review of a summer school in Flow Cytometry for Immunology, showing the diversity of immunology topics' spectrum covered by flow cytometry (K. Psarra et al) complete this special thematic edition of the eJIFCC.

In this issue: Clinical flow cytometry in 2019

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Flow cytometric diagnosis of paroxysmal nocturnal hemoglobinuria: pearls and pitfalls – a critical review article

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ABSTRACT

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare blood disorder characterized by chronic intravascular hemolysis, thromboses in unusual sites and cytopenias related to bone marrow failure. The diagnosis is based on the Flow Cytometric (FCM) detection of peripheral blood cell clones lacking the surface molecules linked to the GPI anchor, which is altered by mutations.

Consensus studies have developed standardized and robust multicolor FCM assays to disclose PNH clones among red cells, neutrophils and monocytes at a high level of sensitivity and accuracy. High-resolution procedures have been also established to detect small PNH clones at a sensitivity level of around 0.01% in red cells and neutrophils. Cell clone type and size have been put into correlation with the clinical presentations of the associated diseases, and recommendations for the clinical follow-up have been established. The recent advent of the therapeutic monoclonal

antibody Eculizumab has dramatically improved both the quality of life and the life expectancy of the affected patients, further increasing the importance of an accurate FCM detection and monitoring of the clones. The technical features of the FCM diagnostic workup and the many critical aspects of the analytical process are discussed here.

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INTRODUCTION

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare, acquired clonal blood disorder of the hematopoietic stem cell characterized by a triad of signs and symptoms: chronic intravascular hemolysis with recurrent crises, thromboses in unusual sites and cytopenias related to bone marrow failure (BMF).

Its name was coined early in the 20th century, but after the extensive research made during the last few decades, nowadays the three key terms characterizing the disease may sound somewhat misleading.

While some 90 percent of patients experience hemoglobinuria at some point of their clinical course (1), hemoglobinuria is seen in about one quarter of cases only at diagnosis (2). Chronic hemolysis with hemoglobinuria occurs not only during nighttime, but 24 hours per day, it is most often asymptomatic and may present as 'paroxysmal' only occasionally (2).

PNH is also known as 'the most vicious acquired thrombophilic state' (3, 4), probably through the free hemoglobin itself (5) and the reduced intravascular nitric oxide, although its multifactorial biological basis still remains partly unknown (6).

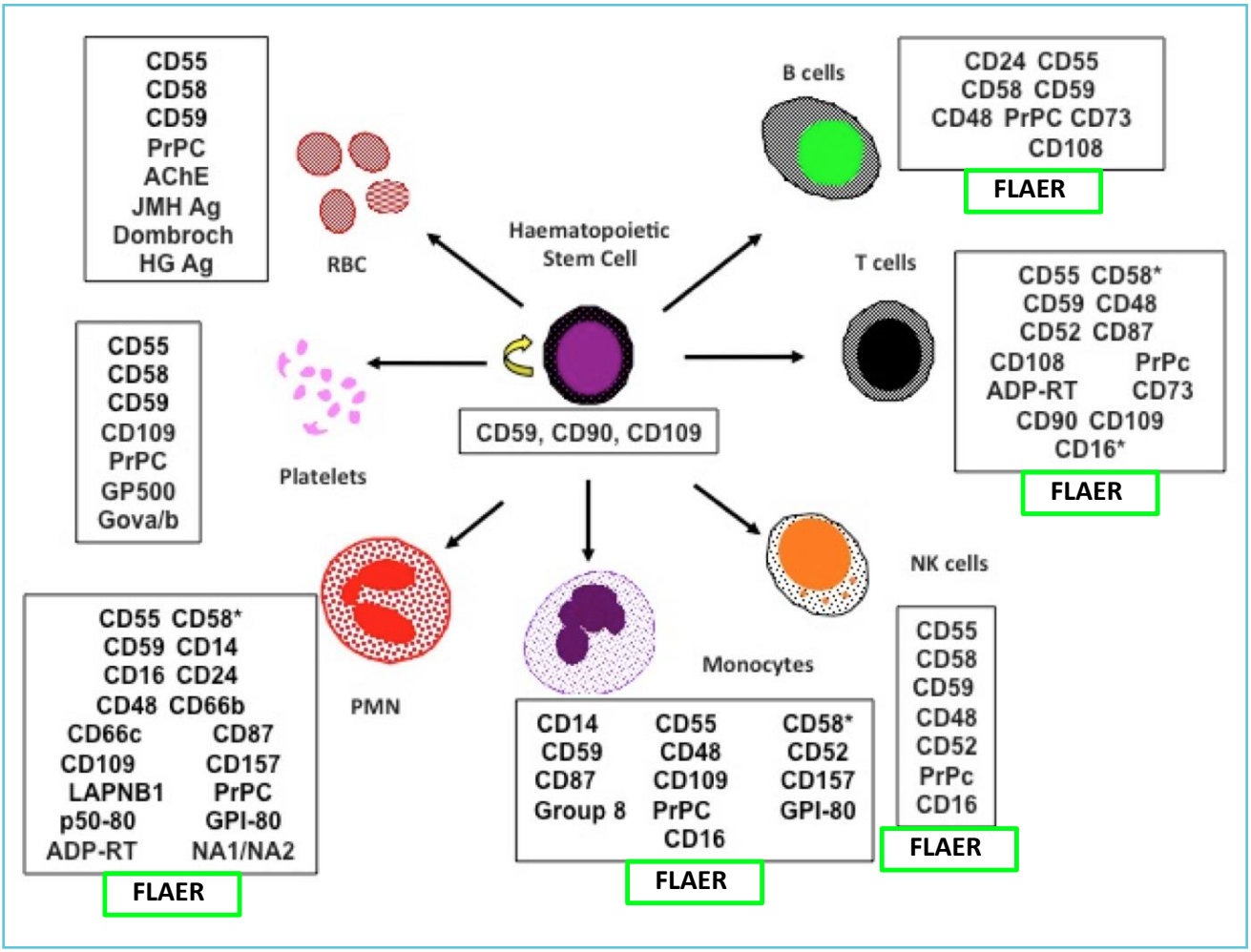
The strong association between PNH and BMF syndromes, including myelodysplasia (MDS) and aplastic anemia (AA), is well known and recommendations have been published to include PNH screening in the clinical workup of the BMF syndromes, MDS and AA (7-9).

A number of other apparently incoherent clinical signs and symptoms are often experienced by the affected patients, namely fatigue, dyspnea, abdominal pain, pulmonary hypertension, renal insufficiency, dysphagia, erectile dysfunction, thus making the clinical assessment long and difficult, with frequent misdiagnosis and mismanagement (1, 10).

Except for Direct Antiglobulin Test (DAT)-negative hemolysis tests and cytopenias, which may focus medical attention, ordinary laboratory assessments usually do not provide specific clues for the diagnosis of PNH (11).

The uncontrolled complement activation, leading to life-threatening hemolysis and thromboses forms the basis of symptomatic PNH. The pathogenic mechanism of PNH resides in the faulty synthesis of the cell membrane protein anchor Glycosyl-Phosphatidyl Inositol (GPI) due to mutations occurring in the PIG-A gene located on chromosome X (12). GPI is the anchor for many functional cell surface glycoproteins (13) (Figure 1): the reduced or absent expression of the GPI-linked complement-regulator molecules on red blood cells (RBC), the Decay Accelerating Factor (DAF, also known as CD55) and the Membrane Inhibitor of Reactive Lysis (MIRL, also known as CD59) are the cause of hemolysis in PNH (2-4, 14). In PNH all blood cell lines deriving from a mutated stem cell carry the same genetic and phenotypic defect, and can coexist as 'PNH clones' along with normal, unmutated counterparts. The flow cytometric (FCM) demonstration of the absence of GPI-linked molecules in a sizable fraction of peripheral blood red cells, neutrophils and monocytes is the cornerstone of the diagnostic process of PNH (15-17). Three types of clinical presentation of PNH are schematically considered: the classical form, associated to overt hemolysis

Figure 1 The major GPI-linked cell surface molecules that can be studied by flow cytometry in the diagnostic process of PNH



FLAER: Fluorescent Aerolysin. Modified from Rotoli B. (13).

and large PNH cell clones; the PNH associated to BMF/AA with hemolysis and usually small clones, and the subclinical PNH, with no or minimal hemolysis and variable signs of BMF. A more extensive discussion on the clinical spectrum of PNH presentations and the associated laboratory features can be found in the recent literature (7, 18-20).

Blood transfusions, anticoagulants and steroids have been the only therapeutic options for PNH for decades (19, 21, 22). Patients with PNH occurring in the context of BMF take advantage from the specific treatment of the underlying bone marrow disorder (7, 19, 23). In recent years, the advent of the complement inhibitory monoclonal antibody Eculizumab has dramatically improved the prognosis and the quality of life of patients with symptomatic PNH (24-28), although the only cure may reside in allogeneic stem cell transplantation in selected cases (25).

Table 1 Frequency of cases showing GPI-deficient cells in blood at diagnosis in 3,938 PNH+ patients according to the reasons for testing in a multicenter study

Medical indications for PNH screening	Frequency of PNH+ cases
Individuals with clinical and biological signs/symptoms of PNH in the absence of a previous hematological disorder (*n* = 3,032)	**8.7%**
Hemoglobinuria (*n* = 73)	47.9%
Hemolytic anemia (*n* = 382)	18.6%
Subtotal hemolysis (n = 455)	*23.3%*
Unexplained cytopenias including anemia (*n* = 393)	22.4%
Unexplained cytopenia without anemia (*n* = 772)	5.1%
Anemia, not otherwise specified (*n* = 468)	3.6%
Subtotal cytopenia (n = 1,633)	*8.8%*
Thrombosis with nonhemolytic anemia and/or other cytopenias (*n* = 73)	13.7%
Thrombosis without anemia and/or other cytopenia (*n* = 800)	0.4%
Subtotal thrombosis (n = 873)	*1.5%*
Iron deficiency (*n* = 57)	0%
Other (*n* = 14)	0%
Subtotal others (n = 71)	*0%*
Patients with hematological disorders (*n* = 906)	**29.8%**
Aplastic/hypoplastic anemia (*n* = 541)	44.9%
Myelodysplastic syndrome (*n* = 261)	9.8%
Subtotal BM failure (n = 802)	*33.3%*
Chronic myeloproliferative neoplasm (*n* = 21)	4.8%
Other hematological and/or immunological disorders (*n* = 78)	0%
Subtotal other non-BM failure disorders (n = 99)	*1.0%*
Total (*n* = 3,938)	**14.3%**

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FLOW CYTOMETRIC DIAGNOSIS OF PNH

The reasons for testing

The classical list of signs and symptoms which may prompt a testing order for PNH includes haemoglobinuria or haemosiderinuria, unexplained DAT-negative haemolysis, aplastic or hypoplastic anaemia, MDS, thrombosis in unusual sites, dystonic symptoms (dysphagia, abdominal pain, erectile dysfunction) (29). However, not all such signs and symptoms have the same diagnostic efficiency in helping the definition of a clinical PNH, especially when occurring separately. In general, when the above listed signs and symptoms combine, the clinical yield increases according to Morado et al. (30) (Table 1). For instance, testing for an underlying PNH in case of thrombosis alone may account for a clinical yield as low as 0.4 to 2.7%, (28, 30), whereas if thrombosis is associated with anemia and/or other cytopenias, the diagnostic efficiency may raise up to about 14% (30).

Preanalytical Issues

The available guidelines on FCM analysis of PNH have not addressed the specific preanalytical requirements of PNH analysis (1) or have provided only very concise indications (15, 31). In the year 2000 guidelines, the maximum blood sample age for analysis was reported as 7 days for RBC and 48 hours for white blood cells (WBC), with the recommendation to refrigerate samples at 4°C if storage time exceeds 24 hours (15). The more recent guidelines (17) just recommend less than 48 hour storage time for RBC with no further details.

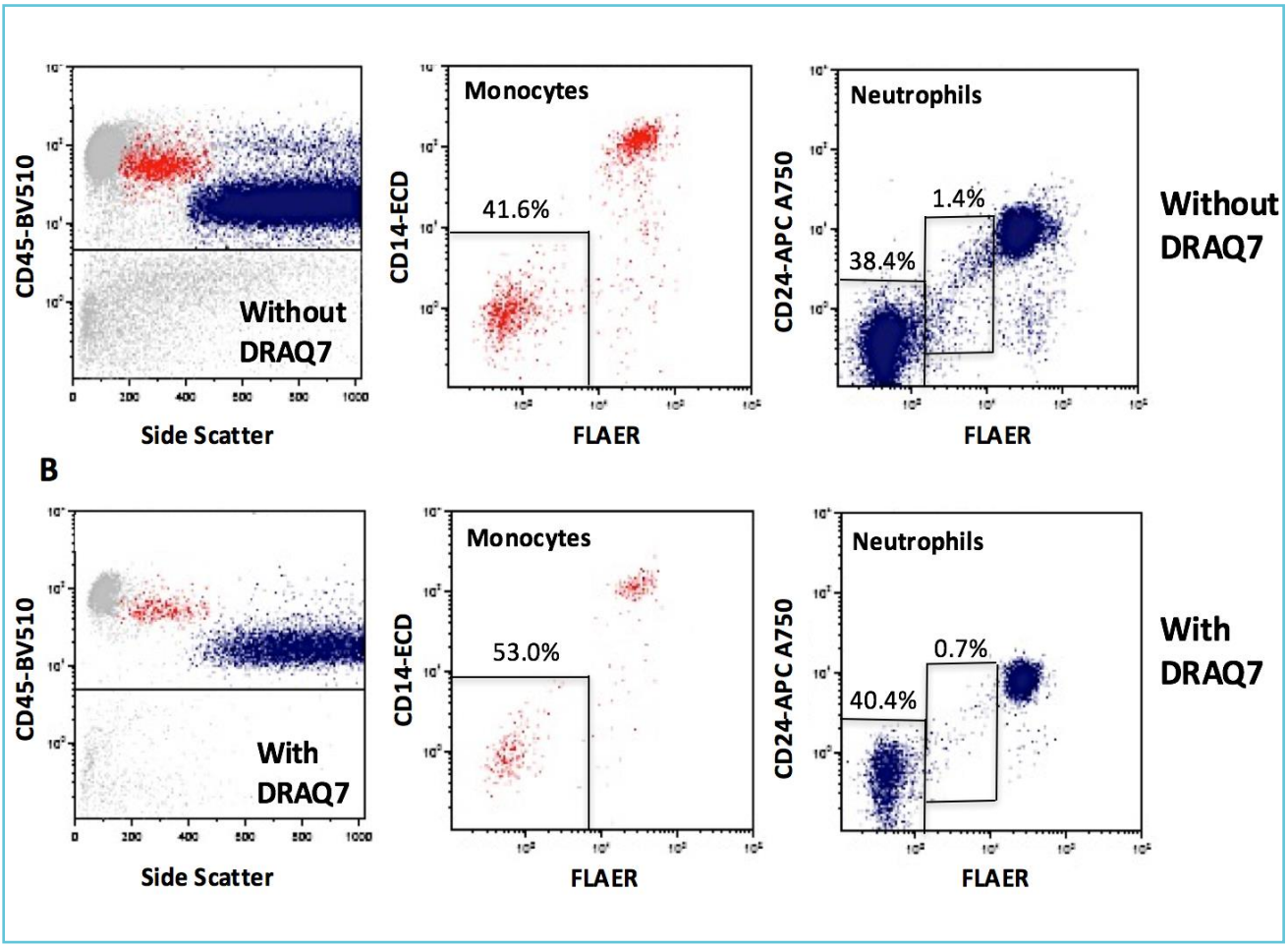
It is important to consider carefully the peculiar analytical context of FCM analysis of PNH,

where the absence rather than the presence of cell surface markers has to be demonstrated. The cell viability in anticoagulated blood samples progressively decreases with storage time, and may introduce unpredictable changes in the cell staining features, both increasing or decreasing artifactually the binding properties of conjugated antibodies or of other reagents.

Pre-analytical procedures, especially ammonium chloride lysis, may kill cells which are already damaged in aged samples. Dead and apoptotic cells can strongly influence PNH clone detection, while also causing non-specific antibody binding, increased autofluorescence and cell aggregation.

To avoid under- or overestimation of clone size a viability dye, i.e. DRAQ7 (32), can be used to exclude dead cells. DRAQ7 is a fluorescent impermeant dye (excited at 630 nm, emitting at >670 nm) that does not enter intact cells. When the cell membrane integrity is compromised DRAQ7 enters the cells and readily binds to nuclei to report cell death, thus improving the assay specificity and reducing artifacts (Figure 2).

Figure 2 **Artifacts induced by dead cells on WBC PNH clones in a suboptimal aged sample with reduced cell viability***



** DRAQ7 counterstaining allows the gating out of dead and apoptotic cells, reducing the non-specific reagent binding, with a marked influence on the calculated PNH clone size.*

The usage of erythrocyte bulk lysis procedures by ammonium chloride buffer before staining has been found useful in reducing the background signal and to increase the resolution between positive and negative cell populations (33, 34).

The FLAER reagent

The *Aeromonas hydrophila* toxin Aerolysin was demonstrated to be a highly specific ligand for the GPI molecule on white blood cells, thus making it a suitable indicator of GPI-deficient leucocytes in PNH (35, 36).

An inactivated non-toxic, fluorochrome-conjugated molecular variant was then developed under the acronym FLAER (FLuorescent AERolysin) and extensively applied as a highly effective non-antibody reagent to be used in FCM studies of PNH leucocytes, along with other conjugated monoclonal antibodies (MoAb) (17, 37-39).

The binding properties of FLAER on blood cells have been accurately studied, and the higher signal resolution obtained by the pre-staining bulk lysis of the sample has been demonstrated (33, 34).

Cell staining protocols

The 2018 ICCS/ESCCA guidelines (17, 31) have provided a fully comprehensive series of technical recommendations on sample preparation, reagent titration, MoAb clone and fluorochrome selection.

Bulk erythrocyte lysis of 1-2 ml of anticoagulated blood with ammonium chloride-containing buffer is useful to enrich cytopenic samples and to concentrate the cell pellet for high-resolution analyses requiring the collection of large numbers of events (33, 34).

For every cell type to be analyzed, gating antibodies are first used to capture the target populations, then GPI-deficient cells are defined in each subset using the appropriate MoAb combination and FLAER, where applicable (Table 2). Several possible staining alternatives have been studied and optimized (17).

It is important to restrict the analysis to mature WBC populations using MoAb reactive with GPI molecules fully expressed by peripheral blood mature cells and not by their progenitors, since these cells usually express GPI-linked markers at a lower density (8).

Table 2 **Examples of validated antibody mixtures for 6-color PNH analysis using recommended fluorochromes and clones, for Becton Dickinson and Beckman-Coulter instrument users***

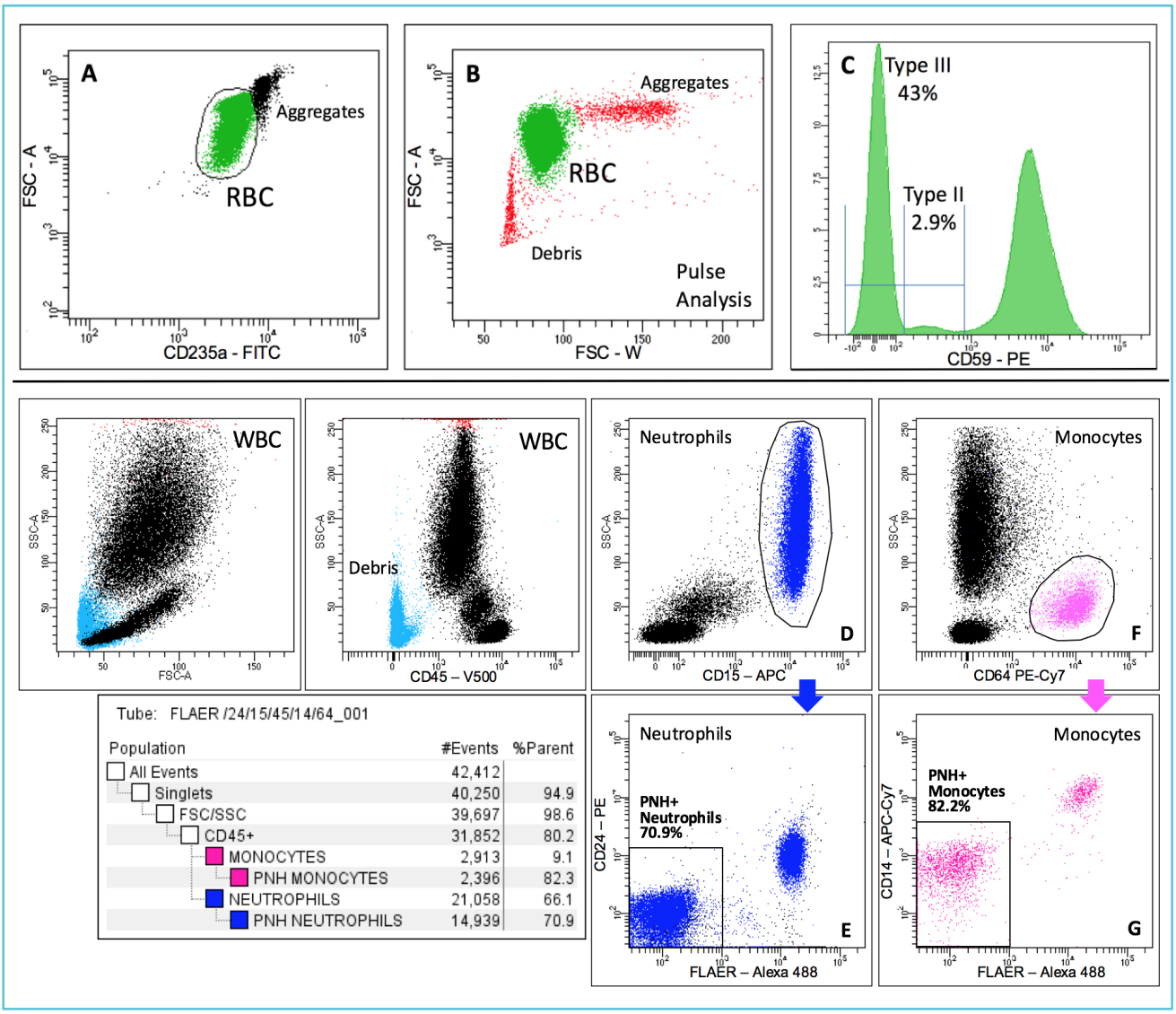
Becton Dickinson FACSCanto (2-Laser)	FLAER-Alexa 488	CD24-PE *(Clones SN3, ML5)*	CD15-PerCP-Cy5.5 *(Clone MEM-158)*	CD64-PE-Cy7 *(Clones 10.1, 22)*	CD14-APC *(Clone MφP9)*	CD45-APCH7 *(Clone 2D1)*
Beckman Coulter Navios (3-Laser)	FLAER-Alexa 488	CD24-PE *(Clones SN3, ALB9)*	CD15-PC5 *(Clone 80H5)*	CD64-PC7 *(Clones 10.1, 22)*	CD14-APC700 *(Clone RM052)*	*CD45-KO (Clone J33)*

** From (17), where several alternative reagent mixtures and clones are described.*
FLAER: FLuorescent AERolysin; Alexa: Alexa-Fluor 488nm fluorochrome; PE: Phycoerythrin; PerCP: Peridinin-Chlorophyll Protein; Cy: Prefix of Cyanine-series fluorochromes; APC: Allophycocyanine and its tandem conjugates; PC5: PE-Cyanine 5 tandem; PC7: PE-Cyanine 7 tandem; KO: Krome Orange.

To determine GPI-deficient RBC clones, of course, a lysis procedure should not be used. RBC are first diluted 1:100 in phosphate buffer (PBS), stained with anti-glycophorin-A (CD235a) for gating and CD59 for GPI-deficiency, then two washes using PBS supplemented with bovine serum albumin (BSA) are mandatory before reading. The FSC-W vs FSC-A pulse analysis of RBC physical parameters can be also of help in defining the RBC gate (Figure 3).

Thiazole orange pre-staining of reticulocytes within the RBC cluster can be also used to measure the

Figure 3 An example of PNH analysis using the recommended guidelines in a highly symptomatic patient, off therapy



Upper panels: *RBC analysis using CD235a for gating (A) and CD59 for PNH clone detection. Type II and Type III clones are detectable (C). In panel (B) the FSC pulse analysis of RBC shows a good discrimination of RBC singlets from debris and aggregates.*

Lower panels: *WBC analysis using CD15 as the gating marker for neutrophils (D) and CD64 as the gating marker for monocytes (F). Panel (E) shows a large PNH neutrophil clone, identified by combining FLAER and CD24. Panel (G) shows a large monocyte clone, identified by FLAER and CD14.*

proportion between normal and GPI-deficient erythropoiesis, which is very useful in evaluating the red cell compartment response to therapy (40).

The state-of-the-art FCM technique to study PNH clones among WBC is a five- to six-color combination that is adjusted slightly according to instrument brand, optical filter sets and laser types.

For WBC analysis whole blood is preferably bulk-lysed and washed twice with PBS+BSA before staining. CD45 and side scatter are used along with CD15 and CD64 to gate neutrophils (with exclusion of eosinophils that express CD15 in lower amount) and monocytes, respectively. FLAER and CD24 is the optimal reagent combination to identify GPI-deficient neutrophils, whereas FLAER and CD14 are used to study PNH monocyte clones (Figure 3).

Lymphocytes are not useful in PNH diagnosis, due to their very long lifespan and to an excess variability in the expression of surface GPI-linked molecules (15). The role of lymphocytes in PNH analysis is currently limited to internal staining controls (17).

Platelets in PNH have a normal lifespan despite expressing the same GPI-molecule deficiency of RBC, and are not included in routine diagnostic procedures (6).

The reported limited availability or the high cost of FLAER in certain countries (17) have prompted the development of non-FLAER based staining protocols, introducing CD157 as a replacement for CD24 and CD14 to determine PNH clones in neutrophils and monocytes, respectively (41, 42). However, the reduced CD157 expression by eosinophil granulocytes may cause artifacts in case of imprecise gating of CD15+ neutrophils (41), and genetic or ethnic variants of CD157 have been described which may account for the detection of false PNH clones in some individuals (43, 44).

Recently, additional multicolor FCM protocols have been described, coupling FLAER to CD157 (7-colors) (45) or adding CD5 and CD19 as a dump channel to reduce background fluorescence (8-colors) (46). The advantages provided by this increase in fluorochrome number and complexity are still to be fully evaluated.

Instrument setup, optimization and gating syntax

Modern digital flow cytometers are equipped with very efficient setup and calibration bead suspensions that should be used to optimize automatically the photomultiplier voltages, the thresholds, the intra- and inter-laser fluorescence spillover compensation, ensuring instrumental consistency with time and comparable results among laboratories using the same type of equipment (47-50). Therefore, if manufacturer's directions are strictly applied, the FCM setup is nowadays easy to standardize, thus making the instrument-related variables almost negligible.

The creation of an appropriate gating syntax, however, is still a manual process that requires skilled operators and sample-to-sample adjustments in order to correct for the unavoidable inter-patient variability in cell cluster positioning (17, 45).

The limits of detection – coping with background events

As mentioned, in PNH analysis the lack rather than the presence of cell surface molecules is the indicator of the disease-related abnormalities. The presence of some GPI-negative elements in the various blood cell subsets of healthy subjects is a well-known phenomenon and represents the assay background (33, 51, 52).

Furthermore, the presence of blasts or other immature cells, as it occurs in MDS, may strongly influence the amount of background events.

This requires a strict check of the analytical process using a cohort of healthy donors to control and minimize the background event frequency, which in turn vary in the different cell populations and in the different clinical settings. Moreover, it is mandatory to pay attention to the numerous biological and staining artifacts that may generate negatively stained cells and false 'PNH events' in the background, such as the presence of autofluorescent events that come as diagonally shaped or 'angle of dangle' parasite events in fluorescence plots (53).

Before the advent of FLAER the high background level in healthy controls and other technical limitations made it difficult to enumerate accurately PNH events below 1% (54). Following the more recent FLAER-based guidelines on high-resolution PNH analysis (16, 17, 41, 45), a clearer picture of the lower limits of detection (LLOD) and of quantitation (LLOQ) of PNH events can be obtained. These issues have been thoroughly discussed and validated in the recent study by Payne (53).

It is important to mention here how the cytometry researchers have tried over the years to translate the concepts of lower limit of blank (LLOB), LLOD and LLOQ, typical of chromatographic or spectrophotometric procedures (55, 56), into a technology based on event accumulation (57). This transition was made - rather simplistically - by analogy with clinical chemistry, but surely without applying the same methodological care, and it is still matter of debate.

With the use of a negative or normal sample, the events falling into the final acquisition window may be taken as the LLOB, and ratioed to their cellular counterpart (the 'clean cell denominator') to obtain the 'blank' level to be subtracted from the analysis of the relevant cell population.

The Poisson's statistics governing rare cellular event analysis mandates the acquisition of at least 100 relevant events to generate measures with the acceptable coefficient of variation of 10%. In the delicate field of high-resolution FCM, however, the collection of 100 events can be sometimes out of reach, so a compromise has been developed, proposing a lower limit of 20 (31), 25 (53) or 30 (58) relevant events for the calculation of LLOD in high-sensitivity PNH and leukemia minimal residual disease analyses, keeping a universally accepted limit of 50 events for the LLOQ. Little attention has been paid however to the inherent variance of such a new approach, which dramatically increases with the lowering of the relevant event number. Despite increasing the acquisition to millions of clean cells, the lower the established minimum level of the detectable relevant events, the wider the confidence interval and the variability of the cytometric measure, according to Poisson's statistics.

In the daily practice, assuming 20 events (31) or 30 events (58) as the minimum size of a relevant cell event cluster for detection, and 50 events as the minimum for quantitation (31, 58), the calculation of LLOD% is: 2,000 (or 3,000) / total number of clean events; and the calculation of LLOQ% is: 5,000 / total number of clean events.

When the same criteria for minimal residual disease studies with high resolution FCM analysis of hematological malignancies are applied (i.e. from 500,000 to >1,000,000 clean cell events as the denominator (58), the median background 'PNH' Type III RBC events in normals are from 3 to 5 per million, with a range from 0 to 17 per million or 0.002-0.007% and a LLOQ around 0.005% (31, 52). Type II RBC events are often difficult to dissect precisely from Type I and Type III populations, moreover they can be generated by artifacts. As a consequence, Type II detection levels are usually not addressed in high-resolution PNH studies (53). For CD15+ neutrophils the average background in normals ranges from 2 to 10 per million or 0 to 0.001%

(8, 31, 52). Similar figures in normal subjects have been consistently reported by other groups (33, 51, 53). For CD64+ monocytes, the low number of events that can be acquired in clinical samples greatly reduces the achievable sensitivity level, with background levels ranging around 0.1% (34, 52).

