ESCCA European Society for Clinical Cell Analysis

ESCCA Annual Conference, Bergen 2019

Detection of GPI-A deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and Bone Marrow Failure Syndromes (BMFS) by Flow Cytometry

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Disclosure

I have no potential conflict of interest

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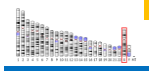
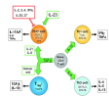
Origin of GPI-AP deficient clones

Initial viral insult → Proinflammatory environment → Immune mediated bone marrow injury

- Reduction of **T regs** (CD4+, CD25 +, FOXP3+)
- Expansion of **Th1** (CD4+, IFN-γ, TNF-α)
- Expansion of **Th17** (CD4+, IL-6, IL-17, TNF-α)
- Expansion of **autoreactive CD8+ cytotoxic T cells**



Preferential survival of PIG-A gene mutated HSC



Clonal expansion with second genetic or epigenetic event

- Heterogeneous group
- Overlapping diseases
- Propensity to develop MDS/AML
- Acquired forms >80%
- Idiopathic forms >75%

Inoue et al. 2003, Young et al. 2006, Parker et al. 2009, Shimamura et al. 2010, Mufti et al. 2018

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Clinical utility of testing for GPI-A deficient clones in PNH

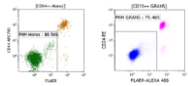
PNH

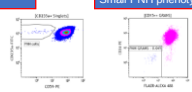
Classic | Aplastic | Subclinical

Hemolytic crisis | Chronic hemolysis | TE

BMFS

No clinical evidence
No laboratory evidence
Small PNH phenotypes





Small / medium / big PNH clone

- level of hemolysis and risk of thrombotic complications
- 20-30% cases can develop pancytopenia

Minor clone (<1%) / cells with PNH phenotype (<0.1%)

- 15-20% can evolve in clinical PNH

Young et al. 2006, Wang et al. 2008, de Latour et al. 2008, Parker et al., 2009, Hill et al. 2012, DeZern et al. 2014, Ristano et al. 2016, Killick et al., 2016, Townsley et al. 2017

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Clinical utility of testing for GPI-A deficient clones in BMFS

BMFS

Inherited | Acquired

5% of AA patients < 40 years have germline mutations

Single cytopenia | Pancytopenia | Single cytopenia | Pancytopenia

Congenital neutropenia (CN)
Diamond Blackfan anemia (DBA)
Shwachman-Diamond Sy (SD)
Amegakaryocytic trombocytopenia
Reticular dysgenesis (TAR)

Fanconi anemia (FA)
Dyskeratosis congenita (DC)

MDS-SLD
MDS-RS-SLD

AA
MDS-MLD
MDS-RS-MLD
MDS-EB
PMF

Hypocellular BM (< 30%): 10-15%

Presence of GPI-deficient clone: high NPV for IBFS

- Presence of GPI-deficient clone is predictive of response to IST and lower incidence of later MDS/AML
- 10-15% of cases show clone evolution

Young et al., 2006, Alter et al. 2010, Shimamura et al., 2010, Dezern et al. 2014, Kael et al. 2016, Stanley et al. 2017, Olsen et al. 2017, Mufti et al. 2018

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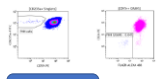
Diagnostic Workflow for iBMFS

Diagnosis of BMF

All cases

Chromosomal Breakage Studies and Lymphocyte Telomere Length

PNH Flow Cytometry



Workout for Acquired BMF

Confirmatory Gene Analysis for FA or DC

Medical history, physical exam., lab. findings suggestive for IBMFS

Multiplex NGS panels

GPI-AP deficiency evaluation by Flow Cytometry - Rule out GPI-A deficiency

DeZern et al., 2014, Alter et al. 2010, Stanely et al. 2017, Mufti et al 2018,

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Diagnostic workflow for AA

FBC: pancytopenia, anemia with reticulocytopenia, makrocytosis.....

