The Future of Laboratory Diagnostics

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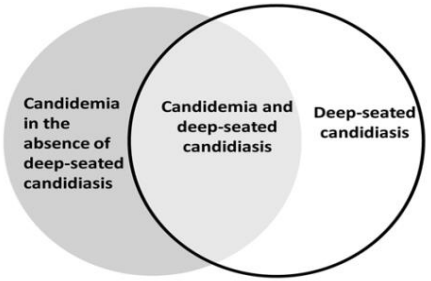
Molecular for all!

Commercially produced PCR assays

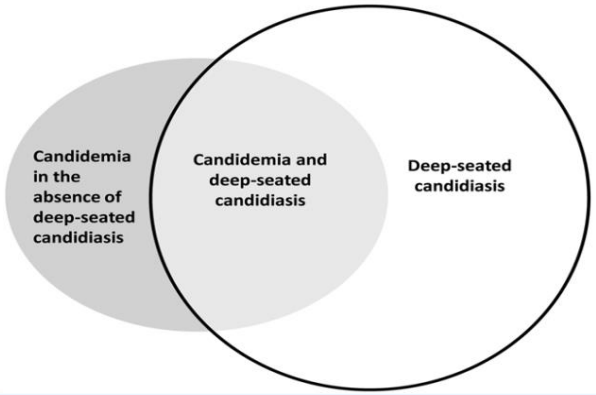
External Quality assurance

Incorporated in definitions

IC- The missing 50%



Improved diagnostics will identify cases of invasive candidiasis, in particular deep-seated candidiasis, that are currently missed by cultures



- BC are analytically sensitive
  - Typical LOD <1cfu/ml
- Clinical limitations
  - Sampling 0.2% of circulation
  - Candidaemia dependent
- BC negativity
  - Burdens below LOD
  - Absence of viable *Candida*
    - Limited sampling
    - Manifestation
  - Transient release of viable cells into the circulation
- Meta-analysis of *Candida* PCR for candidaemia
  - Se: 95% Sp: 92%
  - Avni et al JCM 2011
- Can non-culture diagnostics identify the missing 50%?

Clancy and Nguyen Clin Infect Dis. 2013

24 cases of DS/IC	BC	BDG	PCR	BDG+BC	PCR+BC
Sensitivity (%)	17	62	88	79	98%

T2Candida Assay

- Fully automated
  - Cartridge based DNA concentration/extraction
  - Isothermal PCR amplification of ITS 2
  - Detection and identification of amplicons by magnetic resonance
  - *C. albicans*/*C. tropicalis*, *C. glabrata*/*C. krusei* and *C. parapsilosis*
- Results within 5 hours with ≥120h for BC
- Validation [a]
  - 95% LOD 1-3cfu/ml
  - Sensitivity: 91% Specificity: 98% PPV: 72% NPV: 99.5%
- Meta-analysis [b] 8 studies 2717 pts 525 cases
  - Sensitivity: 91% Specificity: 94%
  - Lower Se: 39% Lower Sp: 59%, 68%
- Lower Sensitivity
  - Impact of antifungal therapy
  - IC in the absence of candidaemia – T2 targets organism
- Cost?

[a] Mylonakis et al. Clin Infect Dis. 2015
[b] Tang et al BMC Infect Dis. 2019

Molecular detection of *C. auris*

- Environmental and clinical culture – limited sensitivity and TAT (> 4 days)
  - Hinders Infection control procedures
- Conventional ID – problematic
- Molecular detection/ID [a] –
  - highly sensitive
  - Accurate ID
  - Rapid - Direct testing of samples (4 hours)
  - Various commercial assays available
  - Fully automated testing using the BD-Max [b]

- Molecular detection of genetic markers of resistance in *Candida* sp
  - Echinocandins
  - Azoles

[a] Leach J Clin Microbiol 2018
[b] Leach J Clin Microbiol 2019

Commercial *Aspergillus* PCR options

Assay	Sample type	Sensitivity (%)	Specificity (%)
Microgen MycAssay Aspergillus	BAL[a]	80-100	88-99
	Serum/Plasma[b]	25-60	83-100
Pathono Asperge			
Ademtech Mycogenie	BAL[e]	54-93	90
	Serum/Plasma[f]	100	85
Bruker Fungiplex Aspergillus	Serum/Plasma[g]	95	90
OLM AspID	BAL[h]	94	77

DNA extraction is rate limiting

International recommendations not followed or available

[a] Torelli et al J Clin Microbiol 2011, Guinea et al Plos One 2013, Orsi et al New Micro 2015
[b] White et al J Clin Microbiol 2011, Pini et al J Clin Microbiol 2015, Danylo et al Med Mycol 2014
[c] Chong GL, et al. J Clin Microbiol 2015, Chong et al JAC 2015,
[d] White et al J Clin Microbiol 2015, White et al J Clin Microbiol 2017
[e] Dannaoui, et al J Clin Microbiol 2017, Guegan et al J Infect 2017; [f] Dannaoui, et al J Clin Microbiol 2017;
[g] White PL, ECCMID 2017; [h] Johnson et al. Mycoses 2018

IA Biomarker – Performance - Blood

- High frequency Screening test
- Exclude fungal disease
  - High sensitivity/NPV
- Beneficial for low incidence disease