PNH clone patterns – diagnostic criteria

The total absence of GPI-linked molecules defines a Type III PNH clone, whereas a partial deficiency defines a Type II PNH clone. It is customary to quantify and report the presence of Type II clones in the RBC population only, while WBC Type II (if detectable) and Type III clones are usually summed up in the reporting, with no further details (31).

RBC PNH clone size may vary remarkably according to the occurrence of hemolytic crises, to the administration of blood transfusions or Eculizumab, so RBC PNH clones may not reflect accurately the disease extent, are often smaller than the respective WBC clones and are not an ideal target for PNH quantitation at diagnosis (38, 59). The established guidelines state that the absence of two GPI-linked molecules must be demonstrated in WBC lineages to corroborate the diagnosis (15-17). Neutrophil and monocyte PNH clones usually tend to be of similar magnitude. In a small percentage of patients, however, marked discrepancies may occur, more often with PNH monocyte clones outnumbering neutrophil clones (20, 31). PNH clone size and type can also change with time, and can both increase or decrease in an unpredictable fashion, thus indicating the need for a follow up with time (20). The correlations between PNH cell clones and the associated clinical pictures - also in pediatric patients - have been extensively reviewed, as well as the recommended criteria for the longitudinal follow-up of small clones (10, 20, 59, 60).

Patients under Eculizumab treatment usually display expanded RBC clones, due to the increased lifespan of GPI-deficient erythrocytes (3, 4, 24, 25, 27). In such patients the abrupt discontinuation of Eculizumab may increase the intensity and the severity of hemolytic crises (25).

The high-resolution PNH analysis and assay precision

Until a few years ago a technical distinction between 'routine diagnostic' and 'high-resolution' PNH analysis was commonplace in the literature and considered in external quality assessment exercises. With the advent of the highly standardized recent protocols such a difference has lost its significance (17), since using the same sensitive laboratory technique both routine diagnostic and high-resolution analysis of PNH clones can be accomplished accurately (62). It is well known that PNH+ patients can have a neutrophil PNH clone of <1% in some 42% of cases (31) (Figure 4), thus stressing the need for the systematic usage of a very sensitive FCM technique. Small PNH clones are more common in patients with BMF syndromes, may change their size with time and seem positively correlated to the response to immunosuppressive treatments (63). The term 'High resolution FCM' refers mainly to the collection of an adequate number of cell events (i.e. from 500,000 to 1-2 million), which enables analyses at a sensitivity levels around 10^{-4} with LLOQ equal or less than 0.005%. This feature can be easily applied to RBC and neutrophil analyses, but such a level of resolution is almost impossible to achieve in the routine with monocytes, due to their low level in peripheral blood.

As expected in any FCM rare event analyses, the lower the PNH clone percentages the worse the assay precision, ranging from 14 to 25% at the lowest limit of sensitivity (31, 52). Moreover, the precision in detecting a very small but well resolved Type III clone is usually better that the

one achieved with larger but less clearly separated Type II clones (53, 61). The acceptable limit of precision for clinical purposes is not yet well established in the literature. Very small PNH clones, however, do not make a diagnosis of clinical PNH and usually do not indicate the need of therapeutic measures, although a strict monitoring also of small clones over the time is mandatory in any instance.

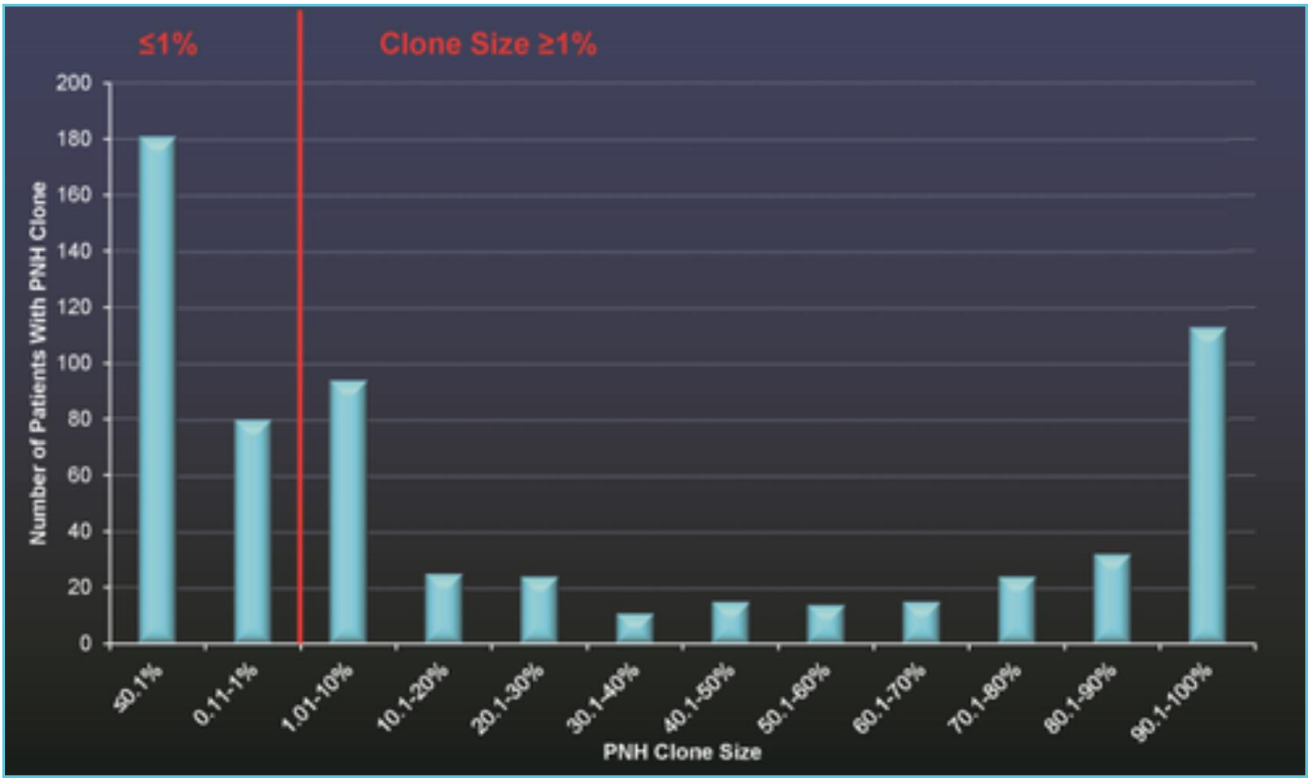
Assay results – reporting PNH analysis

In the reporting of PNH analyses it is important to use a standardized terminology, in order to avoid a common shortcoming, namely the over-diagnosis of clinical PNH when only small GPI-deficient clones are detected. The recommended reporting terminology to be used is: 'Presence of a PNH Clone [specify the cell type(s)] ' in case of cell clones >1%; 'Presence of minor [specify the cell type] PNH population(s)' from 0.1% to 1%; 'Presence of rare GPI-deficient cells [specify the cell type]' when <0.1% (31, 64). The achieved sensitivity level should be specified in the reporting for every cell subset, since it may vary according to the patient's status and the overall cell collection. As mentioned, the presence of Type II clones should be reported for RBC only (31).

Screening tests versus diagnostic confirmation

PNH is a very rare disease, with a diagnostic yield ranging from 6% to 14% under the best reason for testing conditions. This means that testing for PNH will give negative results in the vast majority of the checked cases. In the literature, a primary screening test before a diagnostic confirmation assay in selected patients has been

Figure 4 **PNH clone size distribution in 633 PNH+ patients out of 10,236 screened subjects (clinical yield 6.18%) using neutrophils as clone indicators. 42% of PNH+ patients had a PNH clone of <1%.***



** Reproduced by permission from (31).*

repeatedly proposed to rule out the more numerous negative cases (16, 20, 34, 38, 65). The current 6- or 7-color technical guidelines can be costly, complex and demanding for small peripheral laboratories, thus preventing a diffuse first-level disease surveillance, which should be practiced in every blood transfusion center and hematology laboratory. Due to their favorable features, neutrophils and monocytes may serve better than RBC as the preliminary index population for the screening of new PNH cases (38) in patients with the appropriate reasons for testing (Figure 4). In some countries samples with an initially positive first-level screening test for PNH are then referred to an accredited central facility for diagnostic confirmation, using a state-of-the-art multicolor technique (34). A simplified and inexpensive PNH screening test, which can be performed upon the initiative of the laboratory itself and affordably repeatable over the time, is a cost-effective option especially in resource-restricted countries. This approach proved valuable in disclosing new PNH cases in local studies (20, 34), and can be a practical solution to overcome the disappointing level of disease surveillance reported in some settings (66).

External quality assessment for PNH

The blood stabilization technology implemented by United Kingdom National External Quality Assurance Schemes (UK NEQAS) has been applied in developing stable PNH samples to be used as process control and in external quality assessment studies (67). This advancement has greatly facilitated the diffusion of the FCM analysis of PNH and stimulated the participating centers in selecting the optimal procedures, antibody clones and fluorochromes (62, 68). The periodic surveys made by UK NEQAS, however, still highlight a certain degree of variability in the interpretation and usage of the available guidelines by some participants, which may account for an excess of non-specific assay results in some instances (i.e. PNH clone erroneously detected in healthy donor samples). Two separate PNH schemes - ordinary and high-sensitivity - have been developed initially, and were subsequently merged for the reasons discussed above.

Pifalls and artifacts

Provided the reasons for testing are clinically appropriate and the recommended techniques are applied, the FCM operators involved in PNH analysis may come through a series of technical artifacts and pitfalls with real-life samples, that require an in-depth expertise for the full control of the technology.

Despite CD55 is traditionally included in the list of GPI-linked RBC molecules, Anti-CD55 MoAbs have been progressively dismissed from FCM panels due to the weak antigen expression (17). When RBC are stained with anti-CD59, at least two washing steps are then required to eliminate excess fluorochrome and non-specific MoAb binding, which greatly interfere with positive/negative discrimination (15).

A typical problem with WBC analysis is represented by the appearance of putative small Type II clones, especially in the monocyte gating, often due to errors in mixing the sample with the reagents, to an imprecise gating or to cell adhesion phenomena. Basophils and NK cells that can be erroneously captured in the neutrophil or in the monocyte gate are characteristically stained dimly by FLAER, and can simulate Type II PNH clones. Myeloid blasts that often circulate in patients with BMF syndromes behave the same way, and can be captured in the monocyte gate, especially if they express CD64. Large platelet aggregates can also be dragged into the monocyte gate, and may account for FLAER-negative events (34). This artifact can be effectively prevented by bulk lysis and extensive washings.

Another common problem is represented by the acquisition of an insufficient number of cell

events and by the calculation of improper PNH clone percentages, in the erroneous belief to have achieved a sufficient level of assay sensitivity (62). The latter problem is particularly evident with monocytes, that in some instances cannot be collected in sufficient number for a true high-sensitivity analysis.

CONCLUDING REMARKS

Flow Cytometry is today the laboratory technique of choice to provide an accurate, sensitive, standardized and widely applicable diagnosis of PNH. The diagnostic efficiency largely resides in the appropriateness of the reasons for testing, which may be a major conditioning factor in the clinical workup of this rare and elusive disease.

The state-of-the art multicolor FCM technology is mature, robust, covered by international ISO17043-certified external quality assessment schemes, and enables the detection also of very small PNH clones with good accuracy and precision. The implementation of a diffuse screening network in partnership with central, second-level reference facilities seems today a rational and affordable model to favor the disclosure of new PNH cases.

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In this issue: Clinical flow cytometry in 2019

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Flow cytometric analysis of monocytes polarization and reprogramming from inflammatory to immunosuppressive phase during sepsis

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ABSTRACT

Sepsis outcome is determined by a balance between inflammation and immune suppression. We aimed to evaluate monocytes polarization and reprogramming during these processes.

We analyzed 93 patients with procalcitonin level >0.5 ng/mL (hPCT) and suspected/confirmed sepsis, and 84 controls by analysis of CD14, CD16 and HLA-DR expression on blood monocytes using fluorescent labeled monoclonal antibodies and BD FACS CANTO II. Complete blood cell count, procalcitonin and other biochemical markers were evaluated.

Intermediate monocytes $CD14^{++}CD16^{+}$ increased in hPCT patients (including both positive and negative culture) compared to controls (13.6% ± 0.8 vs 6.2% ± 0.3, $p<0.001$), while classical monocytes $CD14^{++}CD16^{-}$ were significantly reduced (72.5% ± 1.6 vs 82.6% ± 0.7, $p<0.001$). Among hPCT patients having positive microbial culture, the percentage of intermediate

monocytes was significantly higher in septic compared with non-septic/localized-infection patients (17.4% vs 11.5%; p<0.05) whilst the percentage of classical monocytes was lower (68.0% vs 74.5%). Three-four days following the diagnosis of sepsis, HLA-DR expression on monocyte (mHLA-DR) was lower (94.3%) compared to controls (99.4%) (p<0.05). Septic patients with the worst clinical conditions showed higher incidence of secondary infections, long-time hospitalization and lower HLA-DR^{+} monocytes compared to septic patients with better clinical outcome (88.4% vs 98.6%, p=0.05).

The dynamic nature of sepsis correlates with monocytes functional polarization and reprogramming from a pro-inflammatory CD14^{++}CD16^{+} phenotype in non-septic hPCT patients to a decrease of HLA-DR surface expression in hPCT patients with confirmed sepsis, making HLA-DR reduction a marker of immune-paralysis and sepsis outcome.

Analysis of monocytes plasticity opens to new mechanisms responsible for pro/anti-inflammatory responses during sepsis, and new immunotherapies.

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INTRODUCTION

A major defense mechanism against infection and tissue injury is provided by the innate immune system through inflammation (1). Circulating monocytes are critical effectors in the immune response, cross-link innate and adaptive immunity, and are involved in pathogenesis of several inflammatory diseases. Based on the differential expression of LPS receptor, CD14, and FcγIIIR, CD16, three subpopulations are defined: classical monocytes as CD14^{++}CD16^{-}, intermediate monocytes as CD14^{++}CD16^{+} and non-classical monocytes as CD14^{+}CD16^{++} (2). These subsets are associated with specific functions. Classical monocytes show antimicrobial potential, while CD16 positive monocytes are involved in antigen processing and presentation as well as trans-endothelial migration (3). The expansion of the CD16 positive monocytes has been observed in many different types of disorders, especially infection or inflammatory conditions (4).

The host immune response during sepsis is complex and variable over time. The first phase is characterized by an overwhelming inflammation also known as the “cytokine storm”, during which blood monocytes release high levels of pro-inflammatory cytokines (e.g. TNF-α and IL-1) (5). This phase is followed by a stage of immune-suppression in which patients are characterized by persistent inflammation, neutrophilia, lymphopenia and they are more susceptible to secondary infections due to a dysregulation of innate and adaptive immunity (6). In this stage of immune-paralysis, monocytes are functionally deactivated and show decreased release of pro-inflammatory cytokines and decreased antigen presenting capacity because of low HLA-DR surface expression (7).

Human leukocyte antigen DR (HLA-DR) is a cell surface receptor involved in antigen presentation to the adaptive immune system for the purpose of stimulating T cell responses. Many clinical studies have associated the low levels of HLA-DR expression on circulating monocytes with the increased risk of adverse outcome or death of septic patients and increased susceptibility to contracting secondary/nosocomial infections (8-10). For this reason, among all biological parameters, the evaluation of HLA-DR levels on monocytes surface represents the most studied marker to establish their anergy and the general immuno-paralysis state of patients (11-12).

Changes in circulatory cytokines and surface markers can help understanding the mechanisms of response to infection and finding new diagnostic tools. Characterization of cellular component

of the immune system by flow cytometry is a useful tool to evaluate and phenotype immune response during sepsis (10, 11).

In the present study, we investigated the phenotypic and functional changes in different monocyte subsets from inflammatory state to septic state by flow cytometry analysis in order to evaluate monocytes polarization and reprogramming during these processes.

MATERIALS AND METHODS

Patients

The present study has been conducted on 93 patients hospitalized in different medical and surgical wards of the Vito Fazzi Hospital of Lecce (Italy) with high procalcitonin plasmatic levels (PCT > 0.5 ng/mL) and suspected bacterial infection, based on several clinical suspicions. 84 not hospitalized healthy individuals were used as controls.

For each patient, clinical and biological variables were collected. These included demographic characteristics (age and gender), microbiological findings (infection source and the identified microorganisms) and routine markers of inflammation as PCT, C-reactive protein (CRP), complete blood cell count and biochemical markers.

Sepsis diagnosis has been established according to Society of Critical Care Medicine and the European Society of Intensive Care Medicine (13). Written informed consent was obtained from the patients or, if not possible, from their relative as designed by ethic committee.

Hematochemical investigation

Complete blood count was performed on a Sysmex XE-2100 Automatic Hematology Analyzer (Sysmex, Kobe, Japan). Plasmatic levels of PCT were determined by Enzyme-Linked Fluorescence Assay (ELFA) with the VIDAS® B.R.A.H.M.S. PCT™ system (Biomerieux, Marcy-l'Etoile, France), according to manufacturer recommendations and expressed in ng/mL.

Serum levels of CRP were measured immune-turbidometrically on the analyzer Roche/Hitachi MODULAR P, according to manufacturer recommendations (Roche Diagnostic GmbH) and expressed in mg/L.

All others biochemicals parameters analyzed were performed on Modular Cobas® 8000 according to manufacturer directions (Roche Diagnostic GmbH).

Microbiological cultural assay

Microbial cultures were used to determine the type of organism present in different biological specimens derived from different site (respiratory tract, urinary tract, abdomen, and others) according to clinical suspicion and test requested to the laboratory by standardized microbiological cultural assays procedures.

Blood cultures were performed in patients with clinical symptoms of bloodstream infections prior the administration of antimicrobial therapy. For each patient, two bottles sets were used for each septic episode; approximately 10 mL of blood was inoculated in the aerobic and anaerobic bottle (BACT/ALERT Culture Media, Biomerieux, Marcy-l'Etoile,France); the bottles were entered in the BACT/ALERT 3D System for the incubation and measure of the color change in response to shift in pH as a result of rising of CO_2 levels produced by microorganisms. In positive samples, bacteria and yeasts were identified on the Vitek 2 system (Biomerieux, Marcy-l'Etoile, France), according to manufacturer directions.

Flow cytometrical analysis

For monocytes phenotypic analysis, 100µL of EDTA anticoagulated whole blood from patients and controls were incubated with the following combination of monoclonal antibodies: 20 µL of

anti CD45-PerCP (BD, clone 2D1), 20 µL of anti CD14-PE (BD, clone MϕP9), 20 µL of anti CD16-PE-Cy7 (BD, clone B73.1) and 20 µL of anti HLA-DR-APC-Cy7 (BD, clone L243) for 30 minutes in the dark. All samples were stained within 1.5 hours after blood collection.

After staining, red blood cells were lysed for 15-20 minutes with 1:10 dilution of BD FACS™ lysing solution (containing 30.0% diethylene glycol, 9.99% formaldehyde and 3.51% methanol), and centrifugated for 5 minutes at 300xg. Cell pellet was resuspended in 450 µL of BD FACSFlow™ Sheath Fluid solution and acquired on a BD FACS CANTO II flow cytometer.

A minimum of 3500 monocyte events were recorded for each sample based on a gate created on scatter plot of CD45-PerCP vs side scattered light signals. The three monocytes subsets, $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$, were identified by different surface expression of LPS receptor CD14 and the FcγIIIR CD16, among $CD45^{+}$ gated monocytes.

Moreover, in setting conditions, we used also markers for other leucocytes populations (as CD3 for T lymphocytes, CD19 for B lymphocytes or other specific markers of granulocytes lineage in combination with physical and dimensional parameters) and we confirmed the accurate gating of monocytes even in absence of additional markers (data not shown).

For mHLA-DR quantitation, total monocyte events were recorded for each sample on a gate created on scatter plot of CD14-PE vs side scattered light signals. mHLA-DR expression was reported as a percentage of HLA-DR positive monocytes out of the total $CD14^{+}$ monocyte population and as the Mode of Florescence Intensities (MFI) of the analyzed monocyte's population. Samples were collected after 3-4 days from sepsis diagnosis. Data were analyzed by BD Facs DIVA 8.0.1 software (Becton Dickinson).

Statistical analysis

Statistical analysis was performed by MedCalc v19.9.1 statistical software. Data were presented as the mean ± SEM (Standard Error of the Mean). The Student's *t*-test was used for comparison between patients and controls; statistically significant differences were established by *p* value (< 0.05).

RESULTS

The population of 93 hospitalized patients analyzed in the present study was enrolled according to high plasmatic level of procalcitonin (PCT > 0.5 ng/ml, hPCT) while 84 not hospitalized healthy individuals were used as controls. Demographic and clinical characteristics of all individuals are shown in Table 1.

Total white blood cells, monocytes and neutrophils counts were significantly higher in patients compared to controls (Table 1), while lymphocytes count was significantly reduced (Table 1). 24,7% of hPCT patients with negative cultural assay (Cult-NEG, n=23, Table 2) showed a condition defined "strong inflammatory state" characterized by alteration of white blood cells count (13.0 ± 1.6 x 10^9/L, reference range 4.0-10.0 x 10^9/L) and hematological parameters, increased C-reactive protein plasmatic levels (115.0 ± 16.5 mg/L, reference range 0.0-10.0 mg/L), as well as some liver markers (i.e. AST 54.4 ± 18.8 U/L, reference range <45 U/L; GGT 85.9 ± 26.1 U/L, reference range <45 U/L) and total bilirubin (3.0 ± 1.9 mg/ dL, reference range <1.25 mg/dL).

The remaining 75,3% of hPCT patients showed positive cultural assay demonstrating the presence of "infection" in different site as blood, respiratory tract, urinary tract, abdomen, and others (Cult-POS, n=70, Table 2). Among infection-positive patients, 68.6% were infected by Gram negative bacteria, 21,4% by Gram positive bacteria and 10% by Candida.

Table 1 Demographic and clinical characteristics of hPCT patients (procalcitonin > 0.5 ng/mL) and controls

	hPCT patients (n=93)	Controls (n=84)	T-test
Gender	Male n= 53 Female n= 40	Male n= 41 Female n= 43	-
Age (years)	65.9±1.8 62.5 to 69.5 34.2 to 89.0	50.4±1.5 47.3 to 53.4 26.5 to 69.5	-
WBC (10^9 cell/L)	12.9±0.8 11.4 to 14.5 3.0 to 27.0	6.5±0.2 6.2 to 6.8 4.2 to 9.1	p<0.001
Platelets (10^9 cell/L)	220.5±19.7 181.4 to 259.7 28.0 to 520.3	224.5±7.3 210.0 to 239.0 133.2 to 337.0	p=0.85
Monocytes (10^9 cell/L)	0.9±0.07 0.8 to 1.1 0.2 to 2.4	0.5±0.02 0.5 to 0.6 0.3 to 0.8	p<0.001
Neutrophils (10^9 cell/L)	10.6±0.7 9.1 to 12.0 2.0 to 24.0	3.7±0.1 3.4 to 3.9 2.0 to 5.8	p<0.001
Lymphocytes (10^9 cell/L)	1.3±0.08 1.1 to 1.5 0.3 to 2.8	2.1±0.06 2.0 to 2.2 1.2 to 3.0	p<0.001
PCT (ng/mL)	13.6±3.3 7.0 to 20.2 0.5 to 80.0	<0.05	-
CRP (mg/L)	141.9±12.5 116.9 to 166.9 12.9 to 346.0	<10	-

Data are presented as mean ± SEM, 95% CI (mean) and 5-95 percentiles.

Table 2 Grouping of analyzed patients

Analyzed patients			
hPCT patients n=93			Controls n=84
INFLAMMATION (Cult-NEG) n=23	INFECTION (Cult-POS) n=70		
	BC+ n=44	BC- n=26	

hPCT, patients with procalcitonin >0.5ng/mL; Cult-NEG, hPCT patients with negative cultural assay; Cult-POS, hPCT patients with positive cultural assay in different site; BC+, hPCT patients with positive blood culture; BC-, hPCT patients with positive cultural assay in different site.

Bloodstream infection was demonstrated by positive blood culture (BC+, n=44, Table 2) in CultPOS patients with clinical evaluation correlated with sepsis (13).

In a small number of septic patients (n=23), biochemical and hematological parameters were monitored at admission (time 1), at blood culture request (time 2), after 3-4 days from diagnosis (time 3), as well as after 6-7 days from diagnosis (time 4).

The samples were retrospectively categorized into two groups characterized by better clinical outcome (group BO) or worst clinical outcome (group WO) based on time of hospitalization, recovery time from primary infection, appearance of secondary/nosocomial infections and death. Clinical characteristics of subgroups with better and worst clinical outcome are shown in Table 3.

Blood monocyte subsets

Monocytes subsets were differentiated by flow cytometrical analysis on the basis of CD14 and CD16 surface expression.

Intermediate monocytes $CD14^{++}CD16^{+}$ were significantly increased in hPCT patients compared to controls (13.6% ± 0.8 and 6.2% ± 0.3 respectively with $p<0.001$), while classical monocytes $CD14^{++}CD16^{-}$ were significantly reduced (72.5% ± 1.6 vs 82.6% ± 0.7, $p<0.001$). Non-classical monocytes didn't show significant differences between patients and controls (5.5% ± 0.9 and 5.1% ± 0.3 respectively with $p=0.6$).

The differences between subsets of monocytes from hPCT patients with infection (Cult-POS) and with inflammatory state (Cult-NEG) were not significant: in both patient groups, intermediate monocytes remained significantly increased compared to healthy controls (Figure 1) highlighting a pro-inflammatory phenotype for all hPCT patients.

Cult-POS infected patients included both septic patients (BC+) and non-septic patients (BC-)presenting infection in other body sites, excluding bloodstream infection).

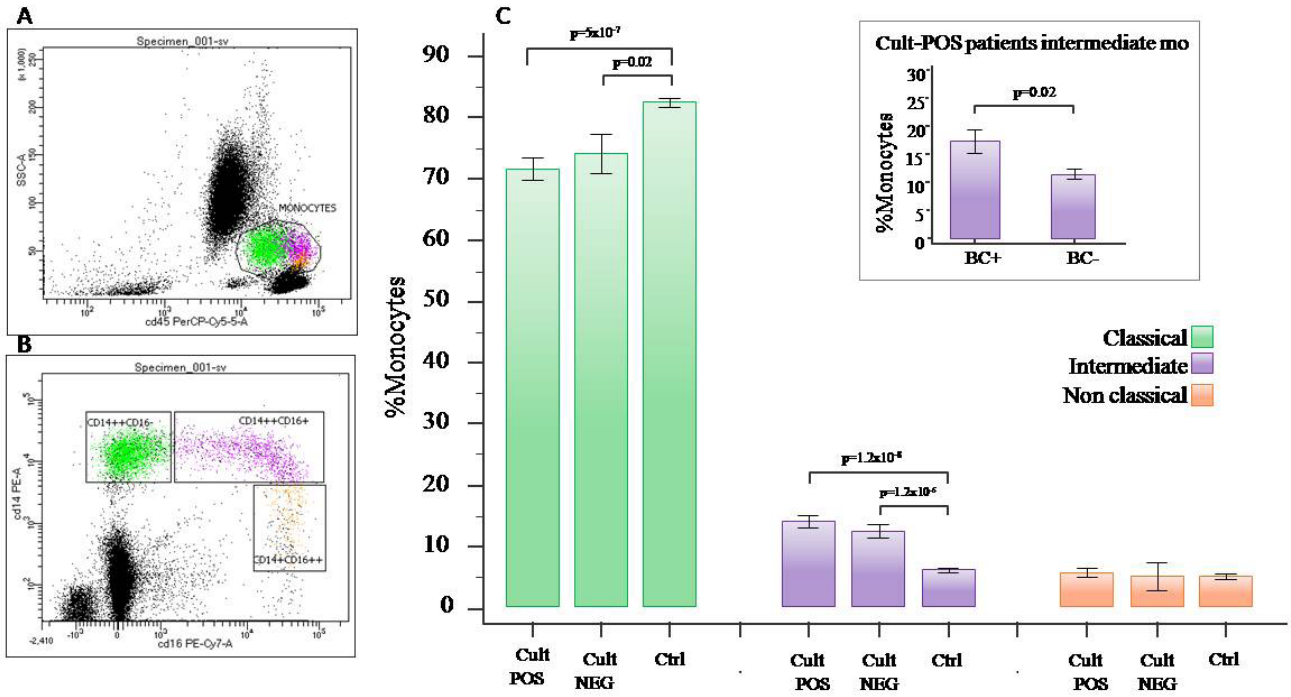
Intermediate monocytes were significantly increased in BC+ compared to BC- (17.4% ± 2.1

Table 3 Clinical characteristics of BC+ patients according to clinical outcome

	Better Outcome, BO (n=13)	Worst Otcome, WO (n=10)	T-test
WBC (10^9 cell/L)			
Time 1	10.2±2.2	11.3±1.5	p=0.72
Time 2	11.5±2.7	14.4±2.8	p=0.47
Time 3	12.1±2.3	16.4±2.9	p=0.25
Time 4	8.4±0.9	14.0±3.0	p=0.99
Platelets (10^9 cell/L)			
Time 1	198.5±30.3	237.1±43.0	p=0.46
Time 2	209.9±32.7	105.9±26.9	***p=0.02***
Time 3	216.3±33.5	113.8±34.1	***p=0.04***
Time 4	291.6±42.6	175.4±46.3	p=0.08
Monocytes (10^9 cell/L)			
Time 1	0.6±0.09	0.5±0.09	p=0.20
Time 2	0.7±0.09	0.7±0.1	p=0.99
Time 3	0.8±0.09	1.1±0.3	p=0.28
Time 4	0.7±0.07	0.9±0.2	p=0.29
Neutrophils (10^9 cell/L)			
Time 1	8.3±2.3	9.4±1.5	p=0.70
Time 2	9.1±2.5	12.7±2.7	p=0.33
Time 3	9.7±2.2	14.2±2.7	p=0.21
Time 4	5.7±0.6	11.6±2.7	p=0.06
Lymphocytes (10^9 cell/L)			
Time 1	1.2±0.2	1.3±0.2	p=0.58
Time 2	1.5±0.4	0.9±0.1	p=0.18
Time 3	1.5±0.2	1.1±0.2	p=0.16
Time 4	1.7±0.2	1.4±0.3	p=0.38
Secondary/nosocomial infections	n=2	n=4	
Death	/	1	

Data are presented as mean ± SEM. Time 1, admission; time 2, at blood culture request; time 3, after 3-4 days from diagnosis; time 4, after 6-7 days from diagnosis.