BM aspirate and trephine biopsy
- aplastic bone marrow, without dysplasia and blast infiltration

Cytogenetics (hampered by insufficient cells in metaphase) / FISH
- 12%: -7/del(7q), del 13q, trisomy 8

SNP-A (do not require dividing cells:
- 19% of unbalanced chromosomal defects

Mutation analysis
- cca 20% associated with myeloid genes typically detected in MDS
- More favorable: PIG-A, BCOR/BCOR1
- Less favorable: DNMT3A, ASXL1, TP53, RUNX1, CSMD1
- MDS associated mutations also occur in healthy older individuals - clinically NOT predictable

GPI-AP deficiency evaluation by Flow Cytometry
- rule out IBMFS (AA)
- prediction of response to IST and risk of transformation to MDS/AML
- clone evolution monitoring

Young NS et al. 2006, Kurtis et al. 2008, Ogawa S. et al. 2016, Shi J et al. 2012, Shimamura et al. 2016, Kordasht et al. 2012, Mufti et al. 2018

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Diagnostic workflow for h-MDS

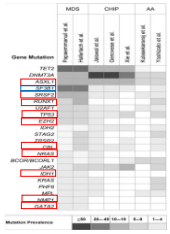
FBC: pancytopenia, anemia with reticulocytopenia, makrocytosis.....

BM aspirate and trephine biopsy
- hypoplastic bone marrow (h-MDS) morphological evidence of dysplasia

Cytogenetics / FISH
54% MDS (-5, 5q, -7, 7q-, +8, 20q-.....)
abnormal cytogenetic clone does not imply the dg. MDS

Mutation analysis
- 35%
- Favorable: SF3B1
- Adverse <5% blasts: NPM1, NRAS, WT1, SRSF2, ASXL1, IDH2, GATA2, TP53, RUNX1, EZH2, PRPF8
- Adverse 5-30%: TP53, RUNX1, EZH2, PRPF8, FLT3, CBL

GPI-AP deficiency evaluation by Flow Cytometry
- prediction of response to IST and risk of transformation to MDS/AML
- clone evolution monitoring



Maciejewski et al. 2004, Gupta et al. 2005, Hosokawa et al. 2012, Holbro et al. 2013, Yoshizato et al. 2015, Mortazavi et al. 2003, Jaiswal et al. 2014, Genovese et al. 2014, Haferlach et al. 2014, Young et al. 2016, Killick et al. 2016, Stanley et al. 2017

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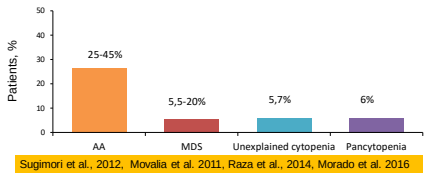
GPI-anchor deficiency in BMFS



Sugimori et al., 2012, Movalia et al. 2011, Raza et al., 2014, Morado et al. 2016

Importance of high sensitivity assay (> 0.01%)

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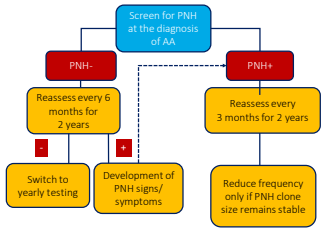
Acquired Aplastic Anaemia

IPIG[1]
"Patients with AA should be screened at diagnosis..."

ICCS[2]
Clinical indications for PNH include: "Evidence of bone marrow failure", including "suspected or proven aplastic or hypoplastic anemia."

BCSH[3]
"Patients should be screened for PNH at the diagnosis of AA."



1. Parker C, et al. 2005, 2. Borowitz MJ, et al 2010 , 3. Killick SB, et al. 2016

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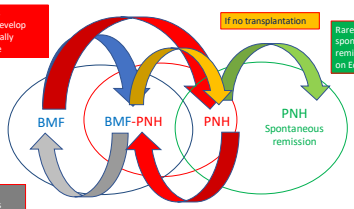
Screening and Monitoring of GPI-anchor deficiency

15-20% of patients with BMFS could develop subclinical or clinically relevant PNH clone

If no transplantation

Rare spontaneous remission on Eculizumab



Sporadic spontaneous remission

20-30% of patients with classical PNH develop pancytopenia

Parker et al. 2005, Schrezenmeir et al. 2009, DeZern AE et al. 2015

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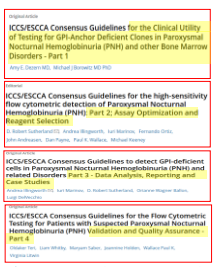
ICCS/ESCCA Guidelines 2018