Parameter	GM-EIA		β-D-Glucan			PCR	
Reference	Leeflang[a]	Pfeiffer[b]	Lamoth[c]	Karageorgopoulo	He[e]	Mengoli[f]	Arvanitis[g]
Sample	Serum	Serum	Plasma/Serum	Plasma/			
Sensitivity	79.3%	79.3%	56.8%	77.1%	77.0%	88.0%	84.0%
Specificity	80.5%	86.3%	97.0%	85.3%	81.3%	75.0%	76.0%

Comparable performance despite limited standardisation

LR-tive: 0.15 = Negative results exclude IA[f]

Combined GM and PCR optimal

[a]Leeflang *et al.* 2009; [b]Pfeiffer *et al.* 2006; [c]Lamoth *et al.* 2012; [d]Karageorgopoulos *et al.* 2011; [e]He *et al.* 2014; [f]Mengoli *et al.* 2009; [g]Arvanitis *et al.* 2014

IA Biomarker – Performance - BAL

- Infrequent test
- Diagnostic: Confirm disease in symptomatic patients
  - High Specificity/PPV

Parameter	GM-EIA		PCR		
Reference	Zuo[a]	Guo[b]	Tuon[c]	Sun[d]	Avni[e]
Sample	BAL	BAL	BAL	BAL	BAL
Sensitivity	83.6%	85.7%	78.4%	79.6%	76.8%
Specificity	89.4%	89.0%	93.7%	94.1%	94.5%

LR+tive: >12

Overall comparable performance

Despite limited standardisation

Combined GM and PCR optimal

[a]Zuo *et al.* 2012; [b]Guo *et al.* 2011;
[c]Tuon 2007; [d]Sun *et al.* 2011; [e]Avni *et al.* 2012;

Direct detection of azole resistance in *Aspergillus*

- Global clinical rate: 3.2% *A. fumigatus* [a]
- Cyp51 mutations
- Mainly applied to tissue and BAL samples [b-e]
- Commercial assays exist - TR34/L98H or TR46/Y121F/T289A [b]
  - Pathonostics Aspergenius Assay
  - BAL 8/11 azole profiles were directly determined
- Blood?
  - Pathonostics Aspergenius Assay
  - Serum 7/14 azole profiles were directly determined [f]
- All culture negative!
  - Delayed diagnosis – delayed appropriate therapy – increased mortality [g]

[a] Van der linden Emerg Infect Dis 2015 [b] Chong GL, *et al. J Clin Microbiol* 2015;53:868–74, [c] Speiss B, *et al. PLoS One* 2014;9:e103113, [d] Zhao Y, *et al. J Antimicrob Chemother* 2013;68:1497–1504, [e] Denning DW, *et al. Clin Infect Dis* 2011;52:1123–9; [f] White PL, *et al. J Clin Microbiol* 2015;53:2115–21; [g] van der Linden et al. Emerg Infect Dis. 2011

A. Fumigatus Cyp51A – Pyrosequencing

- Increasing range of SNP's associated with azole resistance
- Pyrosequencer validation
  - 50 clinical isolates
  - Comparsion with Sanger sequencing
  - 94% agreement
- Direct testing of respiratory samples
- *A. fumigatus* identified
  - H-V culture: 94/326 (29%)
  - 53 resistant isolates
  - Pyrosequencing: 205/326 (63%)
  - 73 resistant
  - Agreement: 25/94 (27%)
  - 11 Pyro-Res/phenotype sensitive
  - 23 Pyro-Sens or failed /phenotype resistant

	cyp51A genotype		Isolate MIC (mg/L)				
Isolate	Sanger sequencing	pyrosequencing	ITC	AMB	VRC	POS	ISA
1	no polymorphisms	no polymorphisms	0.5	0.5	0.25	0.25	NA[a]
2	no polymorphisms	no polymorphisms	0.5	1	0.5	0.125	NA
3	no polymorphisms	no polymorphisms	0.25	0.5	1	0.125	NA
4	no polymorphisms	no polymorphisms	0.25	0.5	1	0.125	1
5	no polymorphisms	no polymorphisms	0.5	1	0.5	0.125	1
6	no polymorphisms	no polymorphisms	0.125	0.25	0.25	0.03	0.5
7	no polymorphisms	no polymorphisms	0.5	0.5	0.25	0.125	NA
8[b]	no polymorphisms	no polymorphisms	>8	0.25	2	1	>8
9	no polymorphisms	no polymorphisms	0.25	2	1	0.06	NA
10	D262Y[c]	no polymorphisms	0.25	0.5	0.5	0.06	NA
11	no polymorphisms	no polymorphisms	0.5	0.5	0.5	0.125	NA
12	G448S	G448S	>8	0.5	8	0.5	8
13	A284T[c]	no polymorphisms	>8	0.5	>8	>8	>8
14	TR34/L98H	TR34/L98H	>8	1	4	0.5	8
15[b]	no polymorphisms	no polymorphisms	>8	1	4	1	8
16	F219V	F219V	>8	0.5	0.5	1	NA
17	G54W	G54W	>8	0.5	1	>8	NA
18	G54W	G54W	>8	0.5	1	>8	NA
19	no polymorphisms	no polymorphisms	0.5	0.5	1	0.25	NA
20	P216H	P216H	>8	0.25	0.5	0.125	1
21[d]	G448S	G448S	>8	2	>8	>8	>8
22[b]	no polymorphisms	no polymorphisms	>8	0.5	0.5	1	NA
23	no polymorphisms	no polymorphisms	1	0.5	1	0.25	NA
24	M220T	M220T	>8	0.25	1	0.5	2
25[b,d]	no polymorphisms	no polymorphisms	>8	1	4	2	4
26	A284T[c]	no polymorphisms	>8	0.5	8	>8	>8
27[d]	no polymorphisms	no polymorphisms	0.5	1	0.25	0.125	NA
28	TR34/L98H	TR34/L98H	>8	1	2	0.5	8
29	TR34/L98H	TR34/L98H	>8	0.5	4	1	8
30	no polymorphisms	no polymorphisms	0.5	0.5	2	0.125	NA
31[e]	no polymorphisms	no polymorphisms	NA	NA	NA	NA	NA
32[e]	no polymorphisms	no polymorphisms	NA	NA	NA	NA	NA
33[f]	TR34/L98H	TR34/L98H	>8	0.5	>8	1	NA
34[f]	M220T	M220T	>8	1	0.5	0.25	NA
35[f]	M220K	M220K	>8	2	2	1	2
36[f]	G54E	G54E	>8	2	0.5	>8	0.5
37[f]	G54R	G54R	>8	1	0.25	2	NA
38[f]	G54R	G54R	>8	2	0.25	1	NA
39[f]	M220T	M220T	>8	1	2	0.5	2
40[f]	G54V	G54V	>8	0.5	0.5	1	NA
41[f]	TR34/L98H	TR34/L98H	>8	0.25	4	0.5	NA
42[f]	G54E	G54E	>8	1	0.25	1	NA
43	F219I	F219I	>8	0.5	0.5	1	2
44	TR34/L98H	TR34/L98H	>8	0.25	4	1	8
45	G448S	G448S	2	1	4	0.25	>8
46[d,g]	TR46/Y121F/T289A	TR46/Y121F/T289A	>8	1	>8	1	NA
47[d,g]	TR46/Y121F/T289A	TR46/Y121F/T289A	>8	1	>8	1	NA
48[b,d]	no polymorphisms	no polymorphisms	>8	0.5	8	2	>8
49	TR34/L98H	TR34/L98H	>8	0.5	4	1	8
50[d]	TR34/L98H	TR34/L98H	>8	1	4	0.5	4