Figure 1 Percentage of monocytes subsets in Cult-POS, Cult-NEG and Ctrl patients



***A**: Flow cytometric analysis of monocytes on a gate created on scatter plot of CD45-PerCP vs side scattered light signals.*

***B:** Monocytes subsets, CD14++CD16-, CD14++CD16+ and CD14+CD16++, are identified by different surface expression of CD14 and CD16, among CD45+ gated monocytes.*

***C:** Bar graph of percentage of three monocytes subsets in Cult-POS, Cult-NEG and healthy control (Ctrl) patients.*

Cult-POS patients showed significantly higher percentage of intermediate monocytes compared to Cult-NEG patients and Ctrl, and lower percentage of classical monocytes.

The difference between blood culture positive and negative Cult-POS patients are showed in the box. (P values are showed in figure; where not indicated, difference was not statistically significant).

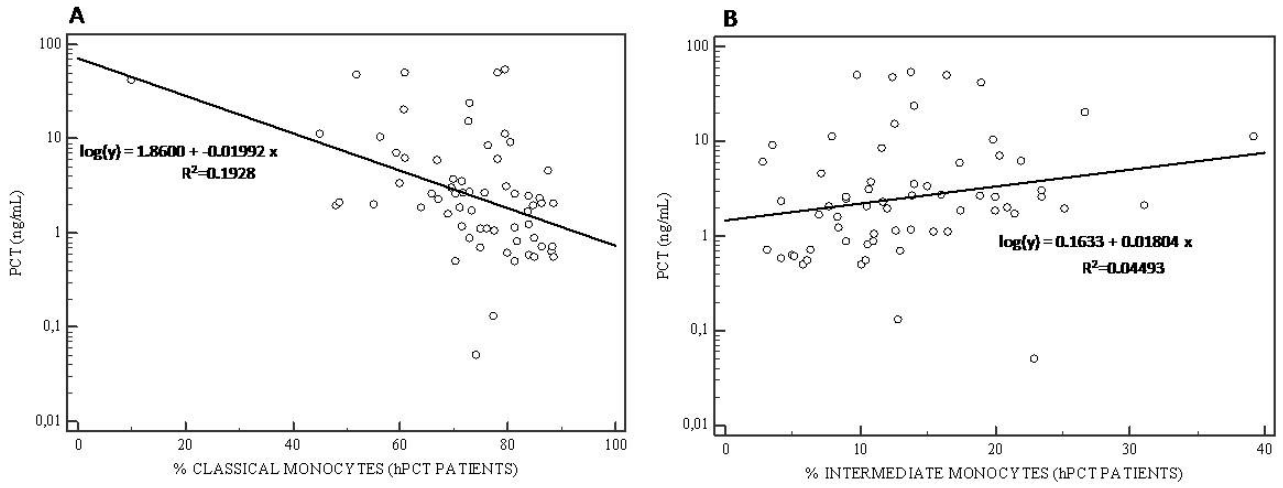
vs 11.5% ± 0.9, respectively with $p<0.05$, as in the Figure 1 box) and compared to the controls (17.4% ± 2.1 vs 6.2% ± 0.3, respectively with $p<0.001$).

According to the role of CD16 positive monocytes in modulating inflammatory response, a positive correlation trend between procalcitonin level and intermediate $CD14^{++}CD16^{+}$ monocytes percentages was observed; on the other hand, classical monocytes showed an opposite trend of correlation with the increase of procalcitonin (Figure 2).

HLA-DR monocytes surface expression in septic patients

HLA-DR monocytes surface (mHLA-DR) expression was analyzed by flow cytometrical analysis (Figure 3 A, B, C). Percentage of mHLA-DR and MFI in non-septic patients (BC-) showed no significant difference compared to controls (99.1% ± 0.3 vs 99.4% ± 0.2 respectively with $p=0.3$). In septic patients, HLA-DR expression on all monocytes subsets was significantly lower (BC+, 94.3% ± 2.2) compared to healthy controls (99.4% ± 0.2, with $p<0.05$) (Figure 3D). As

Figure 2 Serum levels of PCT and percentage of monocytes subsets



A: *Classical monocytes.*
B: *Intermediate monocytes. Intermediate monocytes increased as levels of PCT increased. Classical monocyte show an opposite trend. PCT values are showed in semilogarithmic scale.*

previously reported (11), in septic patients mHLA-DR was estimated 3-4 days after diagnosis of sepsis. mHLA-DR expression in BC+ patients was significantly lower than BC- patients (94.3% ± 2.2 vs 99.1% ± 0.3 with p<0.05) (Figure 3D). No statistical significance was observed for mHLA-DR MFI between BC+, BC- and control patients.

Among septic patients, the subgroup WO showed worst conditions, characterized by higher incidence of secondary/nosocomial infections, often polymicrobial, as well as long time hospitalization (>30 days).

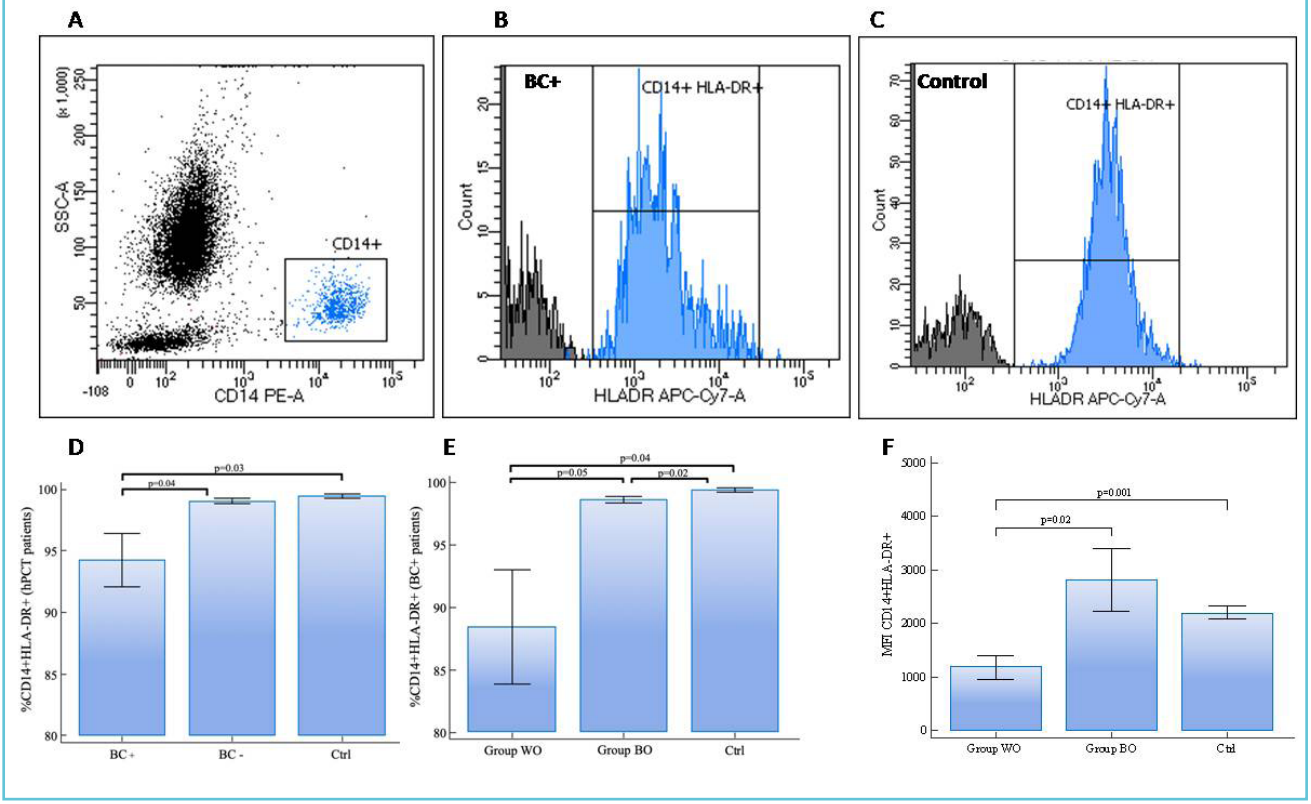
We separately analyzed subgroup WO and, in agreement with the known sepsis-induced immune system alterations, we found that these patients had lower percentage of HLA-DR positive monocytes compared with better outcome patients (BO subgroup) (88.4% ± 4.5 and 98.6% ± 0.3, respectively, with p=0.05, 10% reduction, Figure 3E) associated with lower mHLA-DR MFI (Mode 1193.6 ± 219.6 and 2819.4 ± 591.1, respectively, with p=0.022, 58% reduction) (Figure 3F), and also lower Median of Florescence Intensities (1623.7 ± 416.3 and 2730.8 ± 332.5, respectively, with p=0.05) or Mean of Florescence Intensities (2344.0 ± 590.2 and 3837.4 ± 398.1, respectively, with p=0.05). Similarly, significant 11% reduction of mHLA-DR percentage and 46% reduction of mHLA-DR MFI was obtained by comparing worst condition and control patients (Figure 3E and F).

Interestingly, the worst condition patients didn't show a tendency towards restoration of normal values of white blood cells, platelets, monocytes, neutrophils and lymphocytes counts like better outcome patients (Figure 4, Table 3) as well as BC- patients, which however showed more stable hematological parameters than BC+ (data not shown).

DISCUSSION

Impaired immune response following sepsis originates from delayed restoration of immunologic homeostasis between pro- and anti- inflammatory responses and determines higher risk for adverse outcome including secondary

Figure 3 HLA-DR monocytes surface expression in BC+, BC- and Ctrl patients



A: *Flow cytometric analysis of monocytes on a gate created on scatter plot of CD14-PE vs side scattered light signals.*

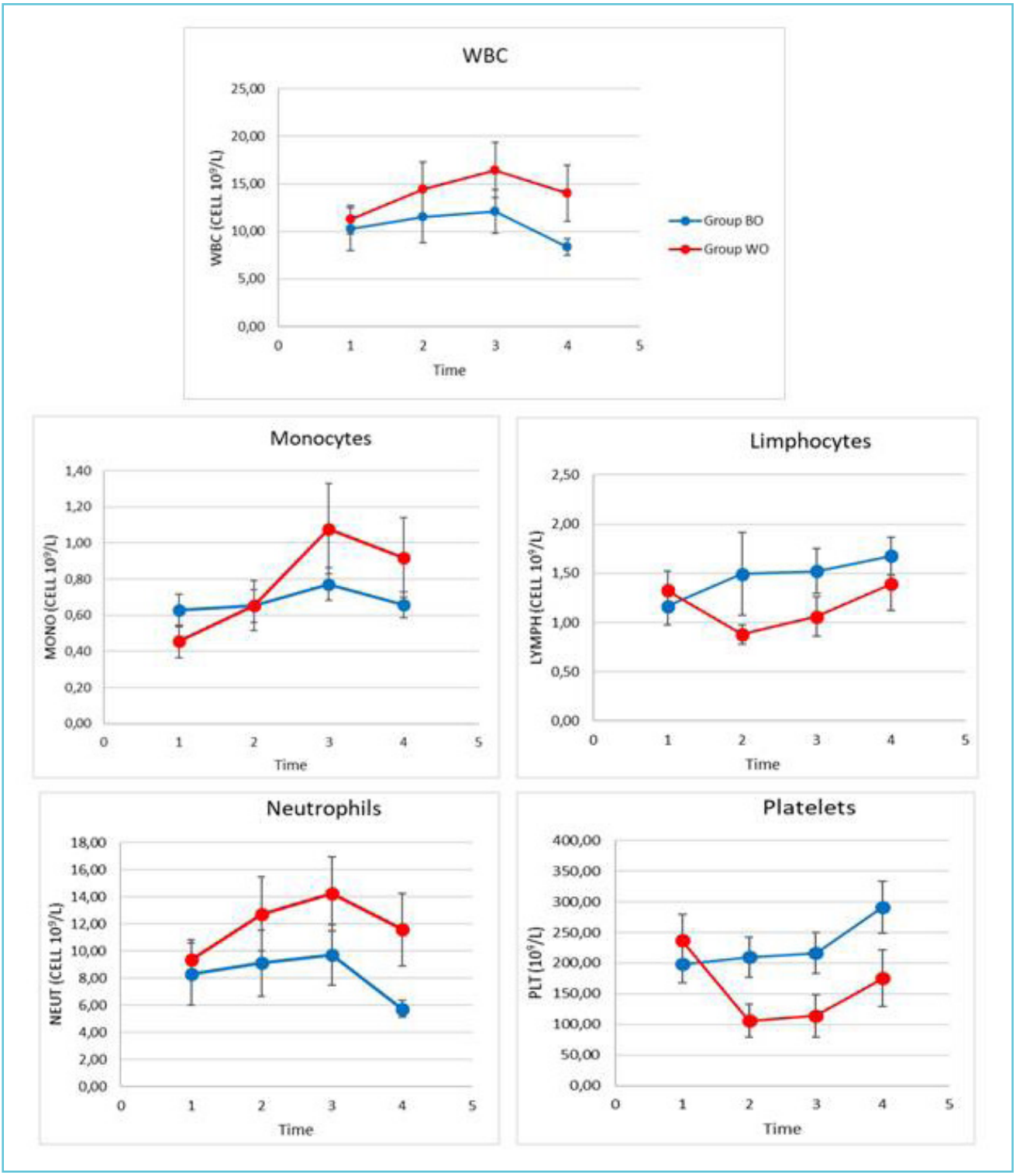
B *and* ***C:*** *Histogram plot of HLA-DR-APC-Cy7 among CD14+ gated monocytes.*

D: *mHLA-DR expression in hPCT-patients; in septic patients (BC+), HLA-DR expression is significantly lower compared to non-septic patients (BC-) and healthy controls (Ctrl).*

E *and* ***F****: subgroup of septic patients presenting worst outcome (BC+, group WO) shows a significant reduction of mHLA-DR percentage and relative MFI compared to patients with better clinical outcome (group BO) and healthy controls (Ctrl). (P values indicated in figure; where not indicated, difference was not statistically significant).*

infection and death (7). The innate immune cells (including monocytes) are involved in the initial immunologic response to critical illness with the adaptative response being more prevalent in the subacute phase of illness. In the present study, we analyzed monocytes polarization and reprogramming from inflammatory to immunosuppressive phase in critical ill patients with high procalcitonin serum levels (>0.5 ng/mL). These patients showed a significant shift to a proinflammatory phenotype of monocytes with the expansion of the $CD14^{++}CD16^{+}$ subpopulation (intermediate monocytes) and the decrease of classical $CD14^{++}CD16^{-}$ monocytes subpopulation (Figure 1C). Intermediate monocytes mainly exert a pro-inflammatory role. $CD16^{+}$ monocytes are recognized to be involved in antigen processing and presentation as well as trans-endothelial migration (3) and to be increased during inflammation (e.g., cancer, sepsis and stroke), infections such as HIV (14-17), tuberculosis (18) and other pathogens (19, 20). Accordingly, in our study, intermediate monocytes were significantly increased during both "strong inflammatory state" (Cult-Neg) and "infection state", identified by a positive microbiological cultural assay

Figure 4 Evaluation of hemato-chemical markers during hospitalization time



Trends values of white blood cells, monocytes, lymphocytes, neutrophils and platelets count in better (BO) and worst (WO) clinical outcome patients. Parameters were monitored at admission (time 1), at blood culture request (time 2), after 3-4 days from diagnosis (time 3) and after 6-7 days from diagnosis (time 4).

(Cult-Pos), compared to controls (Figure 1). In Cult-Pos patients, a positive trend of correlation has been observed for procalcitonin values and percentage of intermediate monocytes, while a negative trend was found for classical monocytes (Figure 2). PCT has already been demonstrated to have diagnostic and prognostic use in shock patients; peak levels have been shown to closely follow those of TNF-α and IL-6 (21). Moreover, we found that the increase of intermediate monocytes was significantly associated with the presence of bloodstream infection (BC+) in Cult-Pos patients (Figure 1, rectangle box) presenting also other clinical signs of sepsis as defined by the third international consensus definition of sepsis (13) on the basis of sequential organ failure assessment (SOFA) score. In hPCT patients analyzed in this study, especially those with infection, monocytes showed to be polarized toward a specific increase of intermediate subpopulations that is indicative of a more pronounced inflammatory environment. Other studies demonstrated that infection triggers expression of CCR2 by intermediate monocytes, which promote their migration into the lesions where increased levels of its ligand CCL2 protein are present; moreover, intermediate monocytes produce TNF-α, thus enhancing the inflammatory response (20). Leukocytes trafficking occurs in presence of increased vascular permeability and endothelial activation during sepsis; these processes are controlled by molecular mediators in the context of the regulation of the pro- anti- inflammatory response (22).

When severe and persistent compensatory anti-inflammatory response follows critical illness, patient is exposed to high risk for adverse outcome. As known, monocytes normally recognize and process the pathogen and present the antigens on their cell surface via human leukocytes antigen HLA-DR molecules; when activated, monocytes secrete proinflammatory cytokines, such as TNF-α, which amplify the immune response. As critical condition progresses, lower mHLA-DR expression determines a reduced monocytes antigen presenting capacity and releasing of pro-inflammatory cytokines in response to bacterial compounds and immune-suppressed state (9, 23, 24). In this setting, we examined mHLA-DR expression in hPCT-patients population of the present study, and we found a significant percentage reduction in septic patients compared to controls and to BC- patients (Figure 3D). Notably, subgroup of septic patients presenting worst outcome (BC+, group WO) showed a significant reduction of mHLA-DR percentage (Figure 3E) and of mHLA-DR quantification as MFI (mode of florescence intensity) which is an indicator of a reduced number of HLA-DR molecule per monocytes (Figure 3F). Time course evaluation of hemato-chemical markers showed worsening of the clinical conditions of WO-patients compared to restoration of same parameters in BO-patients (Figure 4, Table3). Recently, four phenotypes of sepsis have been derived from clustering analysis of multiple data set (25, 26*)*, and currently not included in consensus definition of sepsis (13), which are potentially related to biomarker variation and clinical outcome of patients. Difference between the 4 phenotypes derives from different pattern of organ dysfunction, demographic data and laboratory values: broad differences were observed in the distribution of the host immune response biomarkers across phenotypes; in general there was an increase in the markers of inflammation and of endothelial dysfunction in γ and/or δ high-mortality phenotypes compared with the α or β phenotypes with better clinical outcome (25, 26). From our results, HLA-DR appears to be a good marker for classification of adverse clinical outcome in septic patients. The clinical outcome of the patients includes the successive involvement of adaptive immune response. First available marker of it is the absolute lymphocytes count; in fact, lymphocyte apoptosis

causes lymphopenia during sepsis and has been associated with mortality and secondary infection risk (27-29). We found that lymphocytes absolute count showed a substantial drop in WO-patients (Figure 4), while both BO-patients (BC+) and BC- patients showed a more stable trend.

Further studies are in progress to evaluate degree of T cell dysfunction (i.e. activation in presence of bacterial lysate) and regulatory T cell (i.e. immune-suppressive subset of T cells) in the perspective of better understanding of mechanisms responsible for pro/anti-inflammatory responses during sepsis, and develop new approaches of immuno-monitoring strategies and immunotherapies.

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In this issue: Clinical flow cytometry in 2019

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Evaluation of sample quality as preanalytical error in flow cytometry analysis in childhood acute lymphoblastic leukemia

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ABSTRACT

Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer among children. The intensity of chemotherapy and further therapeutic decisions depend on several prognostic factors, including response to initial treatment by examining peripheral blood (PB), bone marrow (BM) and cerebrospinal fluid (CSF) samples at certain time points. (e.g. day 15 BM). Sample quality is crucial for the correct risk assessment.

Patients and methods

We aimed to explore the rate of inadequate samples as a source of preanalytical error. We retrospectively analyzed flow cytometry results of BM (day 15 and day 33) and CSF samples from children with ALL in different cohorts focusing on PB contamination and viable cell ratio among nucleated cells. We also compared viable cell percentages in native and stabilized CSF samples.

Results

Due to PB contamination (erythroid precursors < 2%) 12.5% of day 15 and 14% of day 33 BM samples were inadequate for flow cytometry risk stratification. Significantly fewer CSF samples had to be considered inadequate for analysis (defined as viable cells < 30%) in the subgroup of stabilized samples compared to native samples. Four of the CSF samples from children with ALL had identifiable malignant cell population despite the low viable cell percentage.

Discussion

Poor sample quality can hamper risk stratification and further therapeutic decision in childhood ALL. Despite low viable cell count malignant cell populations may still be identified in a CSF sample, therefore establishing a certain cut-off point for viable cells is difficult.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common cancer among children still responsible for most deaths from cancer under 20 years of age, despite the tremendous improvement of survival since 1948, when Farber et al. successfully induced remission in children with ALL by administering aminopterin (1,2). ALL can be either B-cell or T-cell type with 85% of the cases being B-cell precursor ALL (BCP-ALL) and 15% T-ALL. The core of modern-day treatment of ALL is still the administration of combined chemotherapy developed by Riehm et al. in the 1970s (3). The intensity of chemotherapy and further therapeutic decisions depend on several prognostic factors including the clinical features of the patients on presentation, the genetic features of the leukemic cells, central nervous system (CNS) status and response to initial treatment (1). Sample quality is crucial in the correct risk assessment. A common preanalytical error can be the contamination of either the bone marrow (BM) or the cerebrospinal fluid (CSF) sample with peripheral blood (PB). The sample must also contain enough viable cells for analysis, carried out either by morphological or by flow cytometric studies (4). According to the AIEOP BFM 2009 protocol evaluation of minimal residual disease (MRD) in the PB on day 8 and in the BM on day 15 and day 33 is important for adjusting treatment intensity (4). The possibility of diluting the BM specimen with PB should be minimized as a diluted sample may result in underestimating residual blast percentage and wrong risk assessment. According to the ALL IC-BFM 2009 Flow MRD SOP a day 15 BM cannot be used for risk stratification if the percentage of the erythroid precursors is below 2% (5), as PB contamination in this case is highly suspected. However, those inadequate samples containing more than 10% pathological blasts can still be reported and used for risk stratification as a Flow High Risk case.

CNS involvement needs administration of intrathecal chemotherapy (6), however, PB contamination can lead to misdiagnosing CNS involvement as the origin of a malignant population detected in a contaminated CSF sample remains unclear. Another pitfall can be the rapid decay of the cells in a CSF sample especially if the CSF sample has to be transferred to a central laboratory e.g. for flow cytometry. As the degradation rate is different in each cell type, long transfers of the sample will affect the qualitative analysis and pathological cell populations might be missed. Low viable cell count is usually not a problem with PB and BM samples but CSF samples are frequently paucicellular, further hampering the analysis. Another hurdle is that there is no consensus on either the minimal number of cells needed for adequate analysis (varying between 100 and 1000 in literature) (7,8), or on the precise definition of a traumatic tap (PB contamination) (9,10,11).

Overall, obtaining correct results in diagnostic and follow-up samples of a childhood ALL or any other leukemia/lymphoma depends on the quality of the BM/CSF sample to a great extent.

We aimed to explore the percentage of inadequate BM/CSF samples when we could not interpret and report the results owing to preanalytical errors.

PATIENTS AND METHODS

Cohort 1a: data of patients with childhood ALL between 2011 and 2018 were analyzed retrospectively. Day 15 bone marrow samples were obtained from 104 patients, 59% of whom were male, 41% were female. 23 patients (20 male, 3 female) had T-ALL (22%), 81 patients (41 male, 40 female) had BCP-ALL. Average age at sampling

Table 1 Antibody panels and clones used for staining the childhood day 15 and day 33 BM samples

A	FITC	PE	PerCP-Cy5.5	APC
Tube 1	CD20 (L27)[1]	CD10 (SS2/36)[3]	CD34 (8G12)[1]	CD19 (SJ25C1)[1]
Tube 2	CD20+CD10 (L27+SS2/36)[1,3]	CD38 (HB7)[1]	CD45 (2D1)[1]	CD19 (SJ25C1)[1]
Tube 3	CD58 (AICD58)[2]	CD10 (SS2/36)[3]	CD45 (2D1)[1]	CD19 (SJ25C1)[1]
Tube 4	cyFXIII-A	CD10 (SS2/36)[3]	CD45 (2D1)[1]	CD19 (SJ25C1)[1]
Tube 5	syto16	CD10 (SS2/36)[3]	CD45 (2D1)[1]	CD19 (SJ25C1)[1]

B	FITC	PE	PerCP-Cy5.5/PE-Cy5.5	PE-Cy7	APC	APC-H7	PB	PO
Tube 1	CD58 (AICD58)[2]	CD123 (SSDCLY107D2)[2]	CD33 (D3HL60.251)[2]	CD19 (J3-119)[2]	CD10 (HI10a)[1]	CD81 (JS-81)[1]	syto40[7]	CD45 (HI30)[5]
Tube 2	syto16[7]	CD66c (KOR-SA3544)[2]	CD34 (8G12)[1]	CD19 (J3-119)[2]	CD10 (HI10a)[1]	CD38 (HB7)[1]	CD20 (2H7)[4]	CD45 (HI30)[5]
Tube 3	cyFXIII-A	CD10 (SS2/36)[3]	CD45 (2D1)[1]	-	CD19 (SJ25C1)[1]	-	-	-

C	FITC	PE	PerCP-Cy5.5	APC
Tube 1	syto16	CD7 (8H8.1)[2]	CD45 (2D1)[1]	CD3 (SK7)[1]
Tube 2	CD4 (SK3)[1]	CD8 (SK1)[1]	CD45 (2D1)[1]	CD3 (SK7)[1]
Tube 3	CD99 (DN16)[6]	CD7 (8H8.1)[2]	CD5 (L17F12)[1]	CD3 (SK7)[1]
Tube 4	nTdT (HT6)[3]	CD7 (8H8.1)[2]	CD3 (SK7)[1]	cyCD3 (SK7)[1]

D	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	PB	PO
Tube 1	syto16[7]	CD99 (3B2-TA8)[5]	CD3 (SK7)[1]	CD7 (8H8.1)[2]	CD1a (HI149)[1]	CD8 (SK1)[1]	CD4 (RPA-T4)[1]	CD45 (HI30)[5]
Tube 2	nTdT (HT6)[3]	CD99 (3B2-TA8)[5]	CD5 (L17F12)[1]	CD7 (8H8.1)[2]	cyCD3 (SK7)[1]	CD3 (SK7)[1]	syto40[7]	CD45 (HI30)[5]

A: *4-colour BCP-ALL panel for MRD detection;* ***B:*** *8-colour BCP-ALL panel for MRD detection. From September, 2017, CD123 was added to Tube 2 (as CD66c+CD123 in FL2) and replaced by CD73+CD304 (clones: ad2 and 12C2, respectively, both manufactured by SONY Biotechnology, San José, CA, USA) in Tube 1;* ***C:*** *4-colour T-ALL panel;* ***D:*** *8-colour T-ALL panel. See Abbreviations for the fluorochromes. Manufacturers: 1: Becton-Dickinson Pharmingen, San José, CA, USA; 2: Beckmann-Coulter, Brea, CA, USA; 3: Dako, Glostrup DK; 4: BioLegend, San Diego, CA, USA; 5: ExBio, Praha, CZ; 6: AbDSerotec, Raleigh, NC, USA; 7: Molecular Probes, Eugene, OR, USA. cyFXIII-A is a homemade antibody (13)*

time of the whole population was 83 months, with a range between 1 and 201 months.

Cohort 1b: day 33 bone marrow samples were analyzed from 90 patients (56% male, 44% female), 13 (14%) T-ALL (11 male, 2 female), 77 (86%) BCP-ALL (39 male, 38 female). Average age in this population was also 83 months, range between 2 and 202 months.

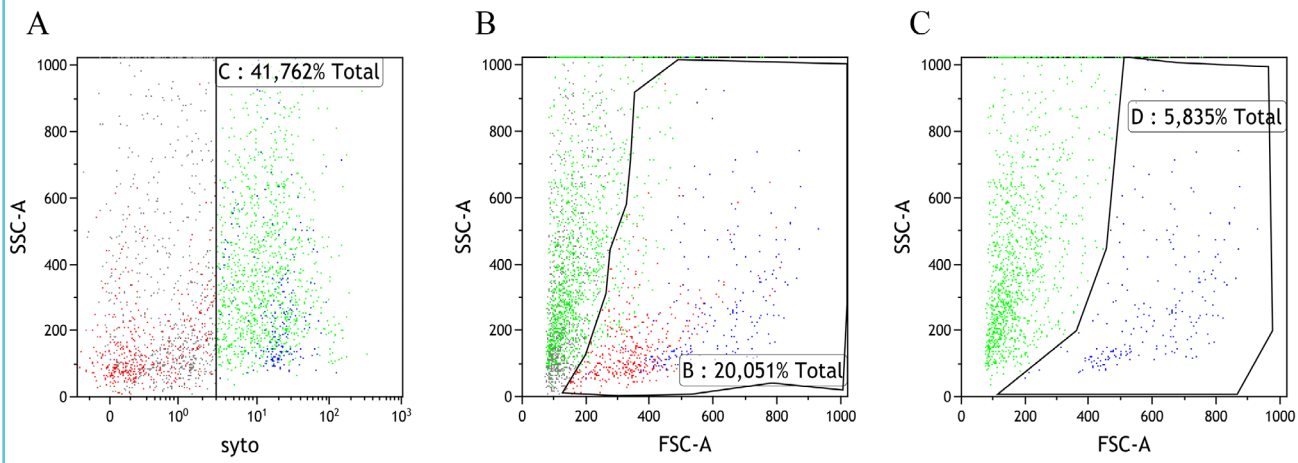
Cohort 2: in the mentioned time period a total of 26 CSF samples were analyzed by flow cytometry from 20 pediatric patients with ALL. The average age was 75 months, range between 7 and 214 months. Twelve patients were male (60%), eight were female (40%). One patient had T-ALL (5%), the others had BCP-ALL (95%). More than one sample was sent from five patients, 3 samples from the T-ALL patient and two samples each from the other four patients.

Cohort 3: fifty-one CSF samples from 47 patients (adults and children) were evaluated and viable cell percentage in native and stabilized samples (TransFix®; Ref. No. TF-CSF-5-25, Caltag Medsystems, Buckingham, UK) were compared. Nineteen of these 47 patients were female (40%), 28 were male (60%). Average age in this group was approximately 43 years with a range of 10 months and 79 years. 29 samples were stabilized, 22 were native.