PROVIDE VALIDATED APPROACHES meeting criteria for high:

- Accuracy / Trueness - EQA, ILC
- Clinical specificity (TN/TN+FP) - > 99%
- Analytical specificity - library of validated MoAbs
- Clinical sensitivity (TP/TP+FN) - > 99%
- Analytical sensitivity/ LOD - 20 events for reproducible detection
- Functional sensitivity/ LOQ - 50 events for reproducible quantification
- Intra-assay imprecision / Repeatability - < 2% / 5%
- Inter-assay imprecision / Reproducibility - < 2% / 5%

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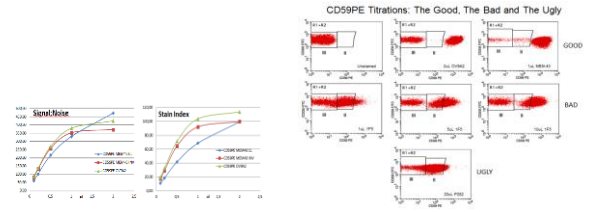
Pre-analytical considerations reagent selection



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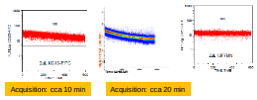
Pre-analytical considerations reagent selection: RBCs

Reagents

Target	Antibody Conjugates	Purpose	Clone and Vendor
RBC	CD235a-FITC	Gating on RBC	10F7MN (eBio) YTH 89.1 (Cedarlane) KC16 (BC) JC159 (DAKO)
	CD59-PE	GPI-linked for RBC	OV9A2 (eBio) MEM-43 (Invitrogen) MEM-43 (EXBIO/Cedarlane)

Panel design

CD235a-FITC/ CD59- PE



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Sutherland DR, Richards SJ, Ortiz F, Nayyar R, Benko M, Marinov I, Illingworth A. Cytometry B 2019 submitted

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Pre-analytical considerations- reagent selection: WBCs

Beckman Coulter

Target	Antibody Conjugates	Purpose	Clone (Vendor)
WBC	FLAER-Alexa488	GPI-linked (Ne + Mo)	NA (Cedarlane)
	CD24-PE CD24-APC	GPI-linked (Ne)	SN3(eBio), ALB9 (BC) SN3 (eBio, EXBIO)
	CD14-PE CD14-APC700	GPI-linked (Mo)	61D3 (eBio), RMO52 (BC), Tuk4 (Invitrogen) RMO52 (BC)
	CD157-PE	GPI-linked (Ne + Mo)	SY11B5 (eBio, EXBIO, BD, BC, Sysmex)
	CD64-PC5 CD64-ECD CD64-PC7	Gating on Monocytes	22 (BC) 22 (BC) 22 (BC), 10.1 (EXBIO)
	CD15-PC5 CD15-PerCP-eF710 CD15-PerCPCy5.5	Gating on Neutrophils	80H5 (BC) MMA (eBio) MEM158 (EXBIO)
	CD45-PC7 CD45-KO CD45-eF450	Debris/unlysed RBC exclusion + pattern recognition	J33 (BC) J33 (BC) 2D1 (eBio)

Panel design

2-laser: 5-color assay (CD157)
FLAER, CD157PE, CD64ECD, CD15PE-Cy5, CD45PE-Cy7

3-laser: 5-color assay (CD157)
FLAER, CD157PE, CD45PerCP-Cy5.5, CD64APC, CD15 (MMA)eF450/v450
FLAER, CD157PE, CD45 PerCP-Cy5.5, CD15 (MMA) APC, CD64 PE-Cy7

3-laser: 6-color assay
With FLAER
FLAER, CD24PE, CD14PerCPCy5.5, CD15APC, CD64PE-Cy7, CD45 APC-Cy7
No FLAER
CD157PE, CD45PerCP, CD64PE-Cy7, CD24APC, CD14APC-Cy7, CD15 (MMA)eF450
CD157PE, CD15PE-Cy5, CD64PE-Cy7, CD24APC, CD14APC-Cy7, CD45KO