Novak-Frazer J Antimicrobial Chemo 2020

PCP PCR – Meta-analyses

Parameter	Study		
	Summah[a]	Fan	Lu
Cases/Total (n/N)	506/2330	606/1793	416/2505
Sensitivity (%)	97	98	99
Specificity (%)	94	91	90
PPV (%)	82	85	66
NPV (%)	99	99	>99
LR +tive	16.2	10.9	9.9
LR -tive	0.03	0.02	0.01
DOR	540	545	990

Summah *et al* 2013 Fan *et al* 2013, Lu *et al* 2011

Commercial PcP PCR and resistance

- Pneumocystis jirovecii [a] -
  - Dihydropteroate synthase mutations = failure in sulfa based prophylaxis
  - Geographical Variation in prevalence
  - Anthropophilic transmission
- Molecular tests are the only non-culture assays with the ability to determine potential resistance
  - ID genetic markers
  - Difficulty with culture
- PneumoGenuis® assay (Pathonostics) [b]
  - Se: 70% Sp:82%
  - DHPS *fas* mutations
  - 89/118 samples successfully amplified
  - 4.5% resistance rate

[a] Hauser et al. Clin Infect Dis 2010
[b] Montesinos et al, In Press

PcP Combination testing

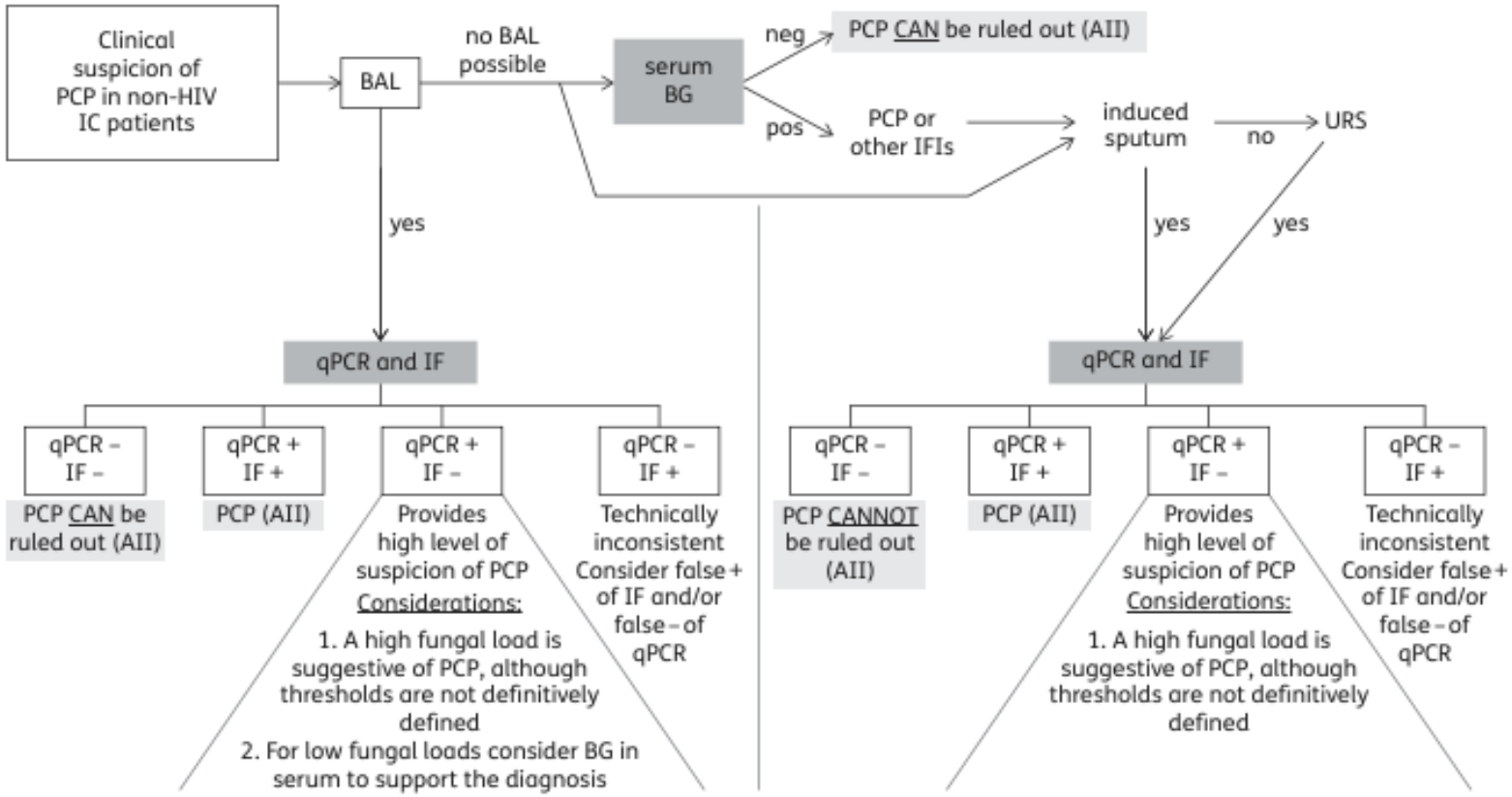


Figure 1. Flow chart for the diagnosis of *Pneumocystis* pneumonia in non-HIV immunocompromised (IC) patients. Biological tests are highlighted in dark grey and recommendations in light grey. BG, β-D-glucan; A-II, level of recommendation; IFI, invasive fungal infection.

[a] Alanio et al. J Antimicrobial Chemother 2016

NOVEL ASSAYS

Pan-fungal biomarker

- Pan-fungal dihexasaccharide
- MALDI-MS
- In sera of pts with IC [a]
- Confirmed in an animal model of IC [a]
- Evaluated in patients with IC (23), IA (15) and **MM (10)** [b]

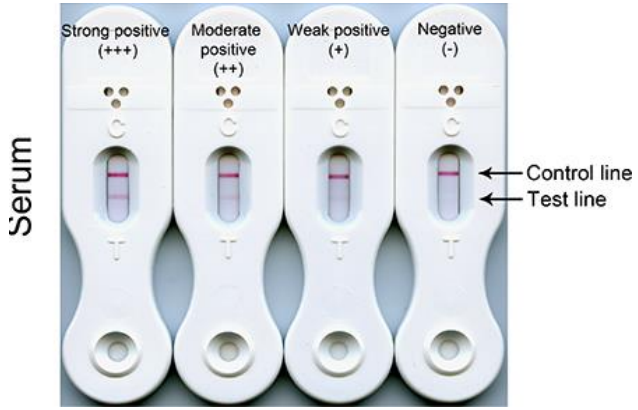
	MS-DS		BDG		Antigen	
Cohort	Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)
IC	**83**	**69**	96	31	39	86
IA	93	76	87	90	**93**	**100**
MM	**90**	N/D	20	N/D	0	N/D

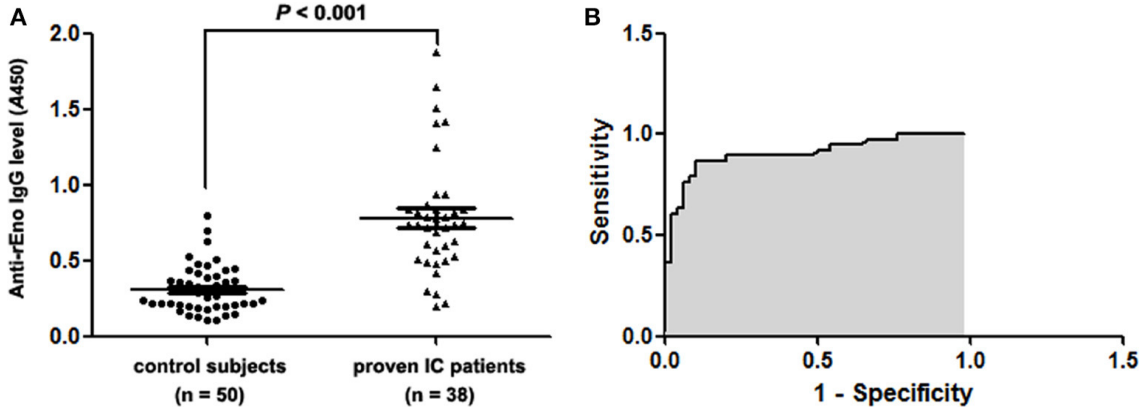
- Persistent MS-DS = poor prognosis in IC
- MS-DS positivity preceded other biomarker in IA

[a] Sendid et al. Clin Microbiol Infect 2015
[b] Marty et al J Clin Microbiol 2016