Flow cytometric measurements were carried out in an 8-colour FACSCanto II flow cytometer, data were analyzed by FACSDiva 8.0.2 software (both by Beckton Dickinson Biosciences, San Jose, CA, USA). Pediatric ALL samples before March, 2013 (regarding BCP-ALL) and September, 2013 (regarding T-ALL) were examined in a 4-colour setting, all samples afterwards were examined by 8-colour setting, labeled in a stain-lyse fashion. The labeling procedure was performed as previously described (12) Antibody panels with clones and manufacturers are summarized in Table 1. *Cohort 2* CSF samples were stained with antibodies based on these panels; due to sample shortage in most cases the whole panels could not be applied. To make the results comparable, the flow cytometer was calibrated daily, using Cytometer Setup and Tracking fluorescent microbeads (Cat No. 641319, Becton Dickinson Biosciences, San Jose, CA, USA) and Autocomp software as recommended by the manufacturer.

Viable cell count in bone marrow samples was evaluated by syto-staining. Duplicates were excluded from all samples before the evaluation of the percentage of syto+ viable cells. Bone marrow samples containing < 2% erythroid precursors were considered contaminated by PB

Figure 1 Syto+FSC/SSC sequential gating of CSF samples to evaluate viable cells



Considerable difference was observed between Syto (A) and FSC/SSC gating (B) in viable cell percentage (example: Sample No. 3 from Cohort 2). Best result was achieved by Syto+FSC/SSC sequential gating (C).

according to the ALL IC-BFM 2009 Flow MRD SOP. In CSF samples viable cell count was evaluated either by syto+FSC/SSC sequential gating (Figure 1) or by FSC/SSC gating if syto was not used. CSF samples containing < 30% of viable cells were considered inadequate for analysis and PB contamination was concluded if the CSF sample contained > 100 red blood cells / microliter (RBC/µL).

RESULTS

Cohort 1a

Peripheral blood contamination was found in 16 (15%) out of 104 day15 BM samples (12 out of 81 BCP-ALL and 4 out of 23 T-ALL). Blast percentages in the BCP-ALL contaminated samples were all below the Flow High Risk limit (10%) therefore these samples were inadequate for flow risk stratification. In the T-ALL subgroup > 10% of residual blasts were found in 3 out of 4 contaminated samples, making them eligible for risk stratification as these patients belonged to the Flow High Risk group. Altogether 13 samples of day15 BMs could not be used for risk stratification out of 104 samples (12.5%) owing to PB contamination.

Cohort 1b

Thirteen samples (14%) out of 90 were contaminated with peripheral blood (11 out of 77 BCP-ALL and 2 out of 13 T-ALL). All these samples contained less than 10% blasts, so they were reported as inadequate.

Cohort 2

RBC count was recorded in 22 out of the 26 samples. Details of this cohort are shown in Table 2. With 100 RBC/µL as cutoff for PB contamination 10 out of 22 (45%) samples were contaminated. Despite the low (< 10) white blood cell count / microliter (WBC/µL) in 11 samples (sample No. 1-7 and 9-12), several thousands of cells (1,840 – 100,000) could be acquired and analyzed by flow cytometry. Four out of the 11 paucicellular samples contained malignant cells (sample No. 9-12), one of them being PB contaminated (sample No. 12). Malignant cells were found in 14 samples from the overall 26, eight (57%) of which were PB-contaminated.

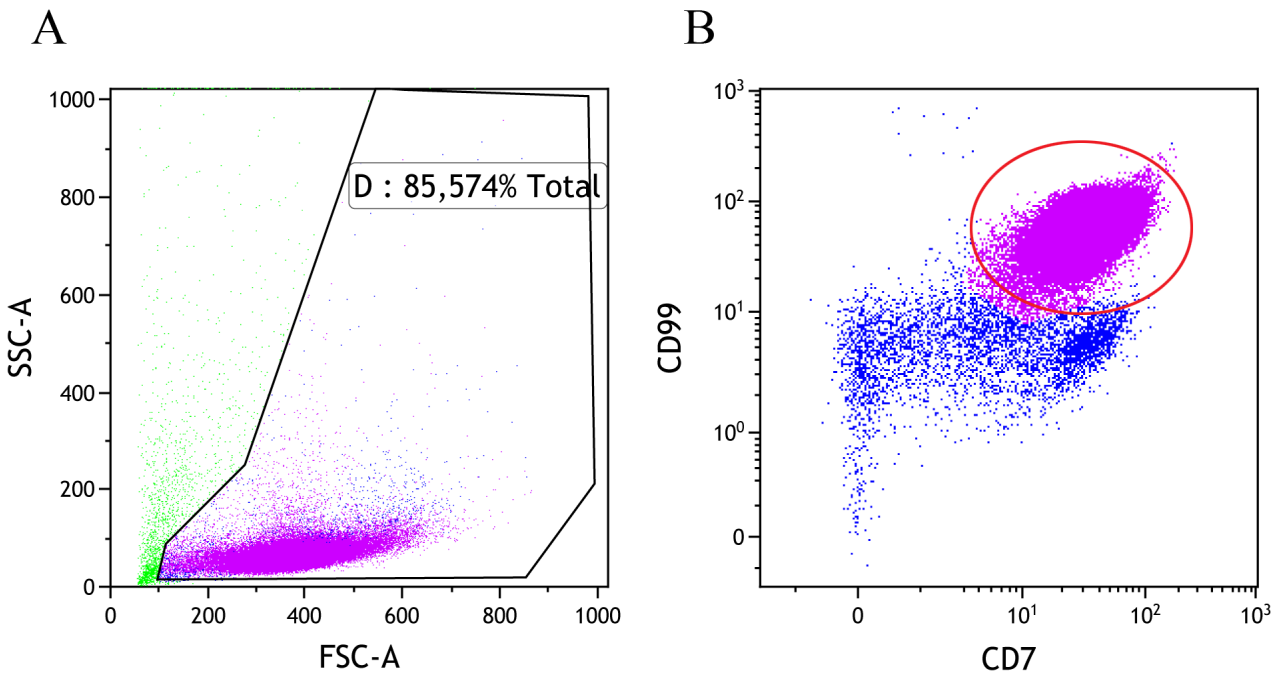
Table 2 CSF data of *Cohort 2**

Sample no.	Viable cells <30%	PB contamination	Pathological cells	No. of events acquired	WBC/µL	RBC/µL
1	**yes**	-	-	10,000	2	11
2	**yes**	-	-	2,829	5	1
3	**yes**	-	-	3,496	6	1
4	**yes**	-	-	5,612	2	1
5	**yes**	-	-	2,024	4	N/A
6	**yes**	-	-	1,840	1	92
7	**yes**	**yes**	-	100,000	7	2,560
8	**yes**	**yes**	**present**	13,961	80	123
9	**yes**	-	**present**	2,576	4	N/A
10	**yes**	-	**present**	4,094	5	5
11	**yes**	-	**present**	9,246	8	8
12	**yes**	**yes**	**present**	3,887	9	1,280
13	**yes**	**yes**	**present**	76,245	336	24,533
14	**yes**	**yes**	**present**	2,576	40	8,533
15	-	**yes**	**present**	300,000	679	1,133
16	-	**yes**	**present**	300,000	12,800	107
17	-	**yes**	**present**	130,893	679	683
18	-	**yes**	**present**	3,082	140	90,453
19	-	**yes**	-	3,703	23	160
20	-	-	-	100,000	623	N/A
21	-	-	-	17,710	179	N/A
22	-	-	-	5,750	180	80

23	-	-	-	2,208	44	67
24	-	-	**present**	137,356	720	5
25	-	-	**present**	2,415	41	32
26	-	-	**present**	2,737	11	61

** Samples 24-26 were from a patient with T-ALL, all other samples were from patients with BCP-ALL. N/A: not assessed.*

Figure 2 **Example of a well-evaluable CSF sample of a patient with T-ALL***



** In Sample 24 (Cohort 2) viable cell percentage was 85.6% (A), among these cells 96% were pathological T lymphoblasts with bright CD99/CD7 expression (purple) (B).*

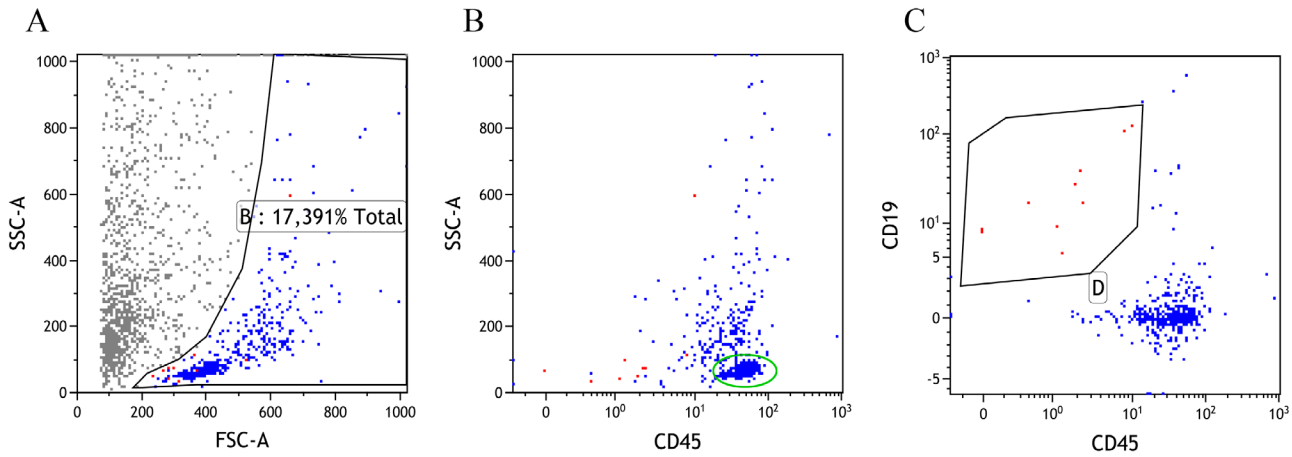
Figure 2 (above) shows dotplots of a non-PB contaminated, well-evaluable sample, with T-ALL blasts expressing bright CD99/CD7.

Eleven (42%) out of the 26 CSF samples were considered inadequate for evaluation due to < 30% of viable cells among the acquired nucleated cells, four of which had identifiable malignant cell population despite of the low percentage of viable cells (sample No. 8-11) (Figure 3).

Cohort 3

Viable cell count was < 30% in 5 out of the 29 CSF samples (17%) in the subgroup with preservative and 9 out of 22 (41%) in the native subgroup (without stabilization), the difference is significant (p=0.05, Fisher's exact test). PB-contamination (> 100 RBC/µL) was found in 8 out of 22 samples (36%) in the native subgroup and in 4 out of 29 samples (14%) in the stabilized samples.

Figure 3 Example of a poorly evaluable CSF sample of a patient with BCP-ALL*



**In Sample 9 (Cohort 2) viable cell percentage was below 30% (A) but a lymphocyte population was clearly visible (green circle) (B), along with malignant cells with CD45-/CD19+ characteristics (red dots, gate D) (C).*

DISCUSSION

Examining BM and CSF samples is essential in the diagnosis and follow-up of leukemias and lymphomas, including childhood ALL. Sample quality is a very important factor in obtaining adequate results. Hemodilution of a BM sample is quite common, His et al. found that 36% of the BM samples were hemodiluted from patients with acute leukemia (14).

According to the ALL IC BFM 2009 Flow MRD SOP a hemodiluted BM sample obtained on day 15 with the percentage of erythroid precursors below 2% is not eligible for flow risk stratification in childhood ALL (5). Such a hemodiluted sample is eligible for risk assessment only if the residual blast percentage is > 10%, meaning Flow High Risk. Those hemodiluted BM samples that contain < 10% residual blasts must not be used for risk stratification and the flow cytometry report must describe the sample as "inadequate".

Several other methods of determining hemodilution in a bone marrow sample have been described (15,16,17). In our cohort (*Cohort 1a*) 12.5% of the day 15 BM samples of children diagnosed with ALL were inadequate for risk assessment that might have hampered treatment adjustments in these cases. Similarly, 14% of the Day 33 BM samples (*Cohort 1b*) were also hemodiluted. Hemodilution can be best avoided if the BM aspiration is done prior to the biopsy and if no more than 1-2 mL of sample is obtained (18).

As hematological malignancies often affect the central nervous system, examination of the CSF is frequently needed. The core of the diagnosis is the identification of malignant cells by conventional cytomorphology in a CSF sample, although up to 60% of the cases can be false-negative (19,20). Flow cytometry has great sensitivity and specificity and is recommended by the National Comprehensive Cancer Network (USA) in conjunction with cytomorphologic studies (6). We could also confirm the utmost importance of flow cytometry, especially in cases of CSF samples with low nucleated cell count (< 10 WBC/µL) when malignant cells could be detected without PB contamination (samples No. 9-11 in *Cohort 2*). Kraan et al. suggested classifying cell clusters of > 25 cells as positive, 10-25 as suspicious and < 10 as negative (21). The cells decay

rapidly in the CSF after sampling with granulocytes and monocytes showing the highest rate of degradation (6). Several studies tried adding different types of cell culture media to improve cell survival with promising results (22,23).

Other methods were also examined, e.g. immediate cooling, minimizing centrifugation steps, aspirating supernatant instead of decanting sample (21,24,25). Preservatives (e.g. TransFix®) may also be used, moreover, TransFix® is recommended for CSF by the British Committee for Standards in Hematology (26). TransFix has been shown to stabilise malignant haematological cells in cerebrospinal fluid, making it possible to determine leucocyte subsets in CSF via flow cytometric analysis 72 hours after lumbar puncture (26,27). Previous studies showed that the use of TransFix/EDTA CSF Sample Storage Tubes prevents cellular loss and enhances flow cytometric detection of leptomeningeal localized hematological malignancies much better than serum-containing medium-filled tubes or untreated tubes, because scatter and antigen expression characteristics of pathological cells are preserved (28,29).

In our *Cohort 3*, significantly better results were achieved regarding the number of reportable results since more than 30% viable cells were detected in the CSF samples when TransFix® was used. However, pathological cells still can be identified in samples with low viable cell percentage, as it happened in the case of four samples (samples No. 8-11) in *Cohort 2*, therefore it is difficult to establish a clear cutoff for viable cell percentage under which the sample is considered inadequate and results are not reported. Reporting these cases should remain the decision of the examiner.

According to Petzold et al. up to 20% of standard lumbar punctures (LPs) are traumatic taps, although there is no consensus about the precise definition (30).

Some authors define a LP to be traumatic if at least 100 red blood cells (RBC) per microliter are present (31), others put the cutoff to 400 RBCs per microliter (32,33). However, according to Gajjar et al. a LP is defined traumatic if > 10 RBCs are present per microliter. They found that blood contamination in CSF in children with acute lymphoblastic leukemia had an adverse effect on treatment outcome (34).

According to Kraan et al. detecting a small malignant cell population in a contaminated CSF sample is diagnostic only if this malignant population is not detected in the peripheral blood obtained simultaneously (21). Te Loo et al. advise against performing a LP in phases of an acute leukemia when the frequency of malignant cells is high in the peripheral blood, as by a traumatic tap even the iatrogenic contamination of the CNS with the malignant cells can occur besides a false-positive CSF result (35).

In our two CSF cohorts, 45% and 24% of the samples were PB contaminated (*Cohort 2* and *Cohort 3* overall, respectively), which is in accordance with results found in the literature (30,36,37).

Limitations of the present results include the retrospective nature of the study, a prospective design would have enabled us to record all quality indicators of the preanalytical phase (38). Furthermore, our results concerning the percentage of reportable CSF samples with or without preservative need to be validated in higher number of samples as well.

In conclusion, poor sample quality can hamper risk stratification and further therapeutic decision in childhood ALL. Despite low viable cell count malignant cell populations may still be identified in a CSF sample, therefore establishing a certain cutoff point is difficult.

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Abbreviations (in alphabetical order)

ALL: *acute lymphoblastic leukemia*

APC: *allophycocyanin*

APC-H7: *allophycocyanin – Hilite® 7*

BCP-ALL: *B-cell precursor acute lymphoblastic leukemia*

CNS: *central nervous system*

CSF: *cerebrospinal fluid*

FITC: *fluorescein-isothiocyanate*

LP: *lumbar puncture*

PB: *peripheral blood*

PE: *Phycoerythrin*

PE-Cy5.5: *Phycoerythrin – cyanine 5.5*

PE-Cy7: *Phycoerythrin – cyanine 7*

PerCP-Cy5.5: *Peridinin-chlorophyll protein – cyanine 5.5*

PB: *Pacific Blue*

PO: *Pacific Orange*

RBC: *red blood cell*

T-ALL: *T-cell acute lymphoblastic leukemia*

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In this issue: Clinical flow cytometry in 2019

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Immune cellular evaluation following newborn screening for severe T and B cell lymphopenia

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ABSTRACT

Newborn screening (NBS) for severe T and/or B cell lymphopenia to identify neonates with severe combined immunodeficiencies (SCID) or agammaglobulinemia rapidly after birth has paved its way into clinical practice. Debate exists on the concept and strategy for rapid verification and stratification of the cellular immune status of positively screened infants. We provide impulses for harmonization of flow cytometric approaches to allow rapid integration in the growing number of immunological laboratories involved in follow-up and subdivision of SCID and non-SCID entities.

INTRODUCTION

The purpose of neonatal screening programs is the early recognition of treatable genetic diseases that manifest with a high rate of morbidity and mortality. While the implementation of newborn screening tests for metabolic disorders traces back to the mid-1960s, suitable technologies to identify severe inborn errors of immune function have emerged only in recent years.

The estimated incidence of primary immunodeficiency diseases (PID) that would require immediate treatment ranges from 2 to 8 per 100,000 live births, making high demands on the effectiveness and availability of screening tests [3].

In comparison with metabolic diseases, the identification of sensitive and traceable biomarkers poses a challenge due to the genetic diversity of pediatric PID patients.

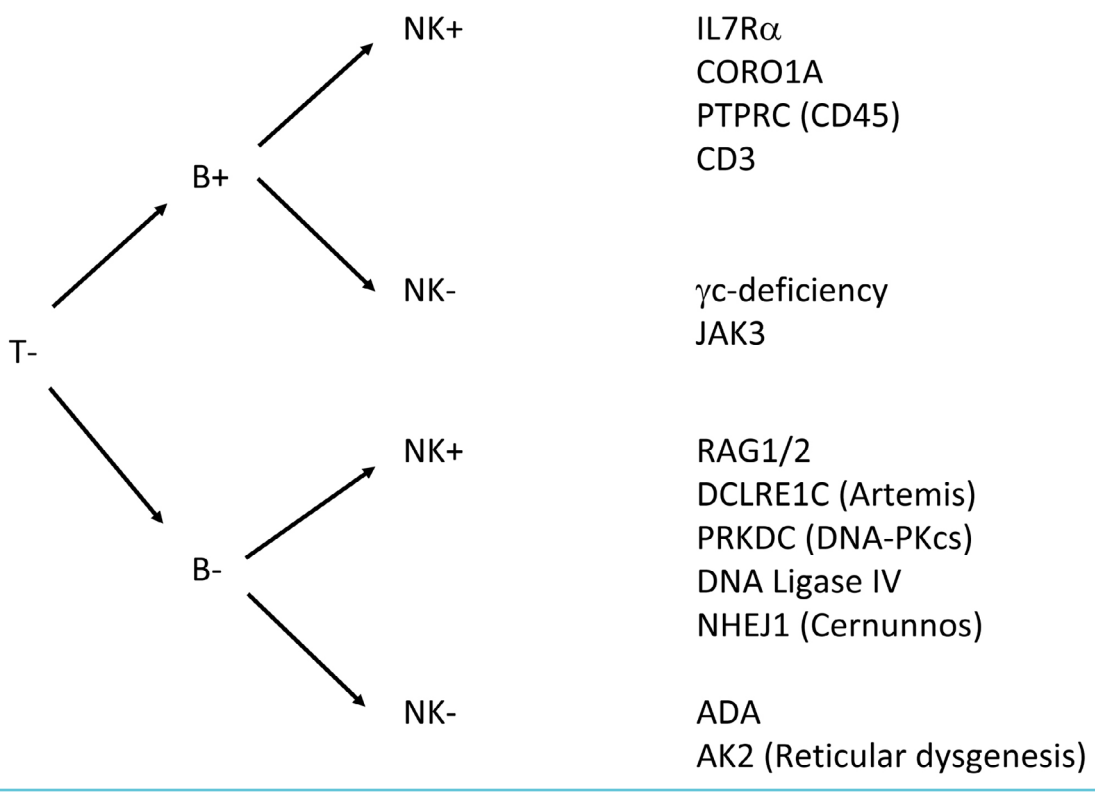
Severe combined immunodeficiency (SCID) is the most severe form of inherited primary immunodeficiency and is a pediatric emergency. Delay in recognizing and detecting SCID can have fatal consequences and also reduces the chances of successful hematopoietic stem cell transplantation (HSCT) [1].

Screening for SCID at birth would prevent children from dying before HSCT can be attempted and would increase the success of HSCT. There is strong evidence to show that SCID fulfills the internationally-established criteria for a condition to be screened for at birth [2].

Severe combined immunodeficiency – a life-threatening group of disorders

SCID is a group of life-threatening immune disorders arising from a variety of genetic defects that lead to the absence of lymphocyte development

Figure 1 T/B/NK-cellular classification of SCID entities



and function [3]. Nearly all patients with SCID have absent T-cells, and are further grouped by the absence or presence of B-cells and NK-cells (Figure 1).

Thus, the absence or severe reduction of functional naïve T and/or B cells at birth would be the preferable biomarker for newborn screening of SCID [4].

The diagnosis of SCID is a pediatric emergency, given that most affected children exhibit extreme susceptibility to bacterial, viral, fungal and opportunistic infections, which are fatal in the first 1-2 years of life without curative treatment.

In most cases, children with SCID appear well at birth and present with recurrent severe infections and failure to thrive at 3-6 months as passively transferred protective maternal immunoglobulins are diminishing.

DIAGNOSTIC CONCEPT AND STRATEGY

Newborn screening algorithm

Normal T-cell development requires production of precursor T-cells in the bone marrow and subsequent processing of T-cells in the thymus. Although SCID can arise from a variety of genetic defects, there is an abnormality of T-cell development in the thymus in all cases. During normal thymic processing, T cells undergo receptor gene splicing and rearrangement, leading to intracellular accumulation of DNA by-products known as T-cell receptor excision circles (TRECs). When used in NBS assays, TRECs are a surrogate marker of newborns' capability to produce T cells, which is severely hampered in SCID patients [4].

TRECs do not replicate in dividing cells and are diluted out upon cellular division. They are therefore only found in recent thymic emigrant

Figure 2 Spectrum of neonatal T cell lymphopenia

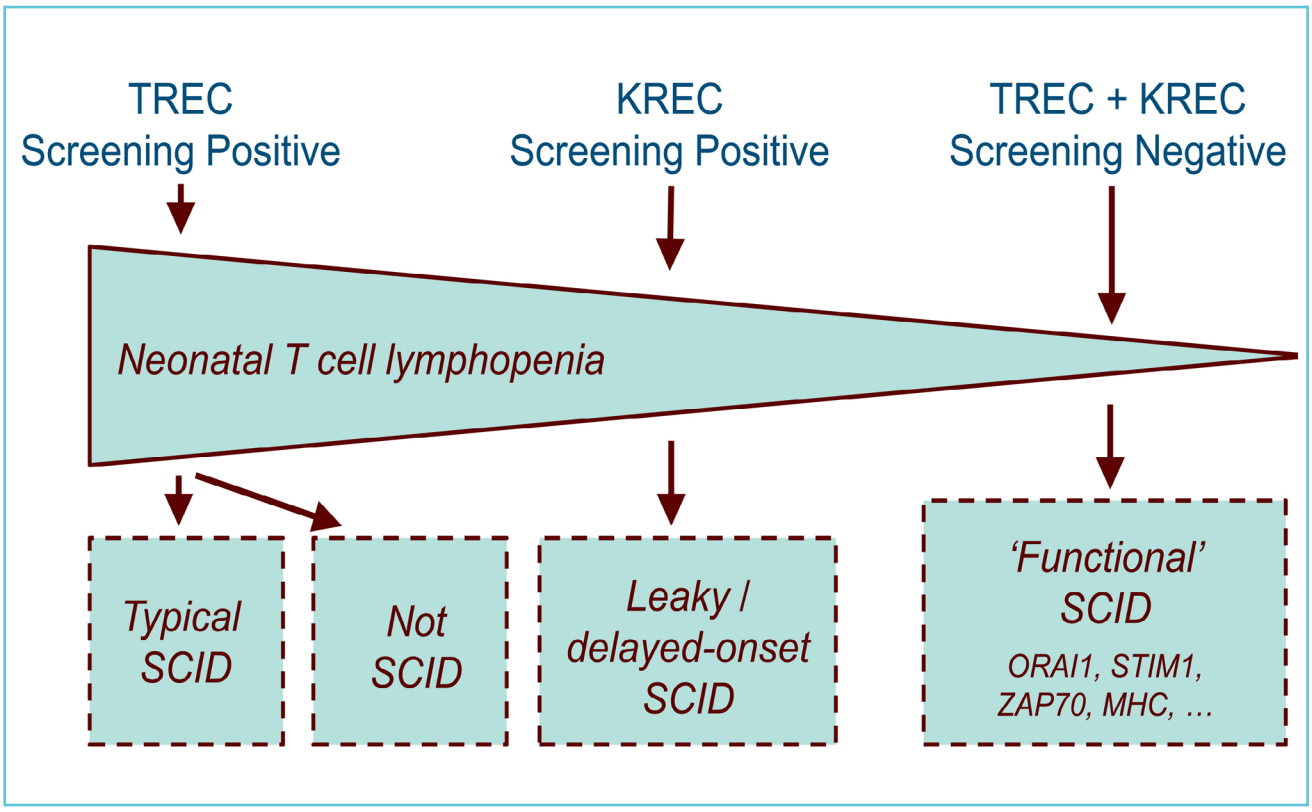
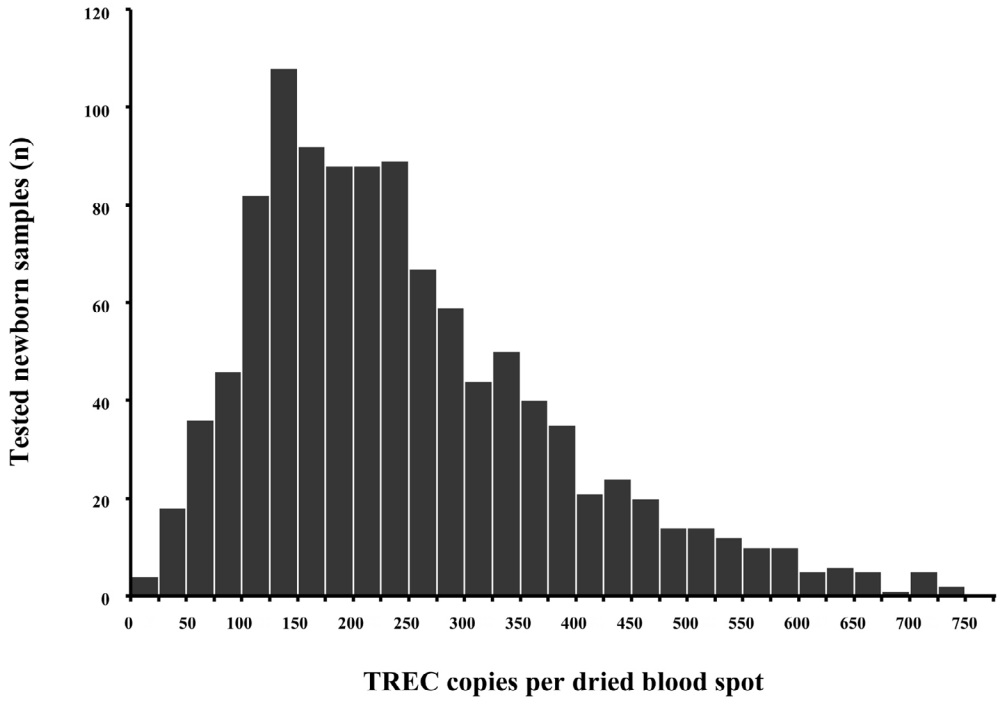
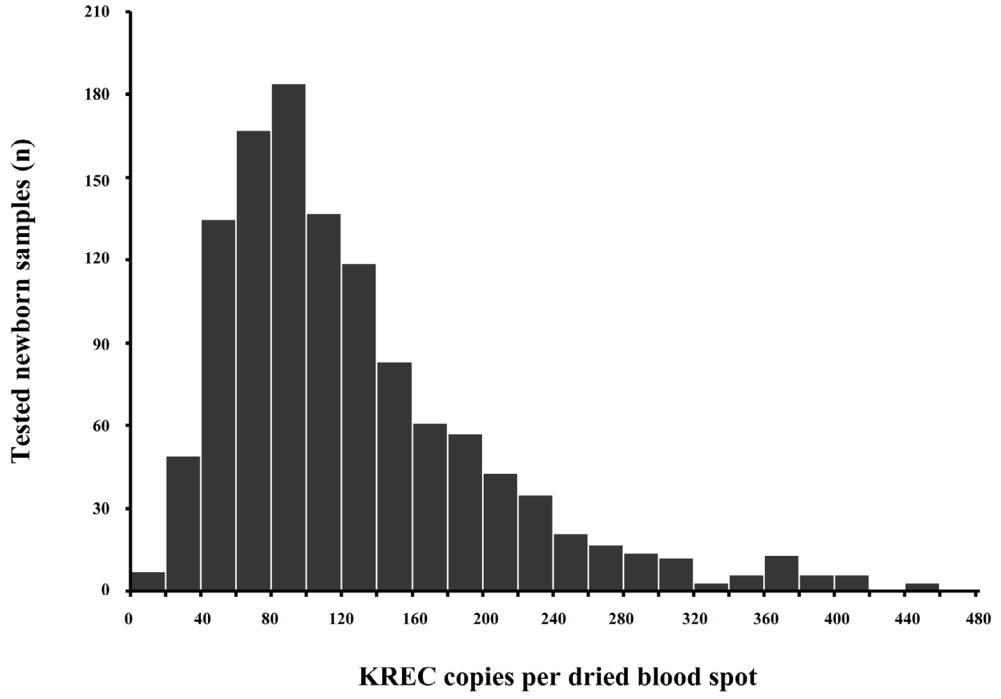


Figure 3 Representative distribution of TREC and KREC copy numbers in neonatal dried blood spot samples



(A)



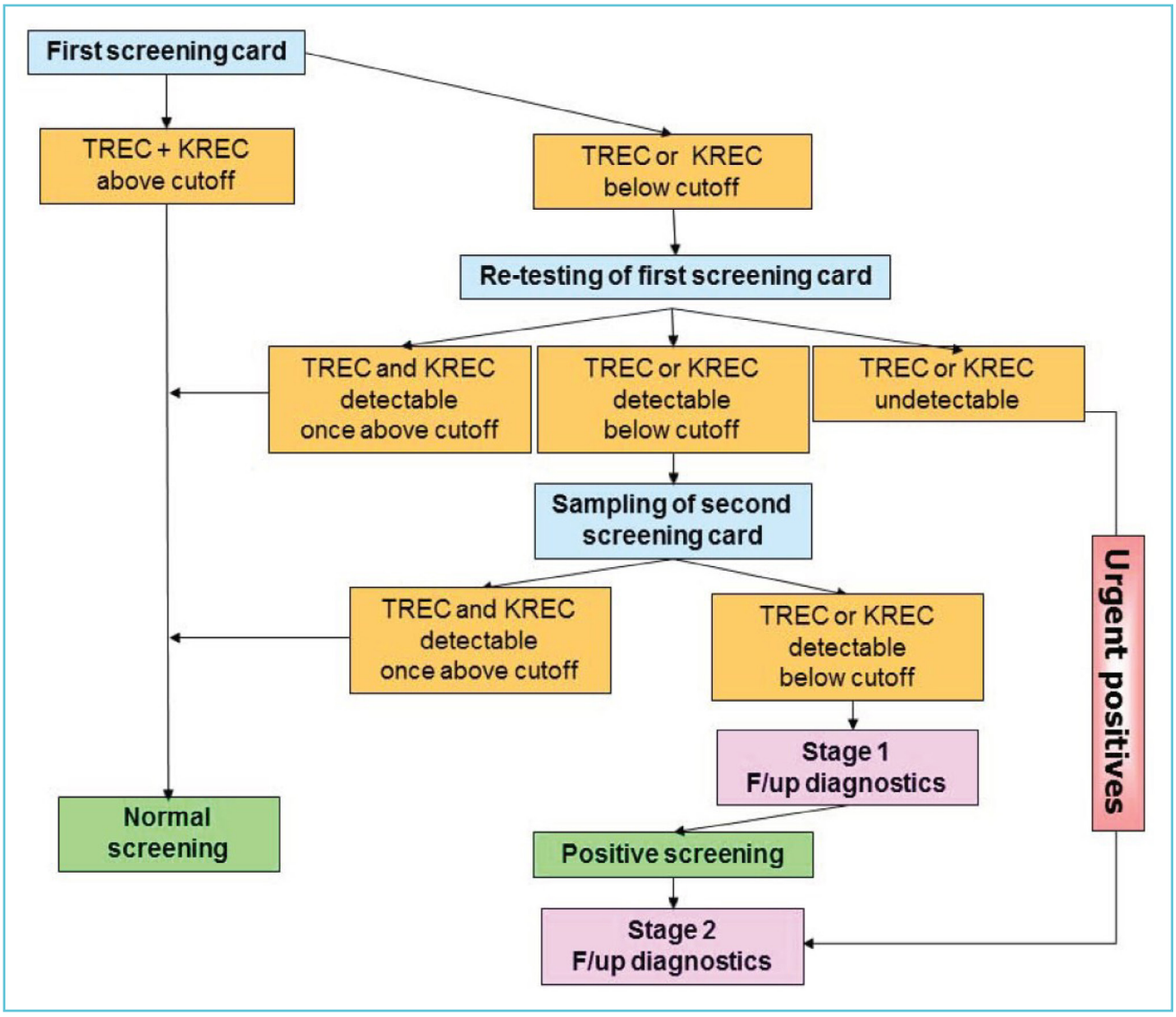
(B)

naïve T-cells. This aspect is important, as in certain conditions such as engraftment of maternal T-cells or expansion of a few oligoclonal T-cells in Omenn syndrome, a substantial amount of T-cells can be found in an infant with SCID. As these T-cells have undergone multiple rounds of cell division, TRECs are diluted and the TREC value is low despite high numbers of T-cells in peripheral blood.