3-laser: 7-color assay (3 GPI markers)
FLAER, CD157PE, CD45PerCP-Cy5.5, CD64PE-Cy7, CD24APC, CD14APC-Cy7, CD15eF450
FLAER, CD157PE, CD15PE-Cy5, CD64PE-Cy7, CD24APC, CD14APC-Cy7, CD45KO

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Pre-analytical considerations- reagent selection: WBCs

Becton Dickinson

Target	Antibody Conjugates	Purpose	Clone (Vendor)
WBC	FLAER-Alexa488	GPI-linked (Ne + Mo)	NA (Cedarlane)
	CD24-PE CD24-APC	GPI-linked (Ne)	SN3 (eBio), ML5 (BD) SN3 (eBio, EXBIO)
	CD14-PE CD14-APC	GPI-linked (Mo)	61D3 (eBio), Tuk4 (Invitrogen) MoP9 (BD)
	CD157-PE	GPI-linked (Ne + Mo)	SY11B5 (eBio, EXBIO, BD, BC, Sysmex)
	CD64-APC CD64-PECy7	Gating on Monocytes	10.1 (BD, eBio) 10.1 (EXBIO), 22 (BC)
	CD15-APC CD15-PerCP-eF710 CD15-PerCPCy5.5	Gating on Neutrophils	H98 (BD) MMA (eBio) MEM 158 (EXBIO)
	CD45-eF450 CD45-PerCP CD45-APC-H7	Debris/unlysed RBC exclusion + pattern recognition	2D1 (eBio) 2D1 (BD) 2D1 (BD)

Panel design

2-laser: 5-color assay (CD157)
FLAER, CD157PE, CD15PerCP-eF710, CD64APC, CD45APC-Cy7

3-laser: 5-color assay (CD157)
FLAER; CD157PE, CD45PerCP-Cy5.5, CD64APC, CD15 (MMA)eF450/v450
FLAER, CD157PE, CD45 PerCP-Cy5.5, CD15 (MMA) APC, CD64 PE-Cy7

3-laser: 6-color assay
With FLAER
FLAER, CD24PE, CD14PerCP-Cy5.5, CD15APC, CD64 PE-Cy7, CD45 APC-Cy7
No FLAER
CD157PE, CD45PerCP, CD64PE-Cy7, CD24APC, CD14APC-Cy7, CD15 (MMA)eF450

3 laser: 7-color assay (3 GPI markers)
FLAER, CD157PE, CD45PerCP-Cy5.5, CD64PE-Cy7, CD24APC, CD14APC-Cy7, CD15eF450

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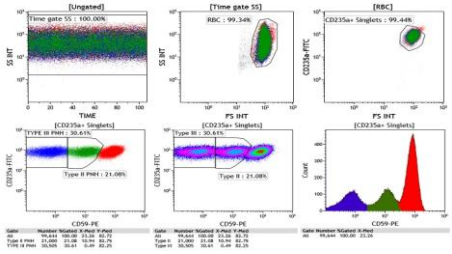
Analytical considerations- RBCs analysis strategy



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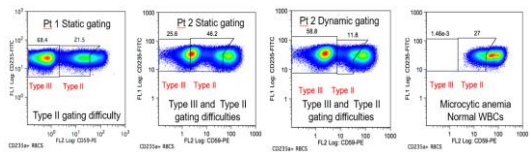
Analytical considerations- RBCs gating difficulties



Sutherland DR, Richards SJ, Ortiz F, Nayyar R, Benko M, Marinov I, Illingworth A. Cytometry B 2019 submitted

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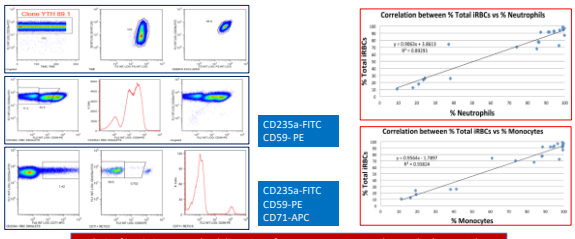
iRBCs (nucleated + reticulocytes) analysis
CD235a/CD59/CD71



Analysis of iRBCs improves the delineation of PNH type III, type II and normal subsets and provides more accurate information concerning the RBC PNH clone size