Lateral flow devices

- Cheap/Simple to perform and interpret/Quick TAT
- Limited throughput/Qualitative (Semi-quant?)/Antigen titres
- Potential POCT
- Revolutionized the detection of CrAg
  - Meta-analysis [a] Serum Se: 98% Sp: 98%
    CSF Se: 99% Sp: 99%
- 2017 CE marked *Aspergillus* LFD – JF5 Monoclonal AB
  - Meta-analysis [b] Serum Se: 68% Sp: 87%
    BAL **Se: 86%** **Sp: 93%**
- *Candida* LFA [c] – IgG against Enolase – Serum
  - Se: 27/38 (71%)
  - Sp: 48/50 (96%)
  - ROC AUC: 0.907





[a] Huang et al. PLOS One 2015
[b] Pan et al J Med Microbiol 2015
[c] He et al Frontiers Microbiol 2016

GIG CYMRU NHS WALES | Iechyd Cyhoeddus Cymru Public Health Wales

Aspergillus LFD/LFA summary

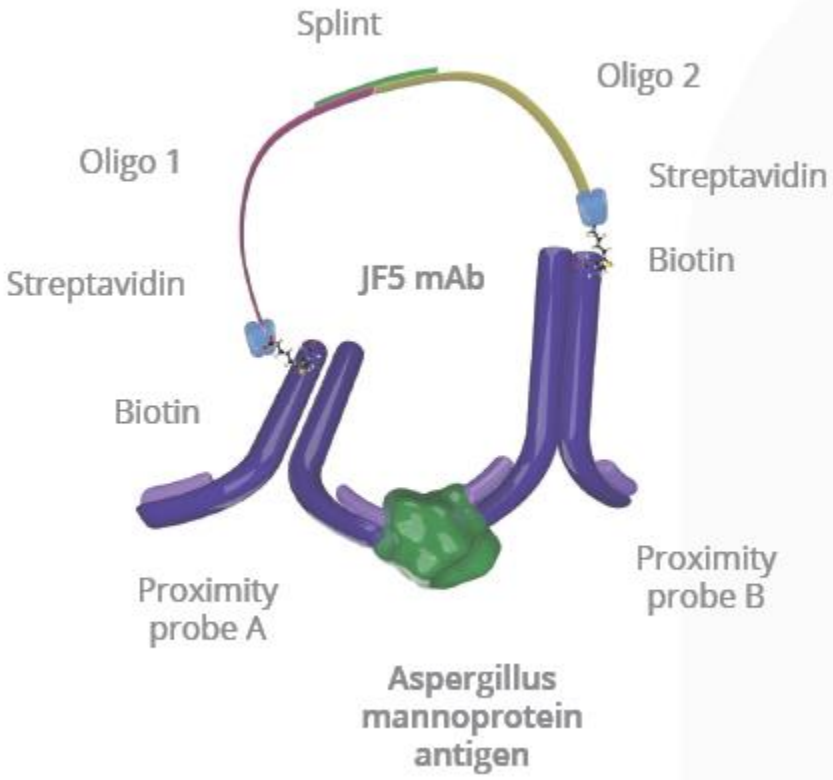
- OLM LFD [a]
- Cube reader
- One MAb
- BAL
  - Se: 86% Sp: 93%
  - LR +tive: 12.3 LR -tive: 0.15
- Serum
  - Se: 68% Sp: 87%
  - LR +tive: 5.2 LR -tive: 0.37
- Antifungal therapy

- IMMY LFA
- Cube reader
- Two MAb
- BAL [b]
  - Se: 92% Sp: 91%
  - LR +tive: 10.2 LR -tive: 0.09
- Serum [c]
  - Se: 97% Sp: 98%
  - LR +tive: 48.5 LR -tive: 0.03
- Antifungal therapy

[a] Pan et al J Med Microbiol 2015
[b] Lass-Florl et al, Clin Microbiol Infect 2019;
[c] White et al J Clin Microbiol 2020

Proximity Ligation Assays

- Specificity of Ab testing
- Sensitivity of molecular testing
- Simulated serum samples
  - LOD 4ng/mL
  - Detection 100 fold > LFD
  - Detection 10 fold > GM
- Clinical evaluation
  - BAL samples
  - 4 cases and 10 controls
  - Se: 100%
  - Sp: 100%



Johnson et al. ECCMID 2015

Other advances

- Improved access to (1-3)-β-D-Glucan assays
  - Fungitell-STAT
    - Low throughput
    - Minimal processing steps
    - Agreement with Fungitell (92%) [a]
    - Se:81% Sp: 90% [a]
  - FungiXpert Beta-D-Glucan
    - Fully automated processing

- T-Cell Immunoassay [b]
  - Isolation of mononuclear cell
  - Lack robustness/practicality
- Dual α-CD28 and α-CD49d
  - Cytokine response
  - *A. fumigatus* antigens
  - ELISA
  - Consistent results
  - 8 hour testing window
  - > 7 fold increase in IL17, IL4 and IL5 – with chronic aspergillosis