As some leaky, variant, or delayed-onset forms of SCID will not be detected at birth based on a single TREC assay, the addition of other screening markers such as kappa-deleting recombination excision circles (KREC), which detect defects of B-cell development, has been proposed and might be considered helpful (Figure 2) [4].

Screening for severe T-cell lymphopenia by TRECs is not standardized and employs different methods, leading to marked differences in cut-offs for the number of newly formed T-cells in the ongoing screening programs in various countries. This, in turn, has resulted in significant

Figure 4 Proposed flow-chart for the follow-up of positive newborn screening results for TRECs and KRECs



difference in the number of patient recalls and diagnostic procedures, including flow cytometry and other cellular testing stages. Typical testing results in a large cohort of healthy newborn using a commercially available screening kit for TRECs and KRECs are depicted in Figure 3 [5].

To ensure adequate follow-up of infants with likely SCID identified by TREC screening and to limit the number of false-positive results at the same time, algorithms have been designed by screening centers together with clinical immunologists [5]. In most cases this will include the following (Figure 4): All infants undergo screening by the TREC (and KREC) assay. If normal, no further intervention is recommended. In infants with TREC levels below the cut-off, the first screening card will be retested for TREC as well as DNA amplification by quantifying beta-actin levels. If the beta-actin level is normal and TRECs are still below the cut-off the primary care provider is contacted for two scenarios: 1) an emergency scenario - if TRECs are undetectable (~1 in 20.000 cases), the infant needs urgent confirmatory testing by flow cytometry and treatment by a clinical immunologist; 2) an intermediate scenario – if TRECs are detectable, yet below the established local cut-off value, re-testing of a second screening card is performed: Tracking of newborns is thus initiated in case of repeated abnormal test results for TREC and/or KREC copy numbers after examination of at least three independent dried blood punches of the first dried blood spot submitted.

Parent and patient interaction

As first action within the tracking procedure, the obstetric unit and the parents of the newborn should be contacted to obtain additional information on the status of the child (Stage 1). While the parents of the newborn should be informed without delay after the examination of the second separate dried blood card, even if the findings are normal, a detailed explanation of the significance of the test results, if they are abnormal again, will be provided only at a specialized immunodeficiency center. The parents will be directed to such a center in the area (Stage 2), and the center will be informed about the patient to be expected.

Subsequently, a specialized treatment center should be selected that is close to home: in the case of suspected severe naive T- and/or B-lymphopenia, intensive hygiene measures and possibly early, strict isolation to prevent opportunistic infections are necessary steps to be taken. In order to perform HSCT or gene therapy, a transfer to a specialized transplant center may be necessary.

Flow cytometric analyses in Stage 1 and Stage 2

Screening for neonatal T and/or B cell lymphopenia reveals not only patients with SCID or agammaglobulinemia, but also genetic, metabolic and other medical conditions associated with low TREC and/or KREC copies in the dried blood spot card. Whereas the most common reasons for low TREC/KREC copies refer to neonates with preterm birth, 22q11 microdeletion syndrome and Trisomy 21, radiosensitivity disorders such as Ataxia telangiectasia (ATM) or Nijmegen breakage syndrome, chylothorax or spina bifida, there is also a fraction of newborns found resulting from side-effects of maternal medication during pregnancy (i.e. azathioprine, methotrexate or Rituximab treatment) [5-7].

Thus, both in testing Stages 1 and 2 there is an imminent need for verification of the cellular immune status of positively screened infants and stratification of SCID, leaky-SCID and non-SCID patients.

In order to decrease patient harm and parental concern, all testing stages will have to be instructed and supervised by a pediatric immunologist, with 24/7 availability. Minimal diagnostic

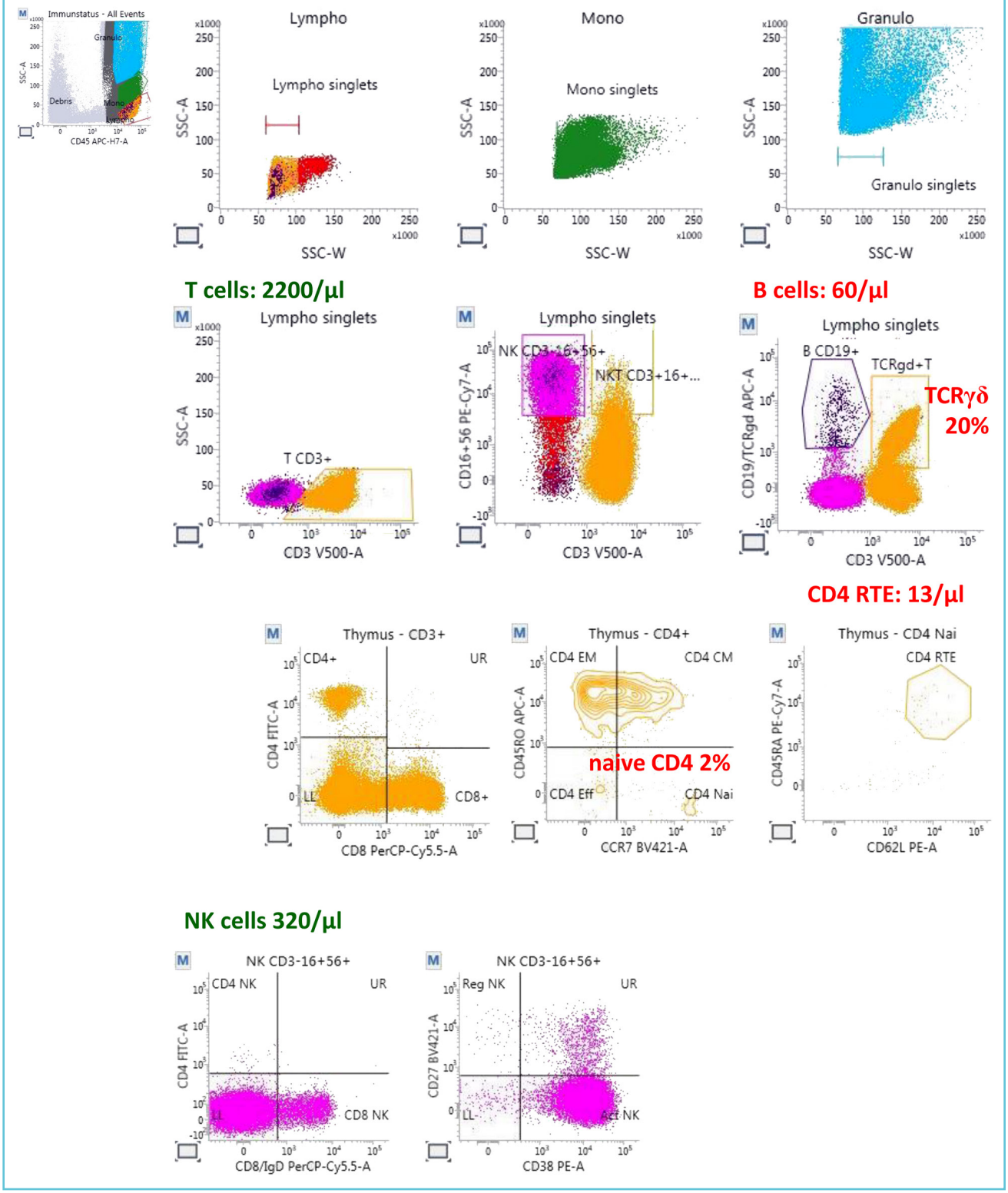
Table 1 Diagnostic recommendation for Stage 1 and Stage 2 centers following positive NBS with TRECs and/or KRECs

Stage 1	
- 24/7 availability of a paediatric immunologist	
- Immediate medical examination and counseling	
- Differential blood count	
- IgM, IgG, IgA, IgE serum levels	
- HIV testing mother and child	
- Flow cytometric analysis based on neonatal reference values for	
	T cells (CD3/CD4/CD8)
	T cell naivety (CD45RA and CD45RO)
	B cells (CD19)
	NK cells (CD3/CD16/CD56)
Stage 2	
- All of the above	
- Additional flow cytometric analyses for	
	T cell naivety (CCR7)
	Recent thymic emigrants (CD4/CD31/CD45RA)
	αβ and γδ T cells (CD3/αβTCR/γδTCR)
- In case of presence of >100 T cells/µl	
	Exclusion of maternal T cells
	Lymphocyte proliferation studies (PHA and/or anti-CD3/CD28)
	Radiosensitivity testing of lymphocytes
	Analysis of the TCR Vbeta repertoire (Omenn-Syndrome)
	ADA and PNP enzyme activity levels
	Whole Exome or Genome Sequencing

Table 2 **Diagnostic consensus of the Primary Immune Deficiency Treatment Consortium**

Typical SCID
- Absence or very low number of T cells (CD3 T cells <300/µL) and no or very low T-cell function (<10% of lower limit of normal) as measured by response to PHA
- Or T cells of maternal origin present
Leaky SCID
- Reduced number of CD3 T cells (CD3 T cells <1000/µL)
- Absence of maternal engraftment
- <30% of lower limit of normal T-cell function (as measured by response to PHA)
Omenn syndrome
- Generalized skin rash
- Absence of maternal engraftment
- Detectable CD3 T cells, ≥300/µL
- Absent or low (≤30% of normal) T-cell proliferation to antigens to which the patient had been exposed
- Or Hepatomegaly, Splenomegaly, Lymphadenopathy
- Or increased IgE level, increased absolute eosinophil count
Reticular dysgenesis
- Absence or very low number of T cells (CD3 T cells <300/µL)
- No or very low (<10% of lower limit of normal) T-cell function (as measured by response to PHA)
- Severe neutropenia (absolute neutrophil count <200/µL)
- Sensorineural deafness and/or absence of granulopoiesis at bone marrow examination and/or a deleterious AK2 mutation

Figure 5 **Immunophenotype of a leaky-SCID (RAG2 mutation) identified by TREC/KREC newborn screening**



testing recommendations for Stage 1 and Stage 2 centers is depicted in Table 1 [8].

There exists a minimal diagnostic consensus, yet of fluid nature and certainly subject to future discussions, for the classification of neonates with typical SCID, leaky SCID, Omenn syndrome, reticular dysgenesis, and idiopathic T cell lymphopenia [8]. These diagnostic criteria are reproduced in Table 2 and should be applied in the cellular and immune-functional testing strategies despite of ongoing genetic analyses.

The aim is to disclose those patients from a HSCT-track that will not benefit from this therapy or might even yield harm during the transplantation conditioning phase, such as seen in individuals with chromosome repair disorders (i.e. ATM). In this context, flow cytometric analyses subsequent to positive NBS with TRECs and/or KRECs will have to rely on best established-practice, or better harmonized and widely-available diagnostic products, and reference values validated for the cell staining/lysis protocol that is applied [Table 1] [9]. As an example, the PID working party of the EuroFlow consortium provided a Primary Immunodeficiency Orientation Tube (PIDOT) with polychromatic flow cytometric markers for classification or T-, B- and NK-cells, thereby fulfilling requirements of Stage 1 testing after NBS [10]. To further endorse harmonization across different labs, such panels will be available in lyophilized antibody format to allow pre-production, storage and uniform staining approaches. Similarly to surface-marker phenotypic staining, there also are protocols available for T/B/NK cell functional analyses of the DNA damage repair capability (γH2AX assay), as well as cellular proliferation upon phytohemagglutinin (PHA) or anti-CD3/28 treatment [11, 12]. Exemplarily, Figure 5 shows testing results of a Stage 2 flow cytometric analysis from a newborn identified with leaky-SCID due to a RAG2-mutation upon positive NBS with TRECs and KRECs.

CONCLUSIONS AND PERSPECTIVE

As newborn screening for severe primary immunodeficiency diseases (PID) - characterized by T and/or B cell lymphopenia - is becoming clinical routine practice in a growing number of countries, there still is debate about the definition of PID and a lack of harmonized approaches to the immune cellular phenotype and functional testing during diagnostic follow-up. In the future, more specific large scale and age-selected studies in the field of neonatal care and primary immunodeficiency diseases are required to provide reliable cornerstones to line clinical decisions upon and provide earliest-possible and safeguarded care for PID patients.

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Flow cytometry in the diagnosis and follow up of human primary immunodeficiencies

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flow cytometry, primary immunodeficiency, diagnostics

ABSTRACT

Primary immunodeficiencies (PID) comprise a group of more than 300 mostly monogenetic disorders of the immune system leading to infection susceptibility and a variety of associated clinical and immunological complications. In a majority of these disorders the absence, disproportions or dysfunction of leucocyte subpopulations or of proteins expressed by these cells are observed. These distinctive features are studied by multicolour flow cytometry and the results are used for diagnosis, follow up, classification and therapy monitoring in patients with PIDs. Although a definite diagnosis almost always relies on genetic analysis in PIDs, the results of flow cytometric diagnostics are pivotal in the initial diagnostic assessment of suspected PID patients and often guide the treating physician to a more selective and efficient genetic diagnostic procedure, even in the era of next generation sequencing technology. Furthermore, phenotypic and functional flow cytometry tests allow

to validate novel genetic variants and the mapping of complex disturbances of the immune system in individual patients in a personalized manner. In this review we give an overview on phenotypic, functional as well as disease/protein specific flow cytometric assays in the diagnosis of PID and highlight diagnostic strategies and specialties for several selected PIDs by way of example.

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INTRODUCTION

The last report of the International Union of Immunological Societies (IUIS) lists 354 inborn errors of immunity resulting in various forms of primary immunodeficiencies (PID), which are subdivided into nine categories:

1. Severe and combined immunodeficiencies (SCID)
2. Combined immunodeficiencies with syndromic features
3. Predominantly antibody deficiencies
4. Immunodysregulatory disorders
5. Defects affecting phagocytes
6. Defects of innate immunity
7. Autoinflammatory disorders
8. Complement deficiencies
9. Phenocopies of PIDs (1,2)

Although PIDs are generally considered as very rare disorders individually, they form a clinically relevant cohort as a group. The major clinical symptom in the majority of PIDs is infection susceptibility, which ranges from specific immunodeficiency to a single pathogen to broad immune failure. However some PIDs are syndromic disorders and many PIDs show additional clinical manifestations caused by immune dysregulation, including autoimmunity, lymphoproliferation, granulomatous inflammation and malignancy disposition. In addition, there is considerable phenotypic variability in many of the monogenetic PID traits and untreated or progressive PID disease causes sequelae, secondary changes and end organ damage.

Finally, secondary immunodeficiency caused by non-immunological disorders, environmental factors and immunosuppressive or ablative therapeutic interventions have to be considered especially in adult patients with manifesting symptoms suspicious for PID. Since most of severe PIDs manifest with first symptoms soon after birth, new born screening programs have recently been successfully established in several countries to detect these forms of PIDs at the earliest time point as possible (see article by Wolf J et al in this issue).

In summary, the complex clinical and immunological presentations of the various PIDs require sensitive and specific diagnostic tests. Flow cytometry emerged as a method of choice for the study of PIDs, since it allows the fast and reliable analysis of almost all branches of the immune system on a single cell level.

The basic clinical and laboratory evaluation of a patient with suspected PID should include a careful clinical history, paying special attention to the family history and the symptoms and features mentioned above, a complete differential blood count, serum immunoglobulin levels, a global complement function test and specific antibody titers for vaccine antigens (e.g. tetanus toxoid).

The individual flow cytometric diagnostic testing will depend on the clinical presentation of the patient and the results of basic laboratory tests. If a defect of adaptive immunity is suspected then usually a basic lymphocyte phenotyping will be performed followed by some more specific testing (e.g. extended phenotype of B-cells in patients with antibody deficiency). The range of applications ranges from phenotypic assays investigating the numbers and proportions of immune cells, functional analysis of

Table 1 Examples of basic and extended phenotypic assays, disease specific surface and intracellular protein analysis and functional assays to study PIDs*

Basic phenotypic analysis		
Test	**Cell populations**	**Indications**
Lymphocyte subpopulations	CD4+ T-cells, CD8+ T-cells, B cells, NK cells	Basic screening for PID, SCID
T-cell subpopulations	CD4+CD45RA+ naïve T-cells, HLA DR+ activated T-cells, CD8 effector cells, γ/δ T-cells, α/β double negative T-cells, regulatory T-cells	SCID, CVID, CID, ALPS
B-cell subpopulations	IgD+CD27- naïve B-cells, IgD+CD27+ non-switched memory, IgD-CD27+ switched memory, transitional B-cells, plasmablasts, CD21low B-cells	primary antibody deficiency, CVID, CID
Extended phenotypic analysis		
Test	**Cell populations**	**Indications**
dendritic cell subpopulations	CD123+ plasmacytoid dendritic cells, CD11c+ myeloid dendritic cells	GATA2 deficiency
regulatory T-cells	CD4+CD25+ FoxP3+ regulatory T-cells	IPEX syndrome
recent thymic emigrants	CD4+CD45RA+CD31+ T-cells	SCID, DGS
TCR repertoire analysis	T-cell Vβ chain variant expression on CD4 and CD8 T-cells	SCID, CID
Functional assays to study PIDs		
Test	**Cell populations**	**Indications**
oxidative burst assay	granulocytes	chronic granulomatous disease, inflammatory bowel disease
T-cell proliferation	CD4+ and CD8+ T-cells after stimulation with PHA, anti-CD3, anti-CD3 and anti-CD28	SCID, CVID, CID

NK cell degranulation	CD107a expression on stimulated or resting NK cells	familial hemophagocytic lymphohistiocytosis
IL-17/IFNγ production	PMA/Ionomycin stimulated T-cells	chronic mucocutaneous candidiasis, Hyper IgE syndrome
Disease specific surface and intracellular protein analysis		
Test	**Cell populations**	**Indications**
BTK	monocytes	X-linked agammaglobulinemia
WASp	lymphocyte subsets	Wiskott Aldrich syndrome
CD40L	activated T-cells	X-linked Hyper IgM syndrome
SAP	lymphocyte subsets	X-linked lymphoproliferative disorder type 1
XIAP	lymphocyte subsets	X-linked lymphoproliferative disorder type 2
Perforin	NK cells	FHL type 2

** ALPS: autoimmune lymphoproliferative syndrome; BTK: bruton tyrosine kinase; CID: combined immunodeficiency; CVID: common variable immunodeficiency; DGS: DiGeorge Syndrome; FHL: familial hemophagocytic lymphohistiocytosis; IPEX: Immune dysregulation, polyendocrinopathy, enteropathy, X linked; SAP: SLAM-associated protein; SCID: severe combined immunodeficiency; TCR: T-cell receptor; WASp: Wiskott Aldrich syndrome protein.*

cellular processes (e.g. proliferation, cytokine secretion, cytotoxicity) to the direct analysis of potentially mutated proteins in disease specific assays (Table 1). Usually these techniques are combined to allow the study of dynamic processes in specific cell subpopulations (e.g. degranulation of NK lymphocytes) or protein expression in specific cell populations after stimulation like e.g. CD40L expression in activated T-cells (see Table 1).

The setup and performance of these diagnostic assays at high and reproducible quality requires a high level of expertise from the laboratory. Reference values for phenotypic analysis of immune cells need to be age adapted and ideally should be determined for each tested parameter and setup individually. Especially for functional tests, in house reference values need to be determined and checked by appropriate tests on a regular basis. Since defined biological control materials for most of the assays will not be commercially available, the parallel testing of healthy controls for each diagnostic procedure is highly recommended. If available, standardized reagents and procedures should be used and followed.

Test results should be reported in context of the clinical presentation and a clear and direct communication between the laboratory staff and clinicians is often very important for the correct interpretation of the results.

To illustrate diagnostic strategies and possibilities of flow cytometry in the diagnostics of PID we will briefly discuss four different clinical scenarios within the field of PID:

1. X-linked (Bruton´s) agammaglobulinemia: a monogenetic PID with a limited clinical and immunological phenotype and some relevant differential diagnosis;
2. Common variable immunodeficiency disorders: a heterogeneous group of patients with primary antibody deficiency without known genetic defect but a clinically and immunologically highly diverse phenotype;
3. GATA2 haploinsufficiency: a monogenetic but clinically diverse trait affecting the cellular phenotype of different hematopoietic cell lineages, resulting in a complex immunopathology and diverse syndromic features;
4. Hemophagocytic lymphohistiocytosis (HLH), an often hyperacute and life threatening condition, in which flow cytometry is a fast and reliable diagnostic tool, which can assist in differentiating diverse primary and secondary causes.

Figure 1A **Basic lymphocyte subset analysis of an XLA deficient patient and a healthy control showing absent CD19+ B-cells (upper right panel)**

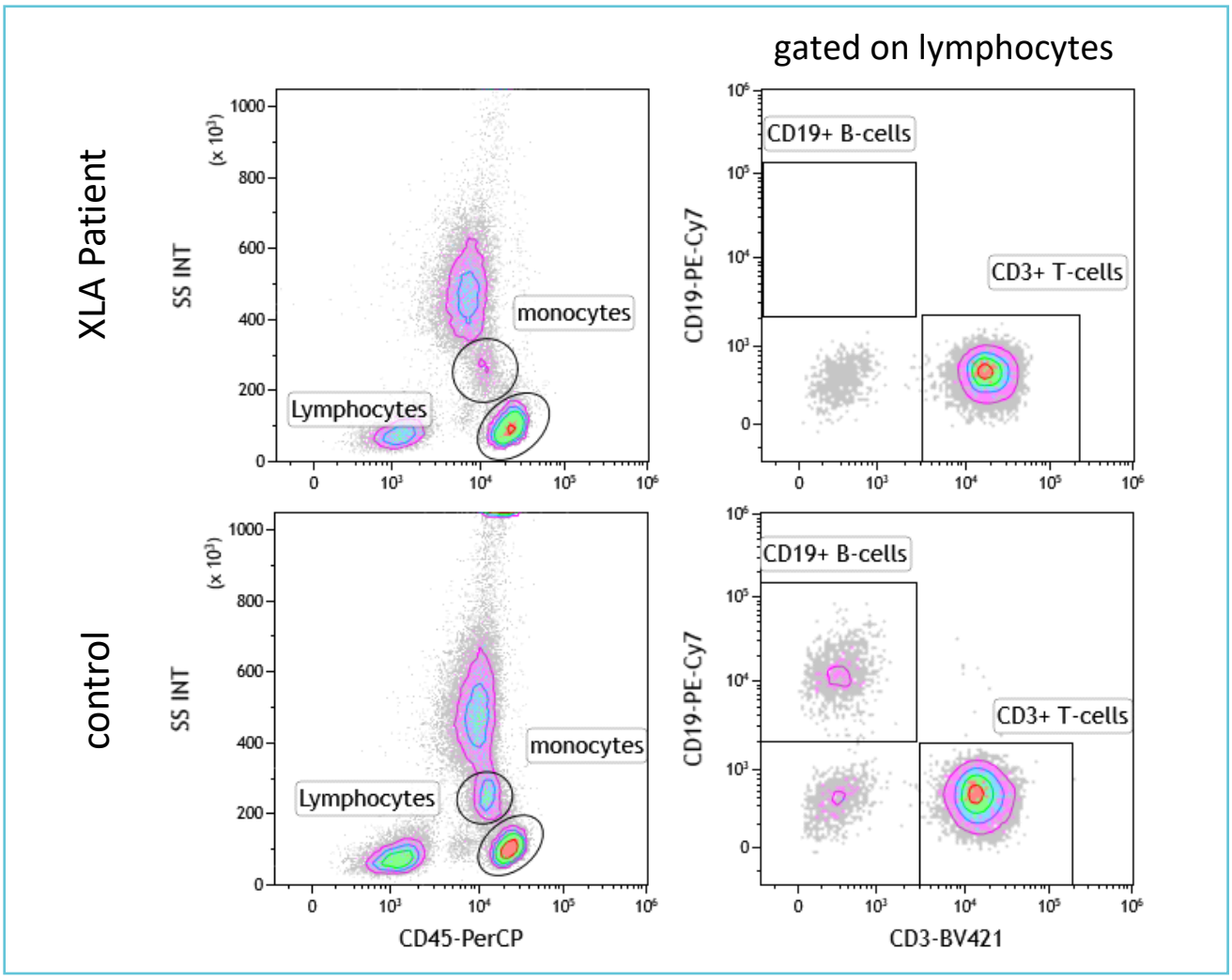
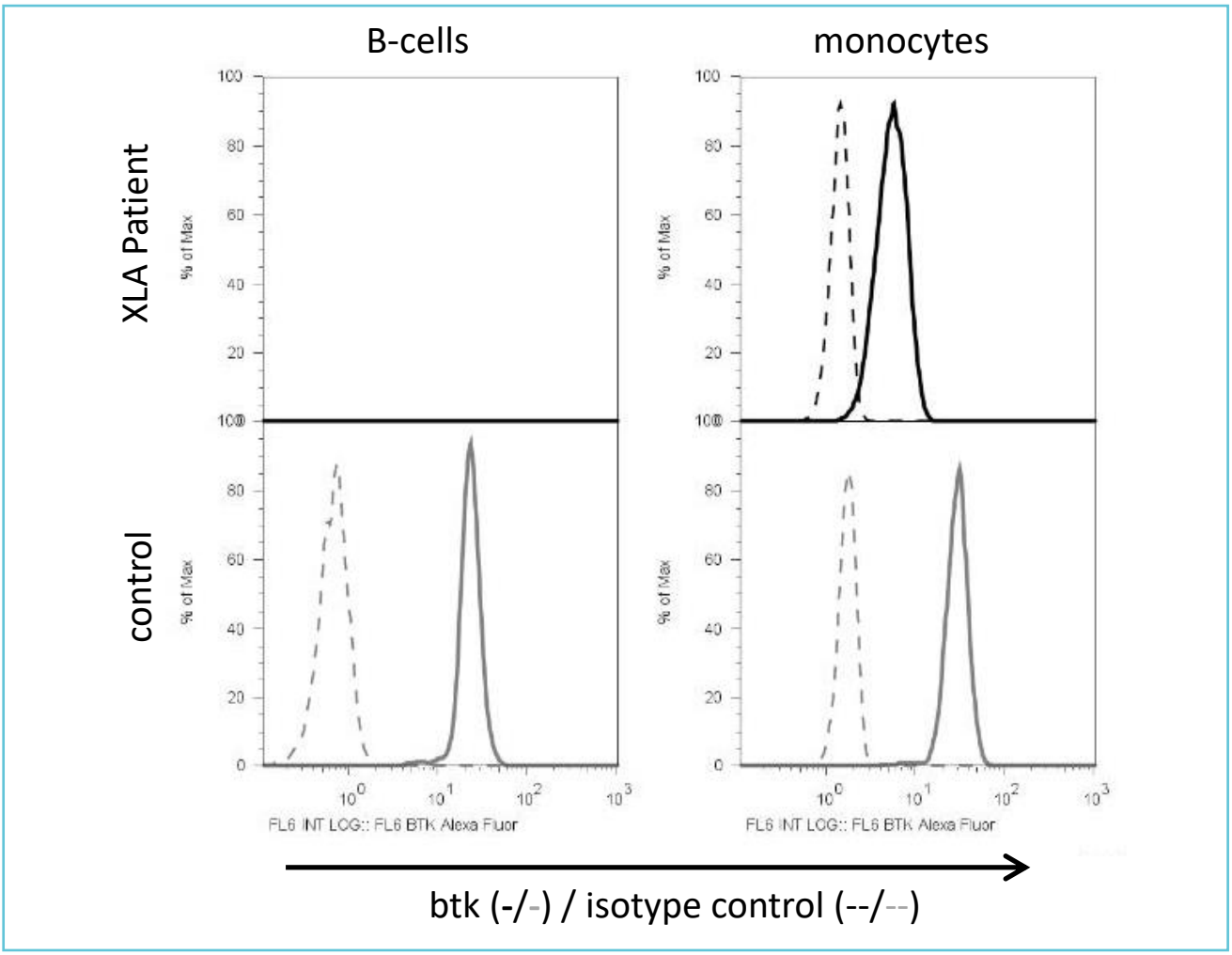


Figure 1B Reduced intracellular BTK expression (solid lines) versus the isotype control (dashed line) analyzed in monocytes (right panels) and B-cells (left panels) of an XLA patient a healthy control



X-LINKED AGAMMAGLOBULINEMIA (XLA)

The pairing of absent or very low cell B-cells and immunoglobulins is summarized in the group of agammaglobulinemias. 80% of affected children are male and most of these suffer from X-linked (Bruton´s) Agammaglobulinemia (XLA).