Sutherland DR, Richards SJ, Ortiz F, Nayyar R, Benko M, Marinov I, Illingworth A. Cytometry B 2019 submitted

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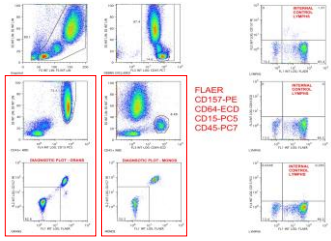
Analytical considerations- WBC analysis strategy
5-c (2 laser) FLAER/CD157 based assay

Rare CD157- non PNH cases (< 0.5%)

- SNP (Arg145Gln)
- Infection (HBV)
- Unknown

Analysis of 2 GPI markers per lineage (Ne, Mo) and RBCs

NO False positive results reported !!!



Sutherland et al. 2014
Marinov et al. 2016
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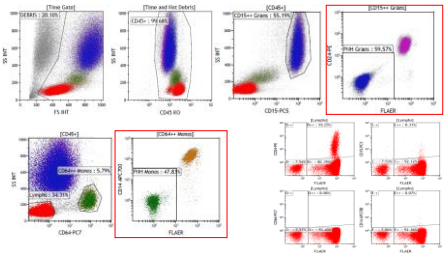
Analytical considerations- WBC analysis strategy
6-c FLAER/CD14/CD24 based assay



Sutherland et al. 2014
Marinov et al. 2016
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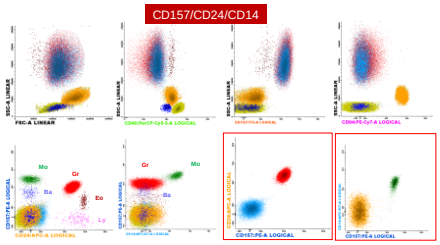
Analytical considerations- WBC analysis strategy
6-c non-FLAER based assay

CD157/CD24/CD14



Marinov et al. 2016
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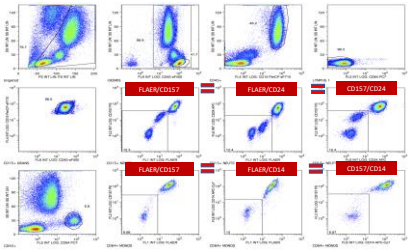
Analytical considerations- WBC analysis strategy
7-c FLAER/CD14/CD24/CD157 based assay



Sutherland DR, Ortiz F, Quest G, Illingworth A, Benko M, Marinov I, Nayyar and Marinov I. Cytometry B 2018
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Post-analytical considerations - reporting

Reporting

- PNH clone present (>LOQ) / detectable (>LOD, <LOQ) / absent (<LOD)
- PNH RBCs: type II , type III , type II+III (%)
- PNH Ne: type II+III
- PNH Mo: type II+III

Terminology CSLI H52-A2[1]

- GPI deficient clone >1%: PNH klon
- GPI deficient clone 0.1% - 1%: minor PNH clone
- GPI deficient clone <0.1%: GPI- phenotype

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TAKE HOME MESSAGES I

- GPI deficiency testing by HS FCM is useful in young patients **to rule out inherited BMFS**
- GPI deficiency testing by HS FCM is important do **detect and follow up PNH phenotypes in acquired BMFS**: 15 - 20% of patients with BMFS could develop subclinical or clinically relevant PNH clone
- GPI deficiency testing by HS FCM is important for **predicting response to IST in AA and h-MDS**
- GPI deficiency testing by HS FCM is important for **predicting progression of AA / h-MDS to MDS/AML**
- GPI deficiency testing by HS FCM is important for the **diagnosis, classification and follow-up of PNH**: 20-30% of patients with classical PNH could develop pancytopenia
- Mandatory analysis of **2 GPI markers on WBC (Ne, Mo) and RBCs**
- Library of **validated reagents** for various HW configurations

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TAKE HOME MESSAGES II

- Report with concern to LOD a LOQ using **uniform terminology** (CSLI H52-A21)
- **Analysis of iRBCs** improves the delineation of PNH type III, type II and normal subsets and provides more accurate information concerning the RBC PNH clone size

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ON BEHALF OF

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