[a] White et al Manuscript in preparation
[b] Lauruschkat et al J Fungi 2021

The impact of Next Generation Sequencing

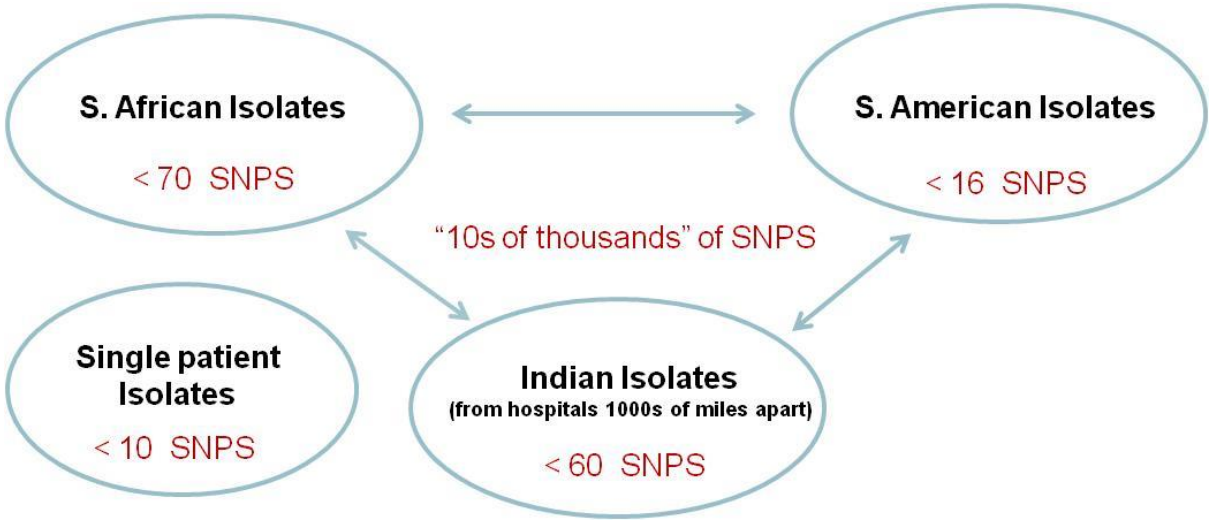
The Mycobiome

- Understanding the composition, interactions and possible pathogenicity of a microbial community within the host
  - e.g. The lung mycobiome
- Molecular assays are more efficient in describing microbial communities than culture
  - NGS of Pan-fungal PCR amplicons will be limited by the presence of the dominant fungi
- Interpretation
  - Patient cohort
  - Molecular approach
  - Sampling technique
- Microbiome versus the Mycobiome
- Candida the dominant species!!!!!! [A]
- Optimization/Bioinformatics/Cost/Genome size

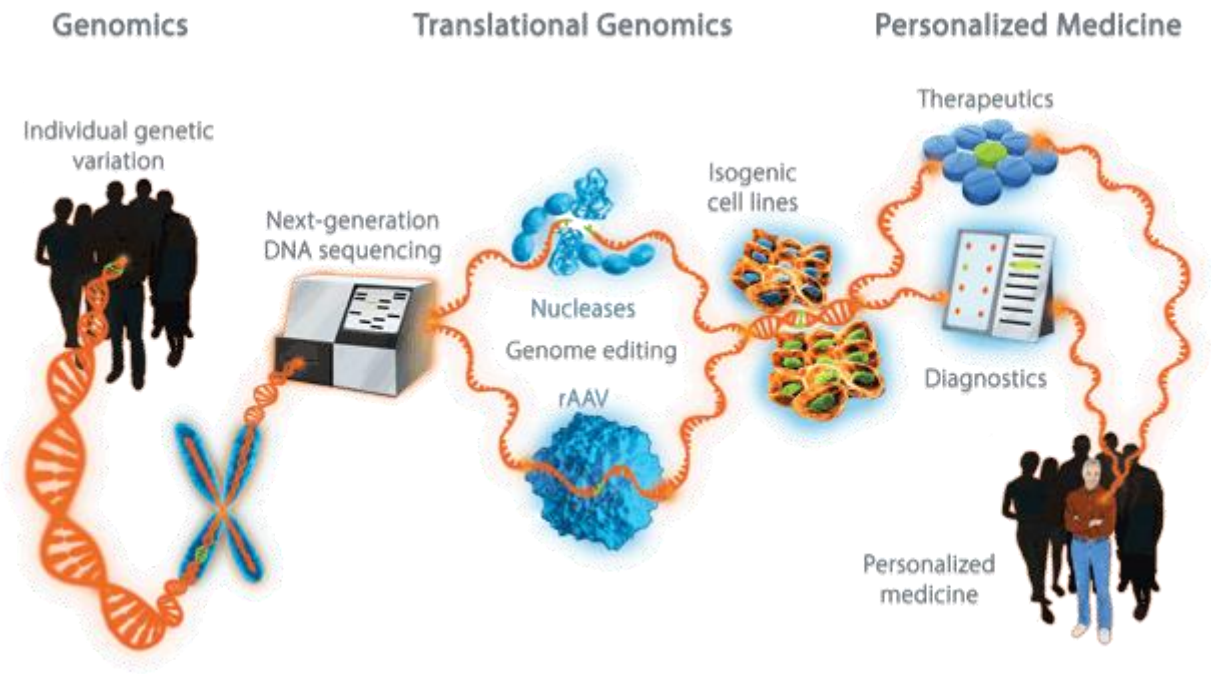
[a] Krause et al. Front Microbiol 2017

The organism

- Screening For mutations associated with resistance
  - *Aspergillus*: TR_{34}/L98H [a]
- Evolutionary studies
  - *Aspergillus*: Genetic divergence – Except for India [a]
  - *Candida auris*: Different clonal populations (S. Africa v Southern Asia/India v S American) [b]
  - Clinical and environmental associations [c]
- Epidemiological investigations?