XLA was first described by OC Bruton in 1952, usually manifests in boys within the first two to five years of life and has a frequency of 1:1.000.000 live births (3,4). XLA is caused by mutations in the *btk* gene encoding for the Bruton Tyrosine Kinase (BTK) on the X-chromosome (5,6).

In developing B-cells in the bone marrow, BTK is important for signalling of the pre B-cell receptor and mutations found in XLA patients generally lead to a developmental block, resulting in severely impaired bone marrow output of B-cells (7).

Typically the patients develop bacterial infections of the respiratory tract, when maternally transferred antibody levels vanish after the sixth month of life (4). Total immunoglobulin levels are typically below 1 g/l but residual amounts of IgG, IgA and IgM may be present especially

in those XLA patients diagnosed after the age of five years (4).

Total lymphocyte numbers are usually normal and flow cytometric analysis of basic lymphocyte subpopulations (T, B, NK) reveals a normal T-cell and NK cell count, but B-cells are usually not detectable or below 1% of lymphocytes (see Table 1; Figure 1A).

In patients with suspected XLA BTK protein expression can be investigated by flow cytometry after intracellular staining in monocytes (8), which also express high levels of BTK and are present in sufficient numbers in patients with XLA (Figure 1B).

Most of the known *btk* mutations impair or abrogate BTK protein expression (9). However normal BTK protein levels do not exclude XLA and in cases where the clinical suspicion is high genetic analysis should be performed. Phosphorylation of BTK Y223 can be studied after pervanadate stimulation (10), providing a method to study the pathogenic relevance of uncertain novel mutations. In female and male patient with a normal *btk* gene autosomal recessive forms of agammaglobulinemias should be considered as differential diagnosis (11).

As these deficiencies affect the pre B-cell receptor complex and lead to characteristic cellular blocks in early B-cell development they could be easily identified by flow cytometry but require a bone marrow sample for analysis and thus are preferably unravelled by genetic analysis.

COMMON VARIABLE IMMUNODEFICIENCY DISORDERS (CVID)

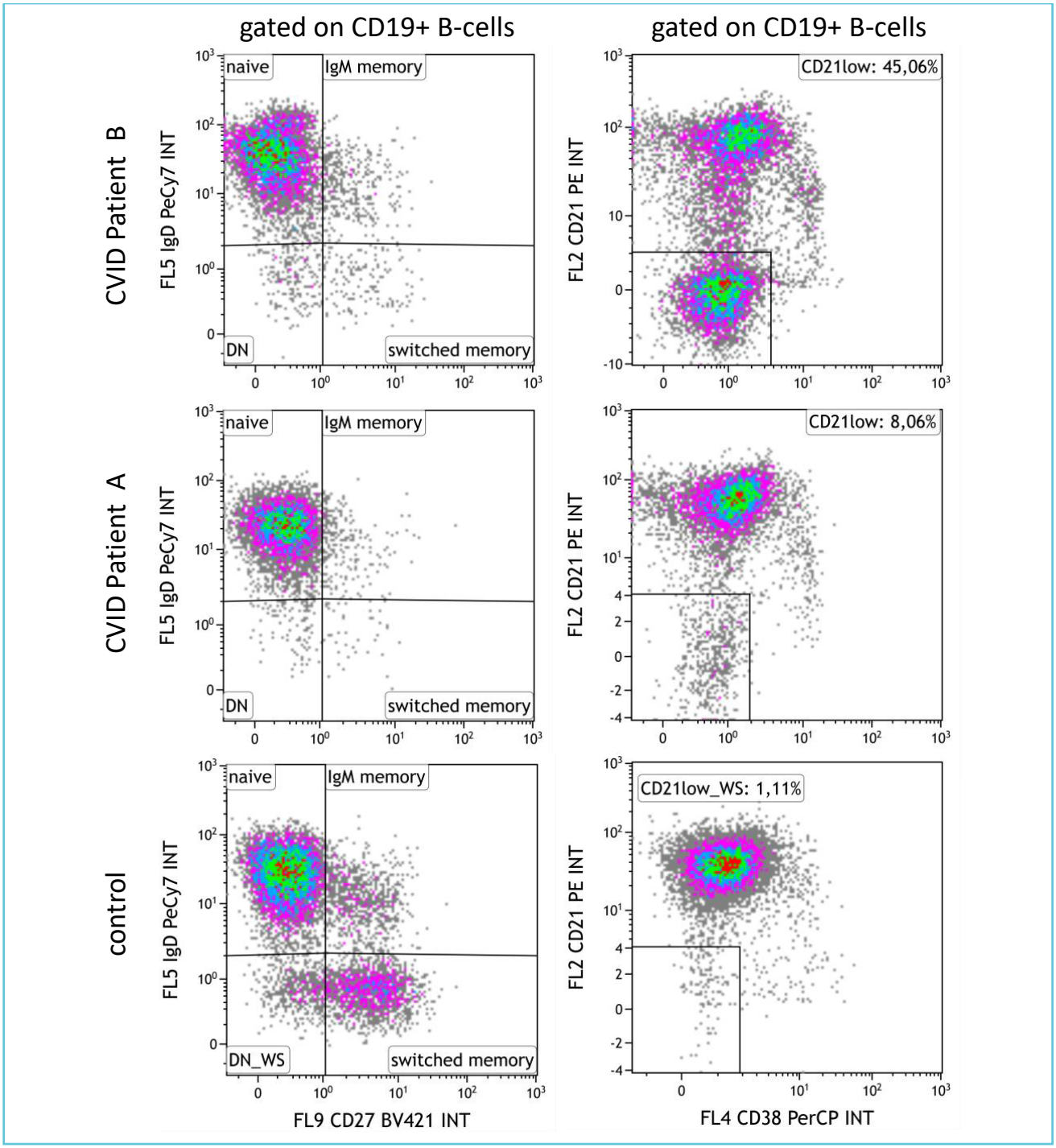
Common variable immunodeficiency disorders comprise the largest group of PID patients in adulthood. It is characterized by hypogammaglobulinemia, recurrent bacterial respiratory tract infections and several associated diseases or sequelae like autoimmune cytopenias, benign lymphoproliferation, granulomatous inflammation, and predisposition for certain malignancies and structural lung disorders.

Unlike most of the other primary immunodeficiencies, which manifest usually in the first year or decade of life, are mostly familial and have a defined monogenetic cause, CVID patients typically are adolescents or young adults when first symptoms occur and usually the cases are sporadic without a family history. As a diagnosis of exclusion CVID serves as a “drop box” for antibody deficiency syndromes of all kind that could not be attributed to any other known PID or other disease state manifesting primarily with hypogammaglobulinemia. Within the past decade it has been recognized that the initial 1999 PAGID / ESID criteria for the definition of CVID (12) need refinement and precision to better harmonize the CVID cohort and avoid misdiagnosis of CVID in patients, who actually suffer from different disorders requiring different care settings and therapy. Interestingly all new proposed diagnostic criteria include now some flow cytometric testing (13–15).

In relation the study of other PIDs, flow cytometric analysis has a special significance in the study of CVID and has contributed vitally to our understanding of these disorders. The cellular hallmark of CVID are severely reduced numbers of switched memory IgD-CD27+ B-cells and plasmablasts (see figure 2), which are observed in a majority of CVID patients (16–18). In subgroups of CVID patients additional B-cell disturbances like expansion of so called CD21low B-cells (see figure 2) or transitional B-cells are observed (17,19). Thus B-cell phenotyping by flow cytometry emerged as a useful tool for pathophysiological studies and the classification of the syndrome (17,20,21).

These studies showed several important significant relations between individual B-cell abnormalities and clinical complications, such as

Figure 2 **B-cell subpopulation analysis in two CVID patients and one healthy control showing naïve, IgM (or non-switched) memory and switched memory B-cells by anti IgD versus anti-CD27 staining (left panels) and CD21low B-cells by anti-CD21 versus anti CD38 staining (right panels). Both patients have reduced memory B-cell subsets and CVID patient B shows an expansion of CD21low B-cells.**



lymphoproliferation, granulomatous inflammation or autoimmune disease (17,20,21).

The early recognition of the development of such complications in individual CVID patients is mandatory, since they have an important impact on the morbidity and mortality (22). However, the B-cell phenotypes so far seem to have limitations as prospective biomarkers in individual CVID patients, likely because changes such as CD21low B-cell or transitional B-cell expansions are not CVID specific and may be only secondary to infections, lymphoproliferation, inflammation or autoimmunity (23–25). Besides B-cells, flow cytometric studies of the T-cell system have been widely used in CVID.

The recent novel definitions of CVID recommend a careful exclusion of severe T-cell deficiencies in patients with suspected CVID to avoid misdiagnosis in patients with LOCID (late on severe combined immunodeficiency) (26) or other forms of combined immunodeficiencies.

The revised criteria for the ESID registry require therefore a T-cell count of >200 / µl with an amount of at least 10% naïve CD4+CD45RA+ T-cells present in adults and/or a normal T-cell proliferation (13). However, the moderate reductions of naïve CD4+CD45RA+ T-cells found in a significant numbers of CVID patients have also been implicated as an alternative way of classification of disease associated pathologies in CVID (27).

In certain cases, flow cytometric screening or targeted analysis for changes in specific cell populations or cellular proteins has been successful to reveal single gene defects, such as ICOS deficiency, CD19 deficiency and BAFF-R deficiency (28–30).

However, these approaches cannot be recommended as routine diagnostics as these monogenetic defects are very rare and not all mutations will result in reduced protein expression and / or complete absence of a specific cell population.

GATA2 DEFICIENCY

In 2010 and 2011 two groups reported two similar novel PIDs, MONOmac syndrome (**mono**cytopenia and **m**ycobacterium **a**vium **c**omplex infections) and DCML (**d**endritic **c**ell, **m**onocyte, B and NK **l**ymphocyte) deficiency (31,32), characterized by autosomal dominant inheritance, certain cellular deficiencies, a variable and diverse susceptibility to infections and a predisposition to myeloid leukemia and infection associated cancers.

Subsequently, heterozygous mutations in the hematopoietic transcription factor *GATA2* have been identified as the genetic cause of the two syndromes (33,34) and several other conditions such as Emberger syndrome (sensorineuronal deafness and primary lymphedema with a predisposition for myelodysplastic syndrome or AML) and familial myelodysplastic syndrome or AML (35,36).

Missense mutations in the zinc-finger 2 domain or deleterious mutations of *GATA2* prevail, leading to functional or genetic GATA2 haploinsufficiency, which is required for hematopoietic stem cell (HSC) homeostasis (37).

In consequence GATA2 deficiency leads to depletion of HSC and especially lymphoid and myeloid precursors. Extrahematopoietic manifestations like thrombotic events, lymphedema or deafness are likely explained by the additional functions of GATA2 in vascular endothelia (38,39). The cellular phenotypes of GATA2 deficiency were studied in larger cohorts of patients and correlated with disease severity (40,41). Although each of the phenotypes is not specific to GATA2 deficiency, the joined appearance of these features is supportive in diagnosis. In particular the combination of monocytopenia, B- and NK cell deficiency (Figure 3A) together with low dendritic cell numbers (Figure 3B) should raise suspicion for GATA2 deficiency in patients with compatible clinical presentations.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH)

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening hyperinflammatory syndrome caused by different inherited and secondary conditions (42).

Primary HLH can be subdivided into the group of familial hemophagocytic lymphohistiocytosis (FHL). These are

- Perforin deficiency, FHL2;
- Munc13-4 deficiency, FHL3;
- Syntaxin 11 deficiency, FHL4 and
- Munc18-2 deficiency FHL-5

and several other monogenetic PIDs (among them are Chediak Higashi Syndrome (CHS), Griscelli syndrome type 2, X-linked lymphoproliferative syndrome type 1 and 2 (XLP1 and XLP2) and others) (43–50).

Secondary HLH occurs in association with viral infections, lymphoma, autoimmune disease, after hematopoietic stem cell transplantation and drug hypersensitivity.

Clinically both primary and secondary HLH may be triggered by viral infections and present with persistent fever, splenomegaly and bi- or trilinear cytopenias and show elevated levels of triglycerides, ferritin and soluble IL-2 receptor in serum (42).

Figure 3A **Basic lymphocyte subset analysis of a GATA2 deficient patient and a healthy control showing absent monocytes (upper left panel) and reduced CD19+ B cells (upper middle panel) and CD16+CD56+ NK cells (upper right panel)**

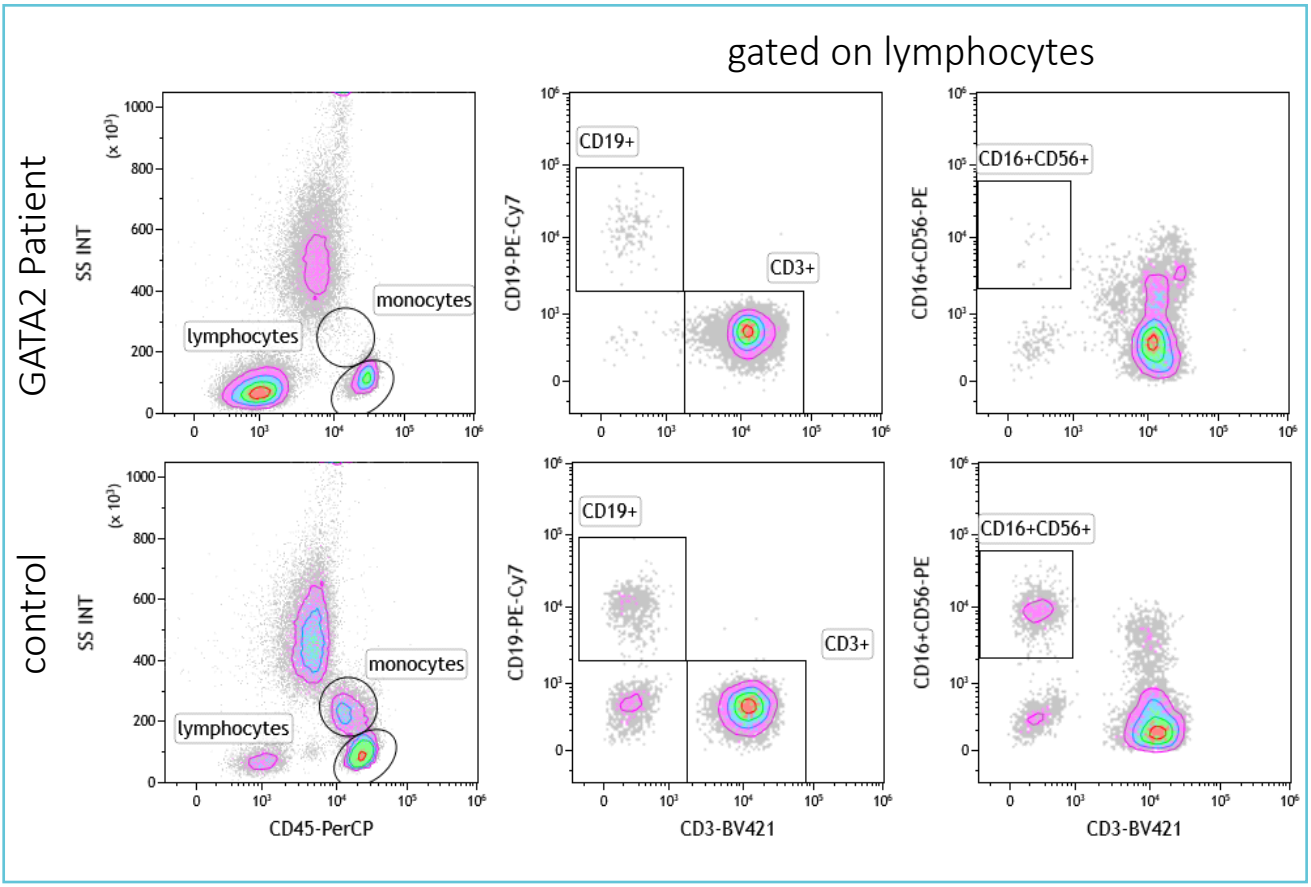
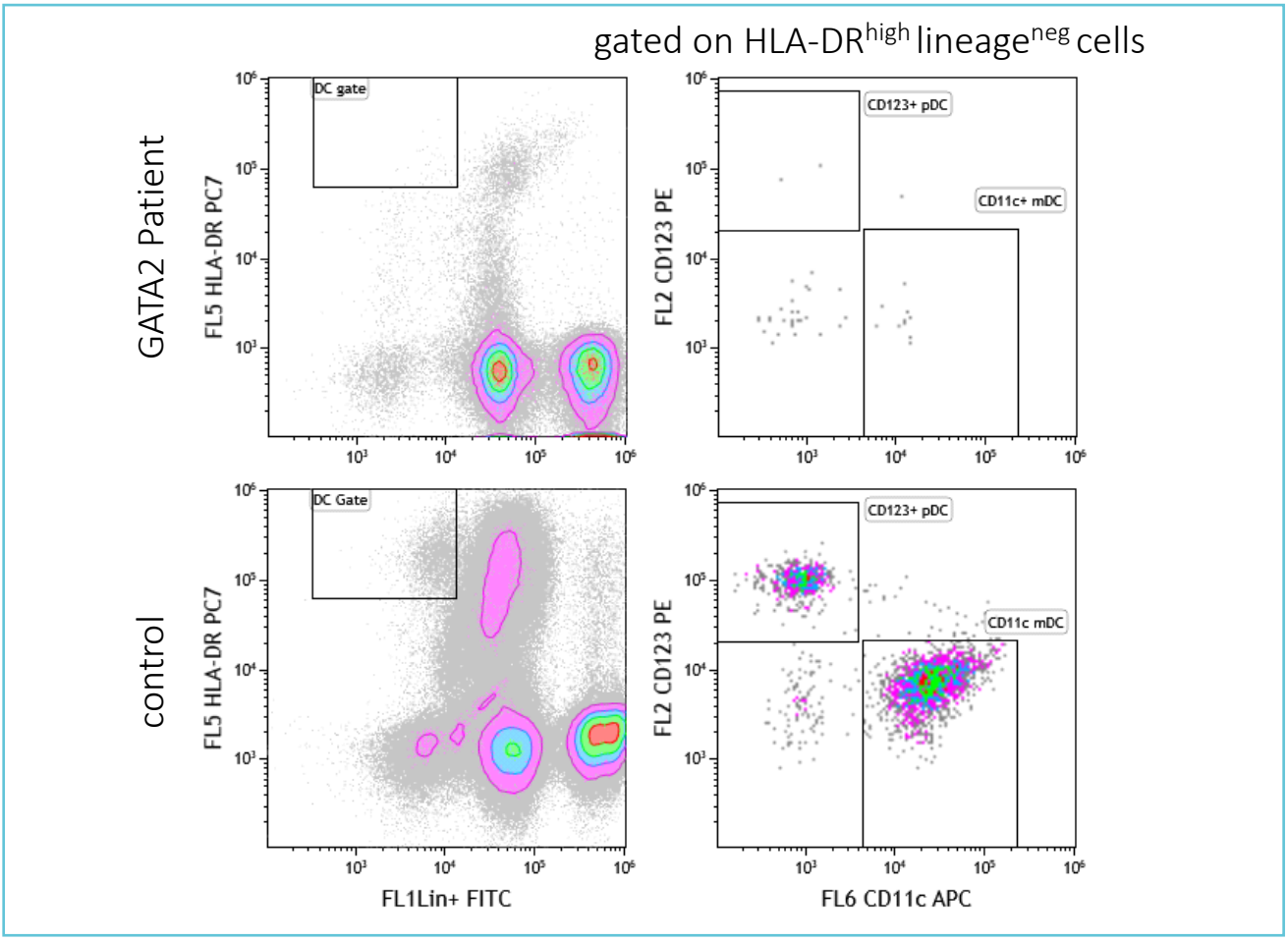


Figure 3B **Analysis of dendritic cell subsets in a GATA2 deficient patient and a healthy control revealing both severely reduced CD123+ lymphoid and CD11c+ myeloid dendritic cells (upper right panel)**



Primary and especially the subgroup of FHL usually manifests early in life whereas secondary HLH may occur at any age. Because the majority of FHL patients require hematopoietic stem cell transplantation, their rapid identification is critical.

FHL is caused by defects in the cytotoxic machinery of T-cells and NK cells and the deficiency of perforin (FHL2) is the prototypic form and the most common in FHL (43).

All other forms of FHL and the closely related disorders like CHS show defects in resting and activated NK cell degranulation, which can be detected by flow cytometric analysis of the cytotoxic granule associated marker CD107a. In XLP1 and XLP2 there is no apparent defect of cytotoxicity (51) but the patients develop HLH triggered by uncontrolled EBV infection due to deficiencies in the intracellular SAP and XIAP proteins (49,50).

A stratified flow cytometric work-up is very helpful in distinguishing the various forms of primary HLH from secondary HLH (51,52). Perforin, and in male patients also SAP and XIAP protein expression are analysed by intracellular flow cytometry (53,54) (Figure 4A).

If Perforin, SAP or XIAP protein expression is abnormal targeted genetic analysis of the respective

encoding genes should be performed. Otherwise degranulation is analysed by CD107a staining of resting NK cells (Figure 4B) to reveal the other primary HLH variants (FHL3-5).

Using a cut-off value of 5% this assay showed a sensitivity of 96% and a specificity of 88% for the detection of an inherited degranulation defect (51).

Additional flow cytometric studies have also been proven useful in the diagnostic work-up of HLH patients.

In cases of normal XIAP protein expression but high clinical suspicion for XLP2 the function of XIAP can be tested by stimulation of monocytes with muramyl dipeptides (L18-MDP) and analysis of TNFa expression by flow cytometry (55).

CONCLUSIONS

Flow cytometry is a highly valuable and versatile applicable diagnostic tool in the diagnostics and study of primary immunodeficiencies.

It has been contributing vitally to our understanding of the pathophysiology of these disorders and these findings have been translated into clinical diagnostic testing at a fast pace.

Given the still growing diversity of known PIDs on the one hand and the rarity of each of these disorders on the other, flow cytometry still proves to be one method of choice as it can be easily adapted to detect novel cellular pathologies in immune cells with manageable effort and costs.

Nevertheless, the diagnostic delay is still a major clinical problem in PIDs that needs to be addressed

Figure 4A **Analysis of intracellular XIAP expression in a patient with suspected X-linked lymphoproliferative syndrome showing severely reduced XIAP expression in NK and T-cells of the patient as compared to control (middle and right panels)**

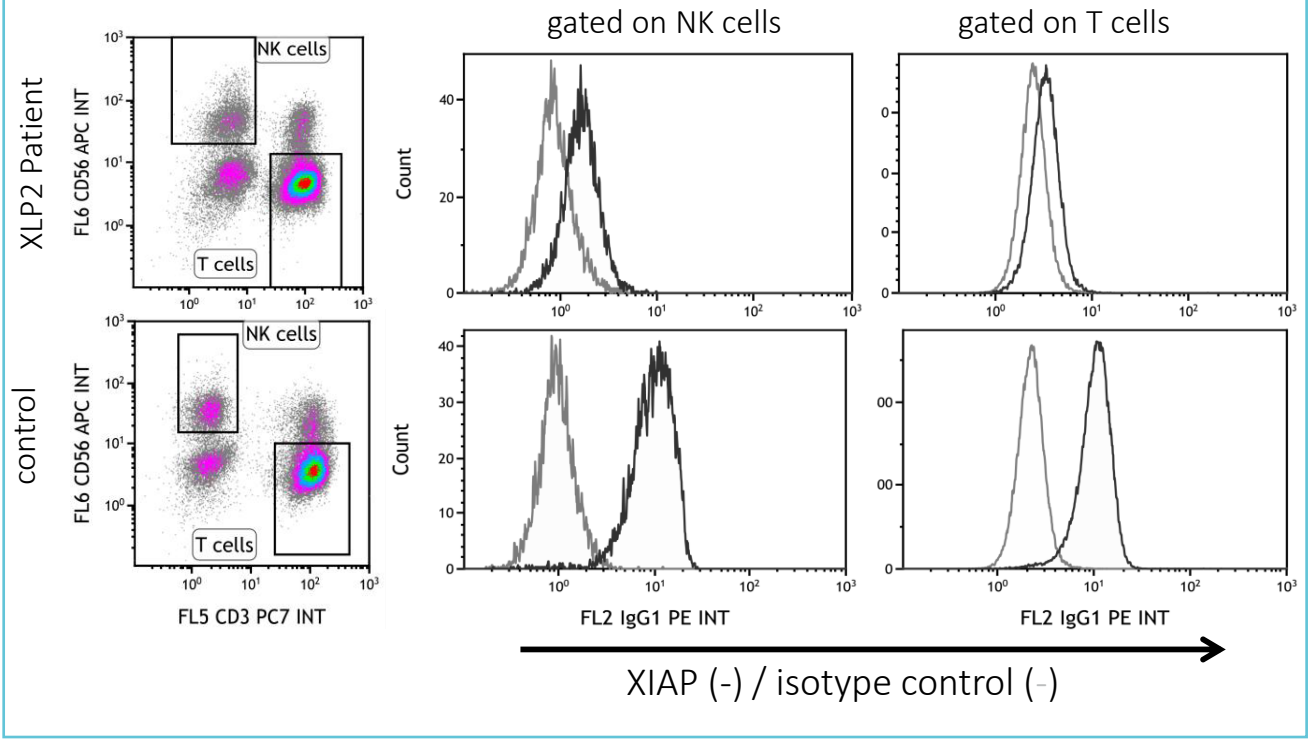
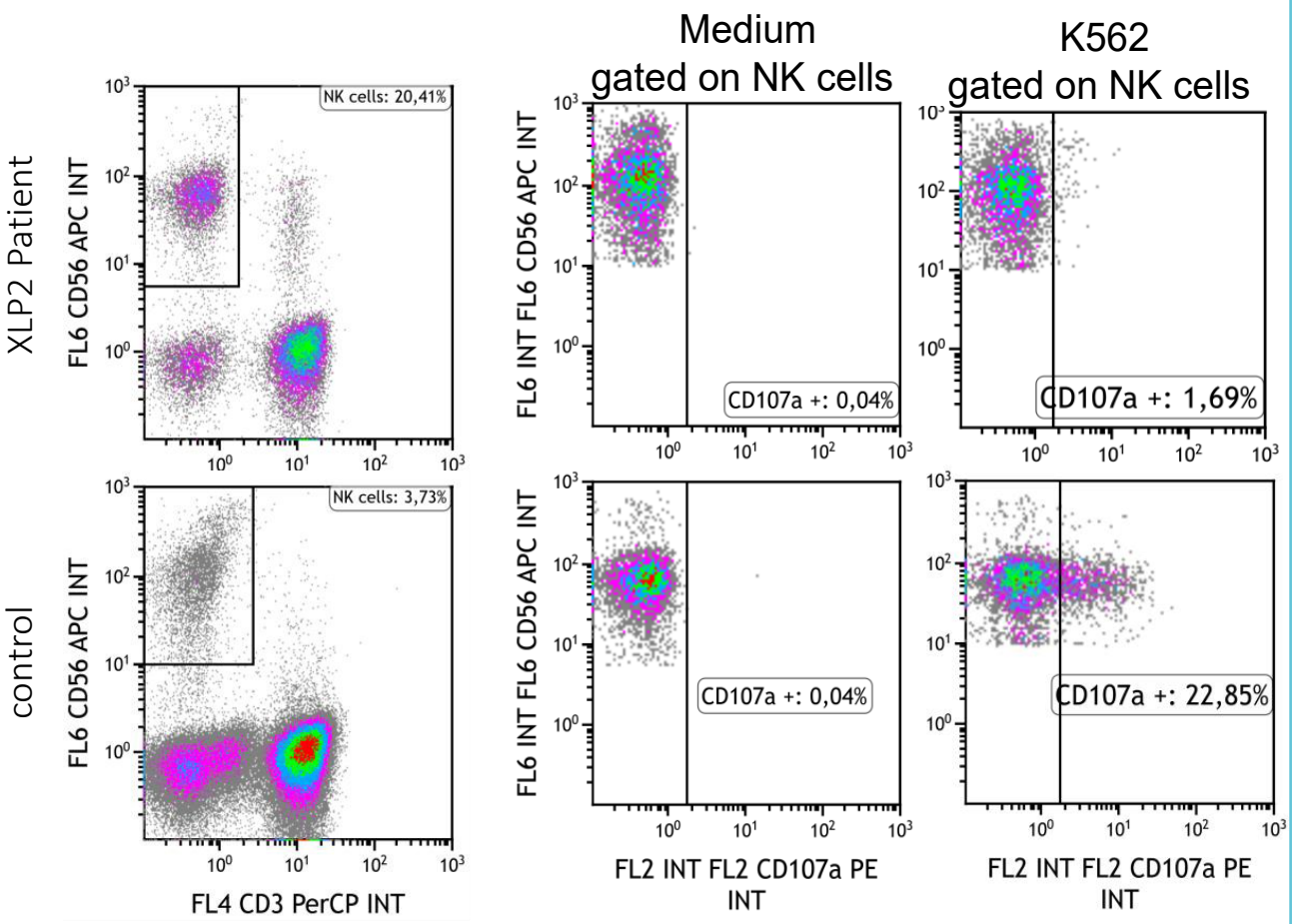


Figure 4B **Analysis of spontaneous degranulation of resting NK cells upon exposure to K562 target cells by staining for the cytotoxic granule associated protein CD107a in a patient with suspected FHL and a control showing impaired CD107a surface expression in the patient, indicating a defect in degranulation (right upper panel)**



by raising awareness and improvement of the flow cytometric diagnostic machinery.