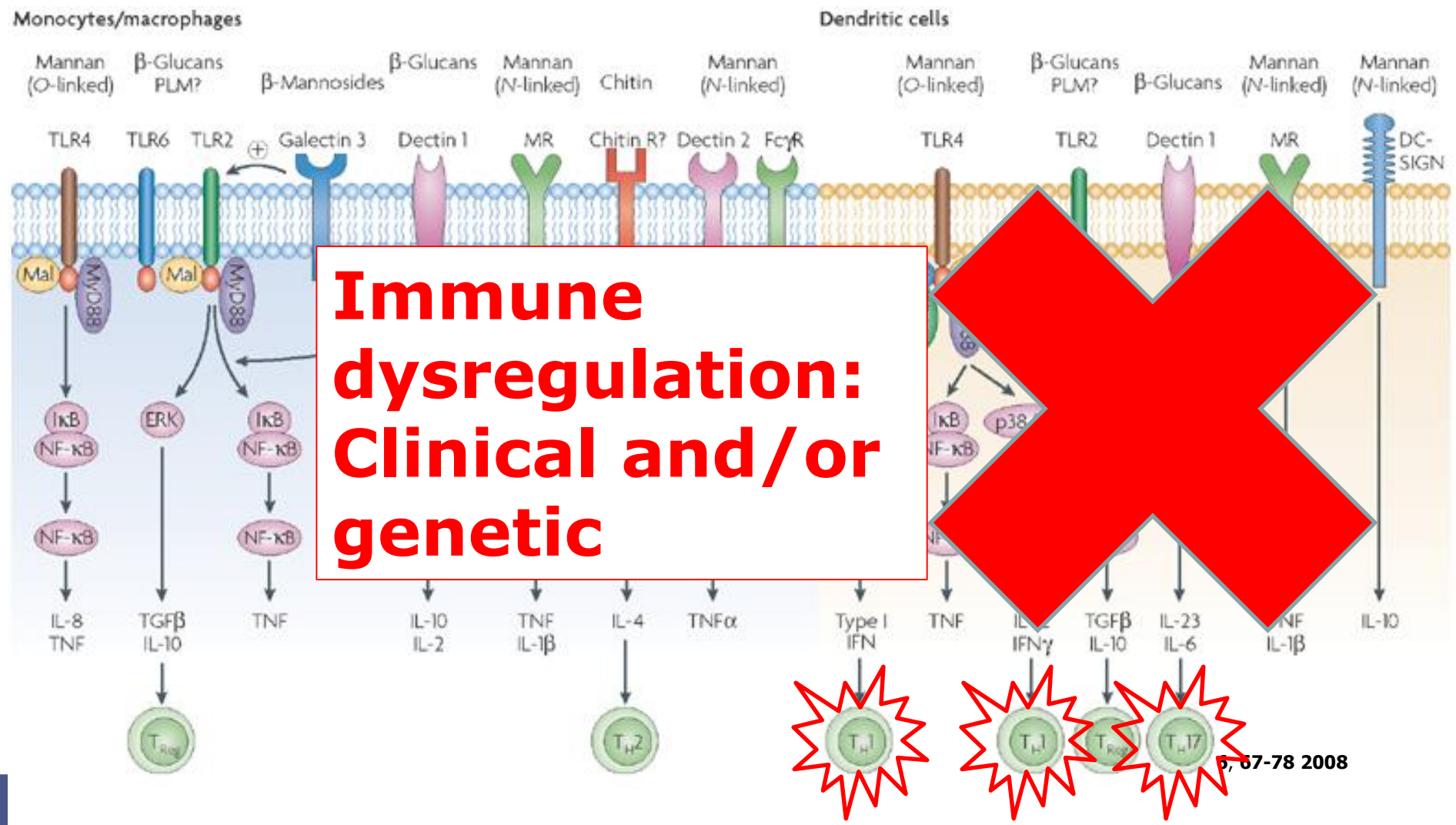
[a] Abdoulrasouli et al 2015
[b] Lochkart et al Clin Infect Dis 2016
[C] Rhodes et al bioRxiv 2021



Predicting the unpredictable

Individual risk stratification of patients at risk of IFD

Innate Immune response to fungi



Immune dysregulation: Clinical and/or genetic

5, 67-78 2008

SNPs associated with IA in haematology

SNPs previously associated with risk of IA in hematopoietic stem cell recipients

Gene	R									
AGER	7									
CLEC7A	8						G/T	0.04[§]	aHR, 2.5	1.0-6.5
									aHR, 3.9	1.5-10.0
	9						C/G	0.28	OR, 4.9	1.5-15.9
CD209	9									
						rs7248637	A/G	0.23	OR, 2.4	1.1-5.2
PTX3	10									
S100B	7	W	DNR	HCT	223 (41)	rs0722	C/T	0.18	aHR, 3.2	1.6-6.2
IFNG	11						C/T	0.48[§]	NR	*P* = .01
IFNG+	12						T/A	0.39	OR, 6.1	1.1-35.5
TLR4						rs4986790—	A/G	0.03		
TLR4	13	W	DNR	HCT	366 (103)	rs4986791[††]	C/T	0.04	HR, 8.0[‡‡]	2.5-25.3
									HR, 2.1[a]	1.0-4.5
TLR1	14						G/C	0.08	OR, 1.2	1.0-1.5
TLR1+	14	W	PT	HCT	127 (22)	rs4833095	A/G	0.35	OR, 1.2	1.0-1.5
TLR6						rs5743810	C/T	0.33		
TLR3	15									
TLR5	16									
CXCL10	17									
PLG	17									
TNFR1	18									

(RAGE) – PRP for glycoproteins

DECTIN 1 – TH17

DC-SIGN – Macrophage internalization of conidia

Pentraxin 3 – Complement Cascade

Interferon

Toll like receptors

Chemokine – IFN-γ – innate and adaptive immunity

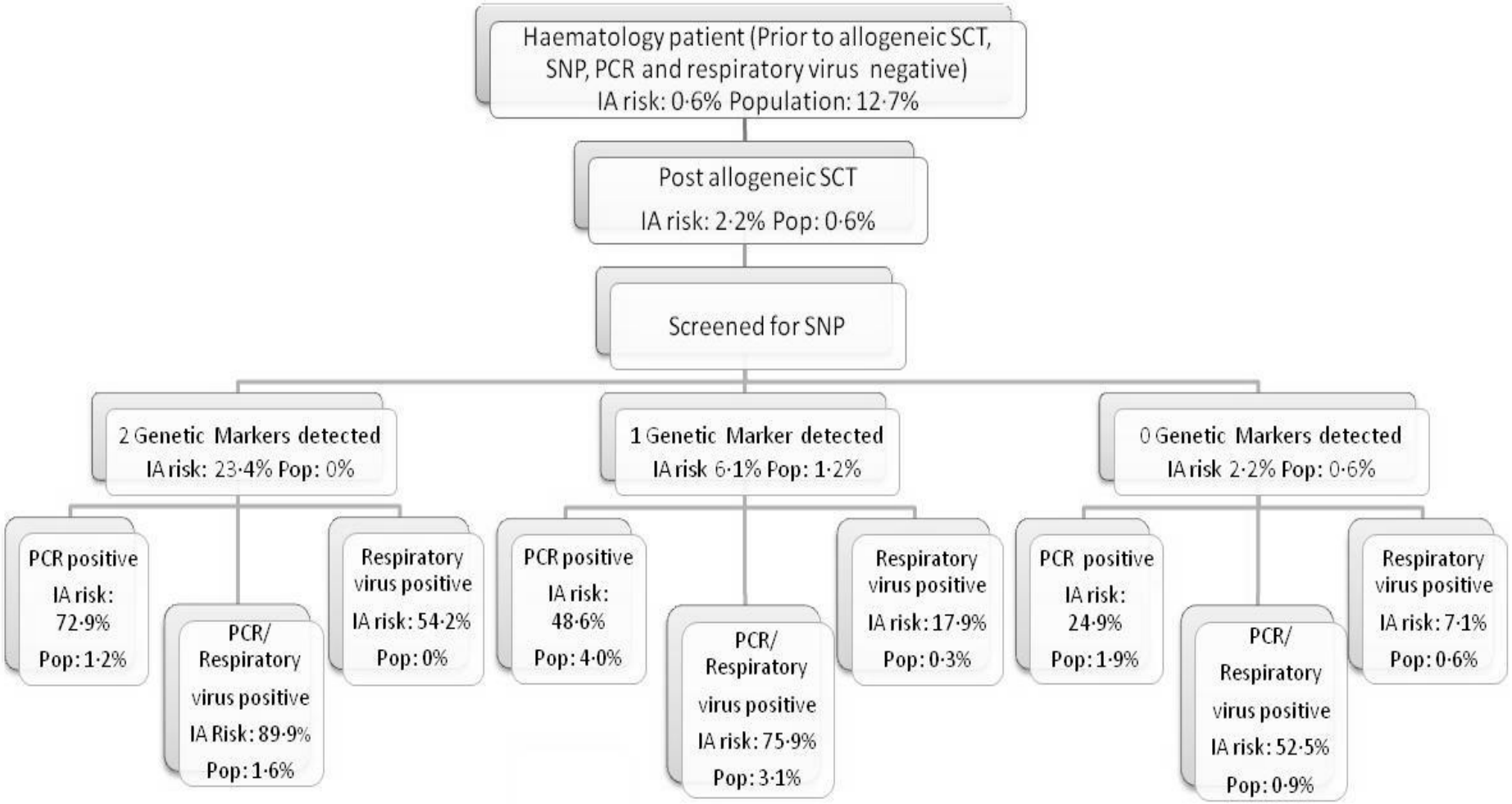
Plasminogen – Plasmin – breakdown of clots, binds to *A. fumigatus*

Tumour necrosis factor receptor

- polymorphisms in immune response genes
  - single nucleotide polymorphisms (SNPs) are frequent
  - Vary with geographical and ethnic background
- >40 SNPs have been linked to genetic susceptibility to fungal infection
  - >20 linked with IA in Haem/HSCT
- Significance determined by Odd ratio – Clinical Interpretation

Fisher CE et al Blood 2017; Cunha and Carvalho Med Mycol 2019

Predicting IA in BMT recipients



White et al J Clin Microbiol 2018

Summary

- Increased availability of molecular diagnostics
  - Detection of resistance
- Combining diagnostic tests is paramount
- POCT tests?
- Novel tests becoming available
  - need further evaluation
- NGS – Targeting the organism, microbiome and the host
- Combining clinical and genetic risk factors may further improve diagnostic strategies
  - Personalised medicine
  - Improve prescribing accuracy
  - Need to automated computation of predicted risk
- Further reading: White PL and Price JS, J Fungi 2021, 7, 41