❖❖❖❖❖

Compliance with ethical standards

Informed consent was obtained from all patients or their legal guardians and the study was done in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

Authors' disclosures

All authors declare they have no conflict of interest related to this manuscript.

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In this issue: Clinical flow cytometry in 2019

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Past, present and future of flow cytometry in breast cancer – a systematic review

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flow cytometry, breast cancer, aneuploidy

ABSTRACT

Breast cancer is the most common malignancy in women worldwide. In this systematic review 28 studies were taken into account, in order to evaluate the role of DNA content and cell cycle phases, measured by flow cytometry in breast cancer. Presence of aneuploidy and S-phase fraction have been extensively studied as a prognostication tool. With the current dawn of the age of intraoperative flow cytometry the present systematic review provide an insight of the current role of flow cytometry in breast cancer and future horizons.

INTRODUCTION

Breast cancer is the most common type of cancer among women according to the World Health Organization (WHO) and affects about 2.1 million women each year [1]. Early detection and screening is of key importance, in order to improve breast cancer outcomes and survival [1,2]. Breast cancer is divided into several subtypes and can either be invasive or non-invasive [Table 1]. In breast cancer diagnosis the next important step is staging for treatment options and prognostic information. In the present study, we performed a systematic review on the value of flow cytometry, presence of aneuploidy and cell cycle fractions, in breast cancer.

FLOW CYTOMETRY

Flow cytometry provides simple, fast and accurate data collection, from a heterogeneous fluid mixture that contains cells or cell particles. Quantification of nuclear DNA content by flow cytometry provides information on ploidy status, DNA Index and % S phase fraction [3,4]. Fresh cells, frozen specimens, ethanol- or formalin-fixed cells, and formalin-fixed, paraffin-embedded tissues can all be examined for these variables [5-7]. Assessment of S-phase fraction has been proved to be a very useful tool for defying high-risk groups of patients in breast cancer [8]. According to another study, in which they focused on the relationship between Chromosomal Instability (CIN) and DNA ploidy in 46 patients with invasive breast carcinoma, DNA ploidy is likely to be determined during the early stages of carcinogenesis [9]. CIN is among the main reason of aneuploidy, an abnormal chromosome number in cancer cells [6]. Generally, the aneuploid chromosome set differs from wild type by only one or a small number of chromosomes [10]. Aneuploidy has been suggested as a cause more than a century and is characterized as the main driver of cancer progression [6]. Flow cytometry can readily identify DNA ploidy. Aneuploidy has been associated with poor prognosis [11]. Fernö et al. proposed to categorize the ploidy of breast cancer cell populations based on DNA Index (DI) distribution as hypodiploid (DI < 0.95), diploid (DI = 0.95−1.04), near-hyperdiploid (DI = 1.05−1.14), hyperdiploid (DI = 1.15−1.91), tetraploid (DI = 1.92−2.04), hypertetraploid (DI ≥ 2.05), and multiploid [2].

Table 1 Types of breast cancer

Noninvasive	Invasive
Ductal carcinoma in situ (DCIS)	Invasive Ductal carcinoma (IDC)
Lobular carcinoma in situ (LCIS)	Invasive Lobular carcinoma (ILC)
	Medullary carcinoma
	Mucinous carcinoma
	Tubular carcinoma
	Papillary carcinoma
	Inflammatory breast cancer

S-phase fraction (SPF) when combined with mitotic activity, had the same prognostic impact as the lymph node status in breat cancer [12]. This type of cancer is heterogenous and clinicopathological features which are currently used for prognostication purpose may fail to predicting the behavior of the tumor in each individual case [13,14]. Thus, investigation for novel prognostic markers is of paramount importance. Current prognostic factors are age, tumor size, histological grade, histopathological type, lymph node status, and mitotic index [12]. Also, the status of estrogen (ER) and progesterone (PgR) receptors, epidermal growth factor receptor status, c-erbB-2 oncogene expression, expression of Ki-67 and other predictors of disease progression have been included in the list [15].The status of axillary lymph nodes is generally recognized as the most powerful prognostic factor in invasive breast carcinoma [16-20]. The presence of internal mammary node metastases also appears to be of great importance in forming the prognosis [16]. In the present study, we set out to investigate the role of flow cytometry in breast cancer.

MATERIAL AND METHODS

The present systematic review has adopted the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [21]. Eligible studies that provided data on Flow cytometry and breast cancer were identified by searching MEDLINE. The following combination of search strings was used in the database search: ''breast cancer'', ''flow cytometry'' and ''DNA content''. No language or other restrictions were imposed. Last literature search was conducted on 1/3/19. Reference lists of all articles that met the inclusion criteria and of relevant review articles were examined to identify studies that may have been missed by the initial database search. All retrieved studies and reference lists were scanned independently by two reviewers (GA and MM).

RESULTS

MEDLINE database search yielded 837 studies. After excluding 2 duplicate studies, the remaining studies were screened for eligibility criteria. After retrieving the full-text version of 50 potentially eligible studies, 22 studies were excluded for not providing data on either diagnostic value or tumor grading. Twenty-eight studies were included [see Table 2 at the end of articles].

The prognostic role of DNA aneuploidy in breast cancer

In favor

In a multivariate analysis, DNA ploidy was significantly associated with long term survival, with 58% of 393 patients having aneuploid tumors. Interestingly in a subgroup of patients with grade 2 tumors (n=195), aneuploidy (n=111) as compared to diploidy (n=84), was an indicator of worse prognosis for Disease-Free Survival (DFS, p=0.011) and Disease-Specific Survival (DSS, p=0.045) [22]. In a study of 584 patients, patients with hypoploid tumors (5.5%), had a 23±8% survival rate at 5 years and no patients were alive at 10 years, compared to the group of patients with diploid and near-diploid tumors with 98±1% survival rate at 5 years and 98±1% after 10 years of follow up. This suggests that DNA hypoploidy (DI<0.95) was a strong, independent prognostic factor of worse survival in short-term clinical outcome in multivariate analysis [8]. Another study, compared a Population Screening (PS) group of 70 patients with Invasive Ductal Carcinoma (IDC) with a 33.3% rate of aneuploidy, to a Hospital group of 225 patients diagnosed at the same period. DNA ploidy was found significant for prognosis in both groups (p=0.016, p=0.015 respectively). The DNA index added prognostic value to Mitotic Activity

Index (MAI) for small tumors and tumors with small nuclei, because a diploid pattern in these cases was correlated with a 95% 10-year survival rate [23]. The prognostic significance of FC DNA analysis in node-negative breast cancer patients was the main interest in another study. Among 155 patients, 41% had aneuploid tumors, and had significantly shorter relapse-free (p=0.0001), as 36% of those relapsed, and shorter overall survival (OS) (p=0.0001), than those with diploid tumors. Crude survival was also significantly lower for the group of patients with aneuploid tumors (p<0.03). Of those with IDC (74%), 41% had aneuploid tumors. These patients with aneuploid tumors and IDC had significantly shorter OS than patients with IDC but diploid tumors, (p<0.002). The multivariate analysis showed ploidy status to be the only significant variable in predicting relapse-free survival (p=0.02), and also the most significant factor in predicting overall survival, (p=0.02) [18].

As for survival, it was found in another study with 565 primary breast cancers from patients treated in the period 1975-1984, that OS was lower for the group of patients with aneuploid tumors (p=0.4). When tumors with a low portion of aneuploidy (DI<1.4) and diploid tumors were combined into one group, the difference in OS and Distant Relapse Free Survival (DRFS) between them and the remaining aneuploid group was increased (p=0.006, p=0.003). In multivariate analysis aneuploidy correlated significantly only with OS (p=0.02) [19]. At the end of a study 42% of patients with diploid tumors had distant metastasis compared to 72% of the aneuploid ones, with a high statistical significance (p<0.001). Also, one-third of the patients with diploid cancers died of the disease, compared to two-thirds of the patients with aneuploid cancers (p<0.001).

With a follow up of 11.5 years, the DNA aneuploidy of the tumor showed a significant association with decreased survival, as 65% of patients with aneuploid tumors had died from breast cancer during the follow-up, in comparison with 33% of those with diploid tumors, (p<0.00) [24]. FC provides additional indirect information on aggressiveness associated with DNA ploidy. Aneuploid tumors in this study had a rate of 47%. This study suggests that tumors with a Ki-67 labeling index of 50% or above are highly proliferative or aneuploid, which means they carry a bad prognosis. Those with lower values require investigation, since aneuploid tumors with a low SPF may also have low Ki-67 indexes. This suggests that the Ki-67 labeling index just reflects the proportion of cells in S-phase, whereas DNA aneuploidy reflects something else in addition, closely associated with a bad prognosis [7].

Aneuploidy was significantly associated with tumors of lacking hormone receptor activity (estrogen receptors and progesterone receptors) [22]. In a study of 807 patients by Stahl et al., 73% of the non-diploid tumors (60%) were ER-positive compared to the 86% of the diploid tumors (p<0.001). DNA ploidy was also significantly correlated with tumor size in this particular study. While more than half of the tumors with a diameter <11 mm were DNA diploid, more than 70% of those larger than 20 mm were non-diploid (p<0.001) [25].

A higher rate of aneuploid tumors was also found in the ER- group, compared with that in the ER+ group, and this difference appeared to be pronounced in patients with negative lymph nodes (N0) than in those with positive lymph nodes (N+), (p<0.05). ER status had a significant effect on OS, but not on Crude Survival (CS) [19]. In another study, focused on the relationship of DNA ploidy level to histology and ER receptor among 155 patients, it was shown that tumors with lower DNA ploidy, tended to be of low grade and ER+ and exhibited a better prognosis (p=0.01). Those with higher DNA ploidy were more likely to be of higher grade, more

anaplastic and ER- [26]. No significant correlation between ploidy and receptor content was found in a study, although there was a slight tendency for diploid tumors to be receptor-positive, (69% vs. 58%) [27]. In node negative patients, aneuploidy was significantly correlated with an unfavorable prognosis for DFS [22].

Patients with a high degree of axillary node involvement (10 or more positive nodes), showed a higher incidence of aneuploidy than patients with lower or zero nodes involved ($p<0.05$). For the group of patients with 3 or more positive nodes (N+), both DRFS and OS were significantly better for patients with diploid tumors [19]. A significant association was also observed between node-negative tumors and DNA diploidy, compared with DNA aneuploidy ($p=0.003$) and between node-positive tumors and DNA hypertetraploidy ($p=0.002$) [28].

A study of 807 patients showed that an increasing number of positive lymph nodes correlated with DNA aneuploidy ($p<0.01$) [25]. Dressler et al., seem to agree, because in their study, it became clear that node-negative tumors were less likely to be aneuploid (49%) vs. node-positive tumors (57%) ($p=0.04$) [29]. Eskelinen et al. found that 50 % of patients with aneuploid tumors had lymph node involvement, compared to 33.3% of diploid ones ($p<0.05$) [24]. In a study, it was shown that aneuploidy was significantly associated with tumors of greater size ($p=0.018$) and a higher grade of differentiation ($p<0.001$) [22]. Aneuploidy which was the 56.6% of all cases in another study, was significantly associated with low-grade carcinoma ($p<0.001$). Also, there was an increasing aneuploidy rate among tumors with a short Doubling Time (DT), ($p=0.009$). Of 11 tumors growing extremely slowly (indefinite DT), 27% were aneuploid [30]. However, Ottesen et al. studied four groups of patients with a DNA aneuploidy rate of 49-90% and observed a relationship between histological grade and ploidy, as tumors with high histological grade associated with DNA diploidy ($p=0.002$) and DNA hyperdiploidy ($p=0.003$). An inverse association was found with DNA hypertetraploidy ($p<0.0001$)[28]. Keyhani-Rofagha et al. also reported that tumors with an aneuploid pattern are more frequently of high histological grade [31]. DNA ploidy measured by FC can be used to predict the aggressiveness of the tumor and patients' survival. Premenopausal patients, had about the same number of diploid and aneuploid tumors, but more than twice as many of the postmenopausal patients had aneuploid tumors than had diploid ones [24]. Ploidy was an additional, independent prognostic factor in postmenopausal patients. Aneuploidy was associated with a significantly lower OS in postmenopausal but not in premenopausal patients. In a study of 114 patients, an association was found between ploidy and age, as significant differences were noted between mean ages for tetraploid compared to all other aneuploid tumors and for multiploid compared to all other tumors. Multiploidy might associate with the menopause [27].

Against

Ploidy status was not an independent prognostic factor in a study of 1831 breast samples in the multivariate analysis, although it reached statistical significance in the univariate analysis, as patients with near-hyperdiploid and diploid tumors had a somewhat similar prognosis, which was a good one. Patients with hypodiploid tumors had a tendency toward poorer prognosis than those with tetraploid, hyperdiploid, hypertetraploid or multiploid [3]. In a study by Bergers et al., among 932 breast cancer patients, DNA ploidy correlated significantly with Mitotic Activity Index, Mean Nuclear Area, steroid receptor status and tumor type, as Medullary and Ductal tumor types were more often DNA non-diploid. No significant correlation was shown with tumor size, lymph node

status, age, and hormonal status. This study suggests that DNA ploidy and DI, as markers of genetic instability, mainly correlate with differentiation and proliferation markers but correlate less with lymph node status as a marker of metastatic potential [4]. In another study of 158 patients, 56.6% of them had aneuploid tumors. Doubling Time (DT) and DNA ploidy correlated well with each other but did not have a correlation at all with axillary node metastasis, or periglandular growth [30].

Taylor et al. showed that there was no significant correlation between ploidy and histological type, tumor size, lymph node involvement or steroid receptor status, in a study of 114 patients, with a 79% of aneuploid tumors [27]. Ploidy had no significant relationship between ER status and DNA content. Also, ploidy by itself yields no significant prognostic information regardless of age, in node-negative breast carcinoma [31].

Noguchi et al. studied the lymph node metastasis versus DNA ploidy as prognostic factors for IDC, among 121 patients, of which 60% had aneuploid tumors. They suggested that DNA ploidy was not an independent prognostic factor in small number of patients [16]. Ploidy status did not predict DFS or OS, maybe because of the small number of patients in their study. All tumors were axillary node-negative, and 56% were aneuploid [15]. DNA ploidy was also not a strong prognostic factor for survival, as there were no statistical difference in survival among breast cancer patients with diploid, or aneuploid tumors after a mean follow up of 4.1 years, in 122 patients [32].

DNA ploidy by itself was not a significant prognostic factor in another study, although all patients with multiploid and hypertetraploid tumors had a recurrence. Ploidy status was correlated significantly with tumor size, histological grade, nuclear gradeand mitotic grade [17].

The role of SPF in breast cancer, as a significant prognostic factor

In favor

In a study of 1985 patients, SPF was a prominent prognostic factor, even after multivariate analysis. SPF, when combined with mitotic activity, had the same prognostic impact as the lymph node status, as both of them correlated with every type of survival [12]. A study of 211 premenopausal node-negative breast cancer, patients found that S+G2/M phase fraction was the only predictor of OS in the univariate analysis. Patients with S+G2/M greater than 9.3% had shorter survival than patients with an S+G2/M equal or less than 9.3%, (p=0.039), suggesting that S+G2/M in premenopausal node-negative carcinoma could be an additional valuable prognostic factor to classify high-risk patients needing adjuvant chemotherapy [33]. In a study amongst 327 breast cancers, SPF had been calculated in 245 of cases in univariate analysis and ranged from 1.0% to 35% (median=5%). Cancers with SPF larger than the median (8.3%) were associated with 65% 5-year survival rate, compared with 86% in those with SPF below or equal to 5%, (p=0.0002) [34].

In a study of 393 patients with IDC, it was found that the SPF had a range of 1.0-27.8 % (median 6.9 %), and was significantly higher (p<0.001) in aneuploid (median 10.8 %; range 3.7–27.8 %) than in diploid tumors (median 4.3 %; range 1.0–12.0 %). Higher SPF values were correlated with advanced disease stage. High SPF exhibited only statistical significance for DSS, but this parameter did not reach statistical significance in the Kaplan-Meier survival, neither in the univariate Cox Analysis [22].

The fraction and percentage of SPF in another study was higher in the group with patients with hypoploid tumors (DI<0.95), which was the group characterized by the worst prognosis with no patients alive after a 10-year follow up.

SPF retained statistical significance in the univariate analysis, however not in the multivariate one [8]. Eskelinenet al. seem to agree that SPF has a prognostic value, as in their study of 117 patients, SPF values greater than 7% were associated more closely with distant metastases or death [24]. SPF correlated significantly with tumor size, histologic grade, nuclear grade, and mitotic rate. SPF was related significantly to the recurrence of disease. However, in the multivariate analysis peritumoral lymphovascular invasion was the most important variable [17]. In a study among 158 patients according to the chi-square test, there was a significant correlation between SPF and pathologic stage of the disease and SPF and tumor size. SPF higher than 7.5% was correlated weakly to axillary lymph node metastasis (p=0.046) but correlated strongly with low histologic grade (p=0.001) and short DT (p=0.02). Also, a highly significant association was observed between SPF and ploidy (p<0.001), as 23% of the tumors with SPF, less than 7.5% were aneuploid, compared to 74% of the tumors with higher proliferation rates [30].

In a study of 807 frozen breast cancer samples, SPF was the only independent factor that was significantly related to nodal status. After a multiple regression analysis it became clear that DNA ploidy, ER status, PR status, lymph node status and tumor size, were all independently related to SPF [25]. According to a study with four groups of patients by Ottesen et al., DNA aneuploid tumors had a median SPF of 11%, compared to 5% for diploid tumors. Testing for difference among DNA diploid and DNA aneuploid SPF showed a significantly higher value (p<0.0001), for the latter. Also, there was a statistical difference between DNA aneuploid SPF in small clinical cancers and DNA aneuploid SPF in screening cancers, 10% and 4% respectively, (p<0.0001) [28]. Significantly lower SPF values in diploid tumors (median=2.6%), as compared to aneuploid tumors (median=10.3%, p<0.0001) were also observed in another study. Receptor-negative tumors had the highest median SPF value (median=12.7%), and receptor-positive tumors had the lowest median SPF value (median=4.6%, p<0.0001). Tumors with ER+/PgR- had intermediate values. When they examined further the relationship between SPF values and receptor status in the two ploidy groups separately, it was again clear that receptor-negative tumors had more often high SPF values, and that the difference was especially in the aneuploid group. Significantly higher SPF values were observed in younger and premenopausal patients, and when these groups were divided by ploidy status, greater SPF differences were found within the aneuploid tumors. When patients were examined by nodal status, node-positive patients with diploid tumors were more likely to have a high SPF tumor, than node-negative patients with diploid tumors, whereas in aneuploid tumors, high SPF was frequent independent of nodal status.

In node-negative patients exclusively, among diploid tumors, there was no a difference in the SPF values in ER-compared to ER+ tumors, but a highly significant difference in aneuploid tumors. For PgR they observed that in both diploid and aneuploid tumors, PgR- tumors were more likely to have a high SPF value. When the node-negative patients were subgrouped according to age or menopausal status, it was only within the aneuploid group that a significantly higher frequency of high S-phase was found in the younger or premenopausal patients[29]. Association of a low (< l0%) S phase with 81% of all diploid and near-diploid tumors compared to only 22% of single aneuploid and tetraploid tumors were highly significant in another study [27].

In a study of 50 patients, patients with grade 3 tumors had significantly higher SPF results in comparison to patients with grade 1 or grade 2 tumors. Also, patients with grade 3 tumors

with a high SPF (equal or less than 15%) were almost more likely to relapse compared to the rest of the group [15]. In a study of 1831 samples of breast cancer, SPF values showed a significant positive correlation with the number of lymph nodes involved ($p<0.001$), tumors size (<0.001), DNA ploidy ($p<0.001$), cathepsin D content (p=00.03), erbB2 ($p<0.001$) and c-myc amplification (p=0.004). SPF also had a significant negative association with age ($p<0.001$), ER- ($p<0.001$) and PgR content ($p<0.001$). SPF values had no significant correlation with int2 amplification, however when diploid and near-hyperdiploid samples were examined on their own, a significant positive correlation was found (p=0.05). SPF greater than 12% was associated with the lowest rate of recurrence-free survival. SPF remained an independent prognostic factor even in the multivariate analysis [3].

Against

SPF of the tumor was not a significant prognostic factor because it didn't associate with survival in a study among 122 patients. However, the follow-up time was limited in this study [32]. In a study of 58 patients with invasive breast cancer, no significant correlation was found between the number of stemlines and intra-tumor variability and SPF [35]. In a study of 106 women who underwent treatment for invasive breast cancer, neither SPF nor DNA index proved to be statistically significant in determining axillary node status. Also, neither SPF nor the DNA index could predict the presence of distant metastasis [36].

Of all the studies that have been taken into account, in 13 of them fresh/frozen samples were used in order to determine the ploidy and SPF fraction of the tumors. Also, paraffin-embedded tissues were used in 11 of the studies, whereas 4 of the total studies used both fresh/frozen and paraffin-embedded specimens and in 2 of the total studies the sample's kind was not mentioned. According to a study by Bergers et al. measurement of DNA ploidy, DNA Index and SPF may be more reliable in paraffin wax sections because the thick slices of specimens provide a more representative sample [35]. Although S-phase measurements were not obtainable in a number of tumors ranging from around 5% from fresh specimens to up to 25-40% in the case of paraffin-embedded ones [37-40]. In a study by Alanen et al., it is concluded that all three types of samples (fresh, ethanol-preserved, formalin-fixed and paraffin-embedded samples) are suitable for the determination of DNA ploidy, DI, and S-phase fraction, although uninterpretable histograms were most often obtained from fresh samples [41]. According to another study by Chen et al., there was 89% agreement in the detection of DNA aneuploidy by flow cytometry in fresh and paraffin-embedded, formalin-fixed tissue; the coefficients of variation of the DNA diploid G**0**/G1 peaks were much wider in the latter [42].

Intraoperative flow cytometry

During the last few years two research groups, one from Tokyo, Japan and the other from Ioannina, Greece working independently have investigated the possible role of flow cytometry for intraoperative usage in brain tumor surgery [43-45]. Shioyama et al. developed a flow cytometry protocol that could evaluate the tumor DNA content within 10 minutes. The authors calculated the malignant index (MI) of the analyzed cells and used thereafter in all analyses [45]. Researchers from Ioannina, developed a quite similar protocol for rapid cell cycle analysis, named Ioannina Protocol. Based on cell cycle fractions, namely G0/G1, S and G2/M phase fraction, brain tumors could be categorized intraoperatively in low and high-grade both in adults and children, glioma margins could be identified and primary central nervous system lymphoma could be identified within 5 minutes

[43,44,46,47]. Cell cycle analysis by propidium-iodine staining of CD56+ (gated) cells could assess the malignacy of pediatric brain tumors [48]. Furthermore, quantification of CD56 expression in pediatric brain tumorscan be an indicator of tumor's grade and aggressiveness [49]. In patients with head and neck lesions intraoperative flow cytometry allowed the identification of neoplastic lesions within 6 minutes with high sensitivity and specificity and when surgical margins were assessed a complete concordance with pathology was reported [50]. Promising results have been reported for other solid masses as breast cancer [51]. Intraoperative flow cytometry provides new horizons during surgical resection of solid tumors in general and could be a novel adjuct to pathology.

CONCLUSION

Breast cancer is the most common cancer and also the primary cause of mortality due to cancer in female around the world. A major part of the literature has been dedicated to defining the long-term outcome of patients, suffering from this type of carcinoma.

Flow cytometry analysis of the DNA pattern of the carcinoma does correlate with well established prognostic factors and has a lot to offer in shaping the prognosis of patients, according to the literature. Flow cytometry analysis provides information as regards to the ploidy of cancer and the percentage of cells in the S-phase, with the last one being a hallmark of cancer. According to many studies, aneuploidy appears to be in a significant relationship with long-term prognosis. Also, aneuploidy correlated significantly with the presence of distant metastases and decreased survival. Intraoperative flow cytometry is a promising novel application and is expected to have a significant impact in breast cancer surgery.

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Abbreviations

N/A: *not available*

S/A: *significantly associated*

A: *associated*

S/R: *significantly related*

N/S: *not significant*

IDC: *invasive or infiltrating ductal carcinoma*

DCIS: *ductal carcinoma in situ*

ILC: *invasive or infiltrating lobular carcinoma*

IMC: *invasive medullary carcinoma*

DFS: *disease-free survival*

DSS: *disease-specific survival*

RFS: *recurrence-free survival*

OS: *overall survival*

CIN: *chromosomal instability*

DNAs: *DNA copy number aberrations*

PF: *prognostic factor*

HSPF: *highly significant prognostic factor*

E/P REC: *estrogen/progesterone receptors*

UP: *unfavorable prognosis*

MAI: *mitotic activity index*

MNA: *mean nuclear area*

IDH: *intratumoral DNA heterogeneity*

AXM: *axillary lymph-node metastases*

IMM: *internal mammary lymph node metastases*

RR: *recurrence rate*

DM: *distant metastases*

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Table 2 **Studies included**

Study	No of patients	Mean age	Cancer type	Study variable	DNA aneuploidy	SPF	Cut off	Sample type	Prognostic value
Pinto et al (2012)	393	59 (23-88)	IDC	DNA ploidy, SPF	58.8 % (43.0 % hyperdip, 6.1 % tetra, 5.1% hypertetra and 4.6 % multi)	median 6.9 % (1-27.8%)	6.1%	Fresh/ Frozen	**DNA ploidy is an independent PF in breast IDC**
Kawauchi et al (2010)	46	57.6	Invasive breast cancer, sporadic tumors	DNA ploidy (CIN), DCNAs	67.4% 69.4% (of 36) were CIN+, and 30.6% were CIN-. 23 Aneu/CIN, and 1 Aneu/CIN-.	N/A	N/A	Frozen	**DNA ploidy was likely to determine the beginning of carcinogenesis**
Chavez-Uribe et al (2007)	584	59 (25-85)	88.1% Ductal type, 9% Lobular type, 2.8% Medullary type, 1% other	DNA ploidy, SPF	GROUPI: (diploid+ near-diploid) with DI =0.96-1.15, GROUPII: (22.9% hyperploid, 8.2% multiploid, 9.4% diploid populations), DI >1.16, GROUPIII: 5,5% hypoploid with DI<0.95.	GROUPI =5.5% GROUPII =8.5%, GROUPIII =10.1%	N/A	Frozen	**DNA hypoploidy (DI<0.95) is an independent PF in long-term prognosis**.
Michels et al (2003)	1984	58	81% IDC, 16% ILC, 3% miscellaneous tumors. 50% G1, 50% G2 80% SR+	DNA ploidy, SPF	50% 10.8% multiploid. (aneuploid: 1.8% hypoploid and 5.8% tetraploid), 10.8%	33% <3% 70.3% <4%	Med CV =3.5%.	Frozen	**SPF is a HSPF**
Martinez-Aribas et al (2002)	181	N/A	152 IDC, 17 ILC, 12 other	DNA ploidy, SPF, Ki67	47%, (DI>1)		Aneuploid +15.8% diploid =9.9%	Fresh and paraffin	**FC provides additional indirect info on aggressiveness ass with DNA ploidy.**
Schmidt et al (1998)	106	57	IDC majority, 6 ILC, 1 inflam., 1 colloid type. 3 in situ exclude	DNA ploidy, SPF, axillary-node status	56% 56% node-negative (66% of those had elevated SPF) 0.01% G1, 40.2% G2, 59.8% G3	High SPF in 72%	SPF =9% Mean SPF =14.1%	NA	**DI is a poor PF** **SPF N/S in AX node status or DM**

Wong et al (1998)	50-46 suitable	50	92% IDC, 2% ILC, 2% tubular, 4% medullary, 33% G1, 22% G2, 39% G3, missing data for 6%	DNA ploidy, SPF, in node-negative	56%	SPF range 0-30%	SPF High >15%	Fresh Frozen	**Ploidy N/S with DFS or OS.** **SPF S/A with relapse**
Wyss-Desserich et al (1997)	57	45	39 DC, 12 LC, 6 other	DNA ploidy, DI, SPF, S+(G2+M)-phase fraction	60%		DI =1.2 SPF =3% S+G2+M-PF =9.3%	Paraffin	**S+(G2+M)PF in premenopausal node(-) is an additional PF**
Bergers et al (1996)	932	60	86% DC, 9% LC, 5% other	DNA ploidy, DI, SPF	62%		SPF =8%, first D =1.8 second DI =2.3	Frozen	**DI and DNA ploidy N/S**
Bergers, Diest, Baak et al (1996)	58	N/A	Invasive breast cancer	DNA ploidy, DI, SPF	Intra-tumor heterogeneity in 53% of frozen and 38% of paraffin cases.	Fresh: range = 9.5-31.6 and 4.5-67.3% (paraffin range = 0-62.7%.	N/A	Fresh frozen and paraffin	**N/S**
Ottesen et al (1995)	148, in 4 groups	53 (38-76) 58 (39-85) 62 (39-88) 61 (50-70)	33 IC/ predominance of DCIS, 52 Clinical IC<15mm, 40 Node-negative IC, 41 Screening IC <15 mm	DNA ploidy, DI, IDH, SPF	74% (18 Hypo, 61 Hyper, 13 Triplo, 52 Hypotetra, 8 Tetra, 14 Hypertetra), 3% tetraploid	ANEUPLOID Med SPF =11% (2-31%)	N/A	Frozen	**SPF A with Ploidy.** **Ploidy S/A with grade and node status.**
Brotheric et al (1995)	110	59+-0,86	8 DCIS, 37 GII, 35 GII, 29 GIII	c-erbB-2 by ΦΨ+ immuno-chemistry. Relationship c-erbB-2 and ploidy		N/A	+threshold for (FC) c-erbB2 expression =3300-3600 molecules /cell	Frozen	**C-erbB2 N/S if assayed by FC.**
Ottesen, Chris-tensen et al (1995)	48	/	IC with DCIS. 15 only DCIS, 17 only IC, 16 cases separate samples from DCIS and IC available.	DNA ploidy, cancer type corr in each case	<u>31 cases with DCIS:</u> 10% tetra, 67% aneuploid (1 Hypo, 5 Hyper, 13 Hypotetra, 7Tetra, 5 Hypertetra) <u>33 cases with IC</u>: 6% 82% aneuploid (1 Hypo, 18 Hyper, 2 Triplo, 14 Hyportetra, 3 Tetra, 4 Hypertetra).	Med SPF for DCIS = 8% (2-38%) AND Med IC=11% (2-28%) Aneuploid SPF= 13% (3-38%).	N/A	Frozen	**DNA ploidy pattern, as detected by FC is established at the preinvasive stage of carcinogenesis.**

Stal et al (1992)	807	50 (40-74)	N/A	DNA ploidy, SPF, nodal status, ER status	60%, 73% receptor negative	mean =8.4% (1-36%)	SPF =5% and 10%	Frozen	**SPF is an independent PF**
Joensuu et al (1992)	327	62.2	Unilateral in-vasive	DNA ploidy, SPF	33-49% 8-21% tetraploid, 2-6% multiploid	8.3% (range 1-35%)	SPF =5%	Paraffin	**DNA Ploidy and SPF S/A with survival**.
Arnernlov et al (1992)	158	65 (42-87)	125 DC, 6 LC, 6 Papillary, 3 Medullary, 12 Mucinous, 7 Tubular	DT, DNA ploidy, SPF	56.6% 6.7% tetraploid	mean SPF =10.7%, med =8.2% 43% patients had <7.5%, and 57% had >7.5%	SPF =7.5% DT = 9 months	Paraffin	**Ploidy SA with grade, size and DT, not with AXM.** **SPF SA stage, size, grade, and ploidy.**.
Ferno et al (1992)	1831	61+-14	N/A	DNA ploidy, DI	60% (1.8% Hypo, 4.4% near-hyper, 35.8% hyper, 4.9% tetra, 7% hypertetra, 5.7% multi)	3 groups by SPF values (<7.0%; 7.0--11.9%, >12%)	N/A	N/A	**SPF S/A with lymph node metastases, age, size, ploidy, E/R REC status,** **SPF and ploidy N/S in RFS in multivariate analysis**.
Bosari et al (1992)	158	N/A	Axillary node-negative breast carcinoma	DNA ploidy, SPF	33% 19% tetra	SPF in 136: mean =7.3. Diploid: 4,6 aneuploid: 11, tetra: 8.1.	SPF =7%	Paraffin	**Ploidy and SPF N/S alone.** **SPF S/R to recurrence.**
Collan et al (1992)	116	N/A	N/A	DNA ploidy, DI, SPF	Lab1: 55% Lab2: 62% Lab3: 54%		DI =1.3 Aneu: Lab+Lab3 =>1G0/ G1 peak, Lab2 =DI>1.0	Paraffin	**DI = more reproducible variable than ploidy**.
Noguchi et al (1991)	121	50.5	Invasive ductal carcinoma, 28 Stage I, 63 Stage II, 30 Stage III	DNA ploidy, regional lymph node metastasis	60% aneuploid	N/A	DI =1.0	Paraffin	**DNA poidy N/S in small series.**
Uyterlinde et al (1991)	PS=70, H=225	50	Invasive ductal	DI, MAI, MPI, MNA,	33.3% 23.3% tetra	N/A		Paraffin	**<u>PS group</u>: DI had additional prognostic value to MAI** . **DNA ploidy S/A both in the H and PS group**.
Keyhani-Rofagha et al (1990)	165	58 (27-81)	150 IDC , 6 LC, 2 MC, 6 colloid, 1tubular	DNA ploidy	57% aneuploid, Mean DI =1.3 (0.73-2.59)	N/A	N/A	Paraffin	**Ploidy alone N/S in node-negative carcinoma.**

Lewis et al (1990)	155	50	74% IDC, 11 LIC, 7 intraductal, 6 papillary, 9 MC, 7 mucinous	DNA ploidy, DI	41% aneuploid. 45% of IDC were aneuploid, 78% of medullary were aneuploid.	N/A	DI =1.0	Paraffin	**Aneu SA with gradeand size.** **Ploidy N/S with age.** **Ploidy SA with relapse and survival.** **DNA ploidy by FC is an powerful PF in node negative patients.**
EskeliN/ Nordling et al (1989)	122	NA	92.% IDC	DNA ploidy, DI, SPF	55% (32% Hyperdip, 20% near-tetra, 3% Hyper-tetra), 10% Multi	Higher in aneuploid than in diploid	DI =1.0-1.049 diploid SPF cut off =8.5%	paraffin	**Ploidy N/S in survival.** **SPF N/S in survival.**
Eskeli N/ Pajarinen et al (1989)	119, 2 excluded	55.7 in diploid, 60.3 in aneupl	N/A	DNA ploidy, DI, SPF	45% (27% Hyperdipl, 14% near-tetra, 4% Hyper-tetra), 17% multiploid	In 54 cases, Sing higher in aneu than diploid	DI=1.0-1.049 diploid SPF cut off =4.8% AND 7%	Paraffin	**Ploidy S/A with metastasis and survival.** **SPF S/A with distant metastasis and size.** **Ploidy can predict aggressiveness and survival.**
Dressler et al (1988)	1331	50	N/A	DNA ploidy, DI, SPF	57% [55% Hyperdipl, 3.7% Hypodipl, 25% tetra, 7.4% Hypertetra, 8.8% muti]	Med SPF in aneu =10.3%, med SPF in dipl =2.6% (p<0.0001)	DI =1.0 diploid SPF med =5.8%	Frozen	**Ploidy and SPF S/A with receptor and nodal status.** **SPF is an important PF.**
Cornelisse et al (1986)	565	57.5 +-14.5	Primary	DNA ploidy, DI	61.6% 9.7% multiploid, 118 p. stage I, 301 p. stage II, 119 p. stage III	N/A		Frozen and Paraffin	**Ploidy is an additional PF in postemenopausal patients.**
Taylor et al (1983)	114	N/A	103 IDC, 18 LC, 2 Papillary, 2 MC, 1 Colloid, 1 Pagets disease +intraductal, 1infiltr mucoid	DNA ploidy, SPF	79% (12% near diploid 42% single aneuploid, 9% tetraploid, 16% multiploid)		SPF =10%	Frozen	**N/S between ploidy and histologic type, tumor size, lymph node involvement or receptor status.** **Ploidy S/A with SPF.** **Ploidy A with age.**
Olszewski et al (1981)	92	NA	75 DC, 6 MC, 5 LC, 2 colloid, 2 Tubular, 2 papillary	DNA ploidy, ER and PgR status	92%	N/A	N/A	Fresh	**Ploidy S/A with grade and ER status.**

In this issue: Clinical flow cytometry in 2019

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Summer school in flow cytometry for immunology: report from a successful ESCCA experience

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ABSTRACT

INTRODUCTION

Immunology is an important scientific discipline in constant development. It has evolved in one of the most important health-related biological sciences and it contributes to a lot of medical specialities. Its study is central to the development of many biological treatments and it constitutes an integral part of personalized medicine.

Cytometry in general, and flow cytometry in particular, plays a central and absolutely fundamental role in either clinical or research-oriented immunology labs. In these last decades, flow cytometry is constantly evolving and offers numerous opportunities to scientist trying to decipher the immunological status of patients or their response to treatments.

Among the goals of the European Society for Clinical Cell Analysis (ESCCA) is the dissemination of education regarding the applications of cytometry. Summer Schools in flow cytometry applications for immunological investigation may represent exceptionally effective educational tools for students and professionals working in cytometry labs in Europe and throughout the world. The educational program of the schools is focused on both cytometric and immunological issues and most importantly on their combination. The environment of the Summer Schools is also crucially important in offering, during the course, the opportunity of friendly interaction between teachers (educators) and students. Greek islands represent the optimal location for such a school. A cool shady room where knowledge is disseminated, followed by exposure to the sun, sea, and good food all together create "The School", as imagined by the ancient Greek philosophers.

The 1st ESCCA Summer School in Flow Cytometry for Immunology, organized by Katherina Psarra and Silvia Della Bella took place in the Greek island of Kos on June 19th-23rd, 2019.

EDUCATIONAL CURRICULUM

A good knowledge of all the innate and adaptive immune cell types is very important. Therefore, immunophenotyping in immunology regarding all cell types, including T and B lymphocytes, NK cells, innate lymphoid cells, dendritic and other myeloid cells, classical and myeloid derived suppressor cells, were thoroughly covered. Typical changes occurring in immunopathologic condition, as in primary immune deficiencies, were demonstrated. Functional assays aimed at assessing essential cell functions, including cell proliferation, apoptosis, cytokine production, cytotoxicity, degranulation, phagocytosis and killing, were also explained and illustrated. An overview of the educational program of the 1st ESCCA Summer School in Flow Cytometry for Immunology is summarized in Table 1, and briefly reported hereafter.

IMMUNOPHENOTYPING IN IMMUNOLOGY

T cells

Immune system is built up in order to distinguish self from non-self, to protect the organism from pathogenic or non-pathogenic elements, which are recognized as foreign and destroyed after having been sensitized (memory), and finally to keep tolerance towards specific autoantigens.

T cells are the key components of the adaptive immune system and mediate what is otherwise known as cellular immunity. Therefore the aim of this educational topic was to understand the main steps of maturation and differentiation of the protagonist, the T cell.

During development, T cell progenitors migrate from the bone marrow to the thymus, where they expand under the influence of IL-7 and begin to express the T cell receptor (TCR). At the stage of full expression of the TCR, the majority of T cells (90%) carry the αβ receptor type on their surface. A small percentage however, <10% carry the γδ receptor type. Studies of the T lymphocyte diversity in immunodeficiencies and in diseases with a pathological immune background offer a better understanding as well a diagnostic tool in immunology. Unlike αβ T cells, γδ T cells display a restricted TCR repertoire. They are located in peripheral blood (PB), intestine, skin, spleen, and lymph nodes where they act as a link between innate and adaptive immunity because they lack precise major histocompatibility complex (MHC) restriction. The γδ TCR recognize non-peptide antigens and they provide a wide range of defense mechanisms against microorganisms.

Further down, trained and differentiated T cells are positively or negatively selected to express either the CD4 or CD8 coreceptor, in addition to

Table 1 Educational program of the ESCCA Summer School in Flow Cytometry for Immunology (June 2019 – Kos Island, Greece)

Immunophenotyping in immunology	T cells
	B cells
	dendritic cells
	NK cells
	innate lymphoid cells
	suppressor cells
Functional assays by flow cytometry	cell proliferation
	cell apoptosis
	cytokine production
	phagocytosis
	oxidative burst
	autophagy
	cytotoxicity
	basophil degradation
	flow x-match
Flow cytometry in health and disease	flow cytometry in primary immunodeficiencies
	BAL immunophenotyping
	circulating tumor cells
	immunomonitoring during treatment with biological drugs

their TCR. The inability to express antigen receptors at any stage leads to apoptosis. Although, the mature T cell pool is commonly recognized on expression of either CD4 or CD8, rare populations of double positive or double negative T cells can be found.

Coming into contact with antigens triggers their differentiation into effector and memory cells. NaïveCD3+CD45RA+CD45RO-CCR7+CD62L+,central memory CD45RA-CD45RO+CCR7+CD62L+, effector memory CD45RA-CD45RO+CCR7-CD62L- and effector T cells CD45RA+CD45RO-CCR7-CD62L-.

Furthermore, distinct populations of CD4+ and CD8+ can be identified based on the type of cytokines that they secrete. During the presentation, the processes and pathways involved in the development of T cell were analysed. The specific receptor-ligand pair interactions which mediate the homing, proliferation, survival, and differentiation were explained, showing flow cytometric data and possible pitfalls during flow cytometry analysis. [1, 2, 3]

B cells

Basic B cell immunophenotyping was covered in a 2 hour lecture, in which we tried to unravel the mysteries of B cell development in the bone marrow and of antigen specific B cell maturation in the spleen and secondary lymphoid organs. Instead of listing the expression patterns of the basic B cell markers, we tried to explain the currently perceived role of each molecule in B cell physiology and to describe its fluctuations throughout the B cell immune response [4]. This way, we provided the participants the information that would give them insight to the function (or the history) of the cells that express each specific marker, rather than strictly define the B cell subset classification, given that the latter is actually a human intervention in a yet to be fully discovered world. However, as practice requires precision, we showed the basic gating strategies for the study of B cells by flow cytometry and referred to common pitfalls and discrepancies in their enumeration [5]. We also mentioned the basic concepts, techniques and some applications of the study of antigen specific B cells [6]. Finally, in order to link the B cell study to clinical practice and to underscore the importance of certain molecules in B cell development, we showed different abnormal B cell patterns in specific defects causing primary immunodeficiencies [7, 8].

Dendritic cells (DCs)

DCs are ubiquitous professional antigen-presenting cells that play a crucial role in initiating and shaping immune responses. The effects of DCs on adaptive immune responses depend partly on functional specialization of distinct DC subsets, and partly on the activation state of DCs, which is largely dictated by environmental signals. In particular, whereas fully mature activated immunostimulatory DCs promote immune responses, immature DCs or DCs matured in immunosuppressive conditions counteract T-cell activation.

After providing an overview on the biology of DCs and the functional specialization of distinct DC subsets, we illustrated the strategies more commonly used for the identification of DCs in the peripheral blood, which represents the most accessible source of human DCs. In fact, because DCs lack unique lineage markers, their identification relies on possible different combinations of positive and negative markers. The importance of characterizing the activatory/inhibitory phenotype of DCs and the pattern of their cytokine production in different clinical settings was explained. We started showing the characterization of peripheral blood DCs (pb-DCs) performed by using a 3-color approach [9], and increased progressively complexity, showing a 6-color [10] and finally an 18-color [11] approach to the study of these cells.

Advantages and disadvantages of these different approaches were discussed. Tips and tricks related to analysis of rare cells, use of polychromatic flow cytometry, and DC-specific behavior were also presented. Finally, in order to introduce multidimensional unsupervised analysis of flow cytometric data, exemplificative t-distributed stochastic neighbor embedding (tSNE) analysis showing different subsets of pbDCs were shown.

Natural killer (NK) and innate lymphoid cells (ILCs)

In the last decade, innate lymphoid cell (ILC) family has grown enormously, demonstrating to be deeply involved not only in fighting viral infection and tumors but also in lymphoid tissue formation, in tissue regeneration and showing to possibly play a key role in many immune and autoimmune diseases [12].

These ILC populations, NK cells excluded, have not yet been unequivocally characterized and many recent studies revealed that they display a high degree of plasticity thus allowing their prompt adaptation to environmental change [13]. For these reasons, we summarized in a simple and unquestionable way what, up to now, we know for sure about ILCs phenotype and functions but most of all we tried to explain how to approach these cells from a rational and critical perspective, fundamental to avoid taking all these “brand new” discoveries for granted [14].

Moreover, we tried to give the students a deep overview of NK cells history starting from their discovery in the late seventies and ending up now in this exiting era of immune therapy based oncological treatments.

As a matter of fact, nowadays, NK cells relevance in human health preservation is showing more and more entirely new and fundamental facets [15,16]

Suppressor cells

Human body has innate immune regulatory mechanisms, but in addition it has evolved an external system of regulatory mechanisms in order to be protected from autoimmunity and to avoid in general immune harm. These cells called suppressor cells are the classical ones, including many subpopulations with different receptors, different mechanisms of action acting at different stages of the immune response.

These classical suppressor cells include:

a) NKT cells, a small population recognizing glycolipids presented by CD1d through polymorphic TCRs and secreting IL-4 and IL-10,

b) Tregs, (natural and inducible Tregs) CD4+CD25 highFOXP3+CD127low acting through direct cell to cell contact, suppressive cytokine secretion or through competition for growth factors linking (tethering),

c) CD8+ T regs functioning during the secondary and memory phases of the immune response,

d) TCRγδ+ T cells showing suppressive function towards TCRαβ+ T cells, antigen presenting cells and granulocytes.

Myeloid derived suppressor cells (MDSCs) are a morphologically, phenotypically and functionally heterogeneous population of immature cells of myeloid origin produced in the bone marrow in inflammatory conditions, including some types of cancer in order to protect the body from the consequences. For educational purposes the cells are ranked into monocytic, granulocytic and immature MDSCs. They have many common and many different characteristics.

MDSCs favor the tumor increase and development through Tregs induction in the tumor microenvironment, through suppressing T cells migration and survival. In addition, MDSCs promote neoangiogenesis or metastasis through soluble factors secretion. [17].

FUNCTIONAL ASSAYS BY FLOW CYTOMETRY

Flow cytometry not only is a critical component in the identification and quantification of immune cells, but it has also emerged as a well established method to evaluate cellular functions that are critical to the activation and regulation of immune responses [18]. A great advantage of functional assays performed by flow cytometry is that they can be combined with simultaneous immunophenotyping. When used in mixed cell populations, as peripheral blood, they allow therefore to assign the function studied to a specific cell type.

Cell proliferation

We started illustrating different methods that can be used to assess cell proliferation by flow cytometry, including assessment of the cell cycle phases, assessment of DNA synthesis, the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay, and measurement of proliferation proteins. Pros and cons of each single approach were discussed, and exemplificative analyses were presented [19-21].

Cell apoptosis

We then outlined the main strategies used to assess cell apoptosis by flow cytometry, based on the assessment of mitochondrial transmembrane potential, activation of caspases, DNA fragmentation, and plasma membrane alterations [22].

We provided detailed information on the most widely used strategy, based on Annexin V staining combined with plasma membrane permeability markers. We illustrated the experimental protocol, and presented exemplificative analyses showing that with this approach a distinction can be made between live, apoptotic, and late apoptotic-necrotic cells [23].

Cytokine production

Finally, we provided an overview of the methods that can be used to assess cytokine production, illustrating the flow cytometric approaches in the general scenario. We illustrated in detail the experimental protocol for intracellular cytokine detection, lingering on the most critical steps of the procedure. Exemplificative analyses were showed and discussed [24]. Other flow cytometric methods to assess cytokine production, including the secretion assay and particle-based multiplexed assays, were also illustrated. Tips and tricks relative to all the functional assays illustrated in this session were discussed.

Phagocytosis

Innate immunity is the body's first line of defense, immediate and non-specific, with no memory. The basic mechanism of non-specific immunity includes the ability to recognize, intake, integrate and intracellular kill "foreign" elements from the "professional" phagocytes of the immune system which are the monocytes/macrophages, the dendritic cells, and the neutrophils.

Phagocytes express on their surface Pattern Recognition Receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) as well as high affinity receptors (Fc receptors) that recognize molecules such as "complement" and antibodies.

The presentation included a detailed explanation of a phagocytosis assay that could be performed in an immunology laboratory. Briefly, it was described how the samples are incubated under the appropriate conditions and the opsonized E. coli labeled with fluorescein bacteria are killed by the phagocytes in the sample. Quantitative and qualitative measurement of the intensity of fluorescent bacteria phagocytosed corresponds to quantitative measurement of phagocytic capacity.

Oxidative burst

For the oxidative burst it was explained how this method is applied for the quantitative measurement of oxygen free radicals produced during the respiratory burst of the cell. Dihydrohodamine is a membrane-permeable colorless substance which, when oxidized by the H2O2 released during the final phase of phagocytosis, converts to fluorescent red pigment rhodamine 1,2,3. The percentage of cells that fluoresce with rhodamine 1,2,3 (R 1,2,3) and therefore have a sufficient oxidation mechanism is measured by flow cytometry.

For both functional tests, it was made sure that certain preanalytical and analytical pit falls were explained as well as the usefulness of these assays as diagnostic tools in an immunology laboratory.

Cytotoxicity and basophil degradation

Secretory lysosome (SL) generation and their exocytosis are complex and articulated processes that need to be finely understood in order to choose the proper protocol when testing a cell population functionality. In this course, after a biomolecular and functional characterization of how, upon different stimulations, degranulation takes place in different cell types, we explained the most used degranulation assays and cytotoxicity tests together with tip and tricks useful to obtain clear and reproducible results. Since degranulation tests are fundamental not only in the diagnosis of some rare primary immune deficiencies like Chediak-Higashi and Griscelli Syndrome but also in the evaluation of allergies, we went deep in detail in the description of basophil activation test (BAT). A fine description of all the different phenotypic and activation markers [25] together with and extended explanation on which should be used in any different occasion was given [26]. Moreover, various usable protocols and the reasons for choosing one or another where discussed together with the students in a friendly and, we hope, constructive atmosphere.

Flow x-match

A very important and old flow cytometry application is crossmatch (XM) for organ (usually renal) transplantation. For this purpose, the donor's cells are incubated with recipient's serum as well as a positive and a negative control. Monoclonal antibodies for T (CD3) and B (CD19) cells are added together with anti-human IgG, in order to determine the donor HLA-specific IgG antibodies in the recipient's serum. The ratio of median fluorescence intensity (MFI) of the IGG expression after the incubation of the potential recipient's serum with the donor's cells to the MFI of IgG after the incubation of a negative serum with the donor's cells is one of the evaluation methods of the result.

It is very important to establish the cut-off value for the distinction between positive and negative crossmatch by performing at least 30 unrelated crossmatches and establishing the mean value plus 2 SDs.

The advantages of flow crossmatch are the following:

a) it is less subjective, quantitative,

b) it detects low titer antibodies (10-250 times more sensitive than CDC-XM),

c) it detects complement activating and non-activating abs (IgG1, IgG2, IgG3 IgG4, IgA, IgM).

The disadvantages are the following:

a) it is not a functional method,

b) Abs detected do not reflect their capacity to activate C. Do they harm the graft in vivo?

c) there are false positive results especially regarding B cells.

Although the positive CDC-XM is an absolute contraindication for transplantation, positive T cell FC-XM is a relative one [27].

FLOW CYTOMETRY IN HEALTH AND DISEASE

Flow cytometry in primary immunodeficiencies

The introduction to primary immunodeficiencies (PIDs) was made by briefly referring to the basic steps of the immune response and by linking their defects to the basic PID groups. We next described the algorithms which are most broadly used for PID diagnosis, starting from basic assays, passing through the more specific immunophenotypic studies and culminating to more elaborate functional tests. Throughout the lecture we tried to raise awareness of PIDs, to deal with some misconceptions concerning them and, therefore, to convince the participants that PIDs are not as rare as widely thought and that a flow cytometrist should be able to recognize, or at least suspect, the basic ones [28, 29, 30].

A brief reference to the follow up of the immune reconstitution post Hematopoietic Stem Cell Transplantation (HSCT) was made during a "surprise" 30 minutes course. The basic principles of HSCT and immune reconstitution were described, with an emphasis on antigen (mostly virus) specific T cells. A more thorough insight to this vast topic would be welcome in future courses.

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) derives from a minimally invasive bronchoscopic technique. Specifically, by inserting the bronchoscope into the appropriate bronchus (the final bronchioles and lung alveoli), normal saline is gradually infused and aspirated. This way, the aspirate is used for the isolation of cells, inhalation particles, infectious agents and soluble non-cellular components. Furthermore a more representative picture of inflammatory and immunological processes at the alveolar level is achieved.

BAL is a diagnostic screening test of choice for sarcoidosis. As a complementary assay, is used for the diagnosis of various lung diseases, such as infections, interstitial diseases and malignancies. As a research tool is used to investigate the immunopathogenicity of lung diseases by contributing to the understanding of the immune and inflammatory mechanisms that prevail in various lung diseases. The BAL study includes macroscopic examination (appearance of the fluid) pathogen detection, cell morphology and immunophenotype.

Thus the presentation summarized the technique the procedure and the possible pitfalls that could occur during the process of BAL

Circulating tumor cells (CTCs)

Circulating tumor cells (CTCs) were first identified in 1869 by Ashworth in the blood of a man with metastatic cancer [31]. CTCs travel through the bloodstream or the lymphatic system and acquire the capacity to colonize in distant organs and finally establish a tumor metastasis [32]. CTCs can be found in the blood of patients as single cells or in groups of two or more adjacent CTCs termed as CTC clusters [33]. The ability of circulating tumor cells (CTCs) to form clusters has been linked to increased metastatic potential [34, 35].

The presence of CTC in the blood stream represents a rare event. However, despite the recent technical advancements, their isolation and detection remain a big challenge [36]. So far, the CellSearch system, is the only FDA-cleared semi-automated system for the isolation and enumeration of CTCs of epithelial origin [37]. CTC status can serve as an indicator to monitor the effectiveness of treatments and guide subsequent therapies [38, 39]. A recent meta-analysis conducted in early breast cancer patients treated by NCT, showed that CTC count

is an independent and quantitative prognostic factor [40].

CTC is a "liquid biopsy" approach for real time monitoring of cancer patients and assessment of treatment efficacy [41]. Molecular analysis of CTC provides significant insights into tumor heterogeneity, mechanisms of metastasis, tumor evolution and treatment resistance [42, 43]. Tumor specific biomarkers based on comprehensive characterization of CTCs could guide clinicians about the decision to prescribe targeted therapies to cancer patients [44, 45]. Using molecular assays, a variety of molecular markers such as multiple gene expression and DNA methylation markers have been detected and quantified in CTCs in various cancer types [46, 47].

In metastatic castration resistant prostate cancer (mCRPC) androgen-receptor splice variant 7 (AR-V7) is a highly promising liquid biopsy predictive biomarker showing primary or acquired resistance to novel androgen receptor signaling inhibitors [48, 49]. A novel multiplex RT-qPCR assay for the simultaneous detection of the androgen receptor (AR) and its splice variants AR-V7 and AR-567es, was recently developed and evaluated in circulating tumor cells (CTCs) and paired plasma-derived extracellular vesicles in mCRPC patients, showing distinct molecular patterns [50].

Checkpoint inhibitor-based immunotherapies have achieved impressive success in the treatment of different cancer types [51]. Abnormally high PD-L1 expression on tumor cells mediates tumor immune escape, and the development of anti-PD-1/PD-L1 antibodies has recently become a hot topic in cancer immunotherapy [52]. PD-L1 is frequently expressed on metastatic cells circulating in the blood of hormone receptor-positive, HER2-negative breast cancer patients [53]. It has been also reported, that PD-L1 over-expression in EpCAM(+) CTC fraction of HNSCC patients provide important prognostic information and represents a significant dynamic liquid biopsy biomarker since may evolve during treatment [44].

DNA methylation is an epigenetic mechanism that cells use to control gene expression [54]. Epigenetic modifications are very important in cancer development, since usually occur at an early stage [55]. The epigenetic silencing of key tumor suppressors and metastasis suppressors has been detected in CTCs by using Methylation specific PCR (MSP) for cystatin M (CST6) [56], Breast Cancer Metastasis Suppressor-1 (BRMS1) [57] and SRY-box containing gene 17 (SOX17) gene promoters. ESR1 epigenetic silencing potentially affects response to endocrine treatment. Mastoraki et.al. have shown that ESR1 methylation in CTCs is strongly associated with lack of response to everolimus/exemestane regimen showing the potential of ESR1 methylation as a liquid biopsy-based biomarker for endocrine treatment efficacy [47].

Immunomonitoring during treatment with biological drugs

Biological drug is a substance that is made from a living organism or its products and is used in the prevention, diagnosis, or treatment of cancer and other diseases. Biological drugs include antibodies, interleukins, and vaccines. Also called biologic agent and biological agent. Examples are:

- Immune check point inhibitors (PD-1, PD-L1, and CTLA-4 targets);
- Immune Cell Therapy (also called Adoptive Cell Therapy or Adoptive Immunotherapy) (TILs, CAR T cells);
- Therapeutic antibodies;
- Immune-Modulating Agents;
- Therapeutic Vaccines.

Regarding Monoclonal antibodies treatment, the cytometrist's tasks are to make a baseline assessment of the relevant antigen on target cells, to set the appropriate reagent protocol to assess monoclonal antibody efficacy, to set protocols to distinguish cell disappearance from antigen modulation, to check for adverse effects of the biological drugs (emergence of malignant clones, disappearance of other types of cells), and to assess reappearance of target cells [58].

CONCLUDING REMARKS

The School was attended by students from Italy, Greece, Czech Republic and Russia. All students greatly appreciated the organization of the course, the quality of all presentations, and the friendly and inclusive atmosphere of the School that offered a nice opportunity to learn and discuss technical and biological issues of the treated themes. All students reported that the Kos School was a very useful experience for their work needs.

The enthusiastic comments received by students indicated that the School was an effective tool for professionals working in cytometry immunology labs in Europe and throughout the world. It should be noted that one of the students is currently doing a one month experience in the research lab of one of the teachers to improve her cytometric and cell sorting skills

Proud of the obtained results, ESCCA will repeat the experience, taking into account important suggestions received by the attendants. Therefore, we are happy to announce that the second edition of the ESCCA Summer School in Flow Cytometry for Immunology will be held in Kos on June 3-7, 2020 (see Table 2, on the following page).

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Table 2 ESCCA Summer School in Flow Cytometry for Immunology (June 3-7, 2020 – Kos Island, Greece)

	6/3/2020	6/4/2020	6/5/2020	6/6/2020	6/7/2020
	Wednesday	Thursday	Friday	Saturday	Sunday
9.00-10.30		**Immunophenotyping in immunology:** Dendritic cells and other myeloid cells Silvia Della Bella (IT)	**Flow cytometry in health and disease:** Distribution of immune cells throughout life Perez Martin (SP)	**Flow cytometry in health and disease:** Flow cytometry in primary immunodeficiencies Marianna Tzanoudaki (GR)	**Functional assays by flow cytometry:** cytotoxicity, basophil degranulation Genny Del Zotto (IT)
10.30-12.00		**Immunophenotyping in immunology:** Suppressor cells Katherina Psarra (GR)	**Functional assays by flow cytometry:** cell proliferation, apoptosis, cytokine production Silvia Della Bella (IT)	**Flow cytometry in health and disease:** Immunomonitoring during treatment with biological drugs Katherina Psarra (GR)	**Concluding remarks**
12.00-13.00		**Presentation and discussion of data from attendees**	**Presentation and discussion of data from attendees**	**Presentation and discussion of data from attendees**	
13.00-14.00		free lunch	free lunch	free lunch	
14.00-15.30	**Immunophenotyping in immunology:** Fluorochrome choices for multi-color flow cytometry Perez Martin (SP)	**Immunophenotyping in immunology:** NK cells and innate lymphoid cells Genny Del Zotto (IT)		**Immunophenotyping in immunology:** Analytical tools for high-dimensional flow cytometry data Silvia Della Bella (IT)	
15.30-17.00	**Immunophenotyping in immunology:** T cells Katherina Psarra Alexandra Fleva (GR)	**Functional assays by flow cytometry:** phagocytosis, oxidative burst, autophagy Alexandra Fleva (GR)	**visit to Asklipieio**	**Functional assays by flow cytometry:** microvescicles Genny Del Zotto (IT)	
17.00-18.30	**Immunophenotyping in immunology:** B cells Marianna Tzanoudaki (GR)	**Flow cytometry in health and disease:** Circulating tumor cells Areti Strati (GR)		**Functional assays by flow cytometry:** Phosphoflow in immunology testing	

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