Aspergillus Diagnostics

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27th September 2018

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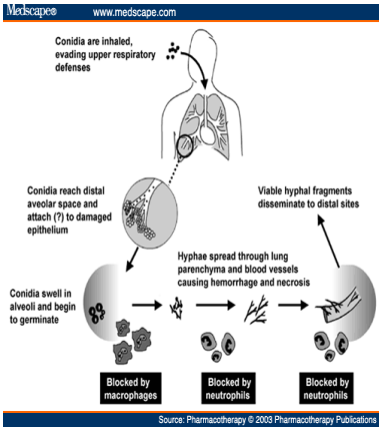
Aspergillosis – Disease process



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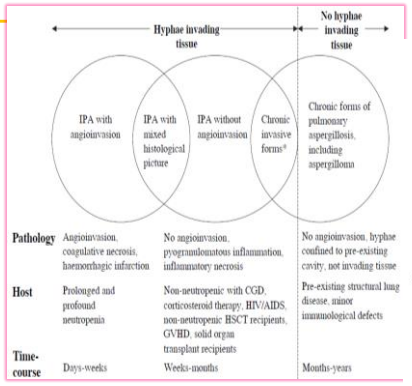
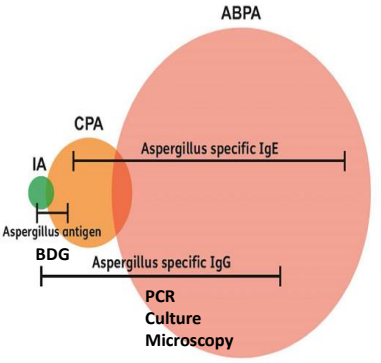
Aspergillus Manifestations – Testing options





Hope et al 2015; Page et al 2015

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Conventional Approaches

Reference standard

- Culture of fungi from deep or sterile anatomical site
- Histological/Microscopic evidence of fungal elements in tissue
- Highly specific / Poor sensitivity

De Pauw et al 2008

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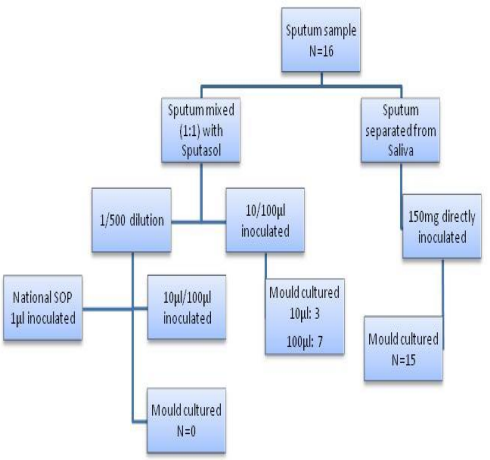
Culture

- Benefits
  - Applied to wide range of specimens
  - Broad-detection range
  - Isolate available for further adjunctive testing
    - Identification
    - Susceptibility
    - Typing
    - NGS
- Limitations
  - Sensitivity (<50%)
  - Turn around time

Optimization

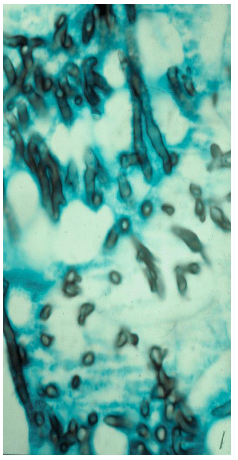
- Sample type
  - Sputum vs BAL
- Testing frequency
- Dilution factor
- Inoculation volume
- Media composition
  - Media B+ vs SDA
  - Improved recovery of yeasts and moulds



Fraczek et al 2014, Denning et al 2016; Pashley et al 2012; Nagano et al 2008

Microscopy

- Benefits
  - Applied to wide range of specimens
  - Part of culture ID
  - Broad-detection range
  - Rapid
- Limitations
  - Sensitivity
  - Limited differentiation
    - Aspergillus from hyaline moulds
  - No isolate for further adjunctive testing
  - Requires experienced operative
- Optical brighteners (Calcofluor)/Histological stains (Silver stains/PAS)



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Biomarker Approaches for IA

- Molecular
- Serology
  - β-D-Glucan
  - Antigen Assays
    - LFD
    - Sandwich ELISA
  - Antibody assays

- Rapid results
- High Sensitivity
- High Specificity

- Performance may be limited to specific cohorts
- False positives
- False negatives
- Variable performance

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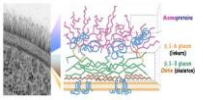
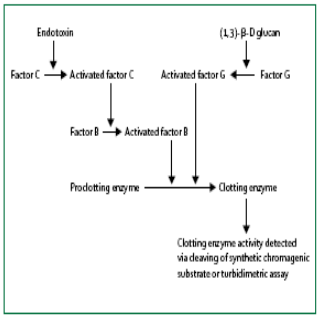
GM-ELISA

- Aspergillosis
- Sandwich ELISA assay (galactomannan EIA)
  - Monoclonal antibody
  - Galactofuran side-chain
- Serum (plasma) and BAL
- False positives
  - Antibiotics, Blood products (Plasmalyte)
  - TPN, Foods, Paediatrics
  - Cross reactivity (e.g Penicillium sp, Fusarium sp)
- False negatives
  - AFT
  - Disease with limited (angio)invasion
- Recommended in guidelines (e.g. EORTC/MSG, IDSA, ECIL)
- First FDA Clinical biomarker for IA



β-D-Glucan

- Broad fungal biomarker
  - Except Cryptococcus and Mucorales
    - Differentiation of IFD?
  - 6 different commercial tests
    - Heterogenous data :
      - retrospective vs. prospective
      - ICU vs haematology
      - Candida versus mould
    - Different cut offs
  - Serum/Plasma only
  - Sensitivity, specificity variable but NPV high
    - False-positives: - bacteraemia, antibiotics, surgical gauze, haemodialysis, Crohn's disease, Burns, CF
    - Interference: Bilirubin, haemoglobin, triglycerides
  - Utility data limited
  - Recommended in guidelines (IDSA, EORTC/MSG, NICE)



Molecular tests

- Benefits
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legatives

Assay	Sample type	Sensitivity (%)	Specificity (%)
Microgen MycAssay Aspergillus	BAL[a]	80-100	88-99
	Serum/Plasma[b]	25-60	83-100
Pathonostics Aspergenius			1
			1
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

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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							itis[g]
Sample	Serum	Serum	Plasma/Serum	Plasma/Serum	Plasma/Serum	Blood	Blood
Sensitivity	79.3%	79.3%	56.8%				
Specificity	80.5%	86.3%	97.0%				

Comparable performance despite limited standardisation

PCR LR-tive: 0.15 = Negative results exclude Ia[f]
Isolated testing?

[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

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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

BDG?

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

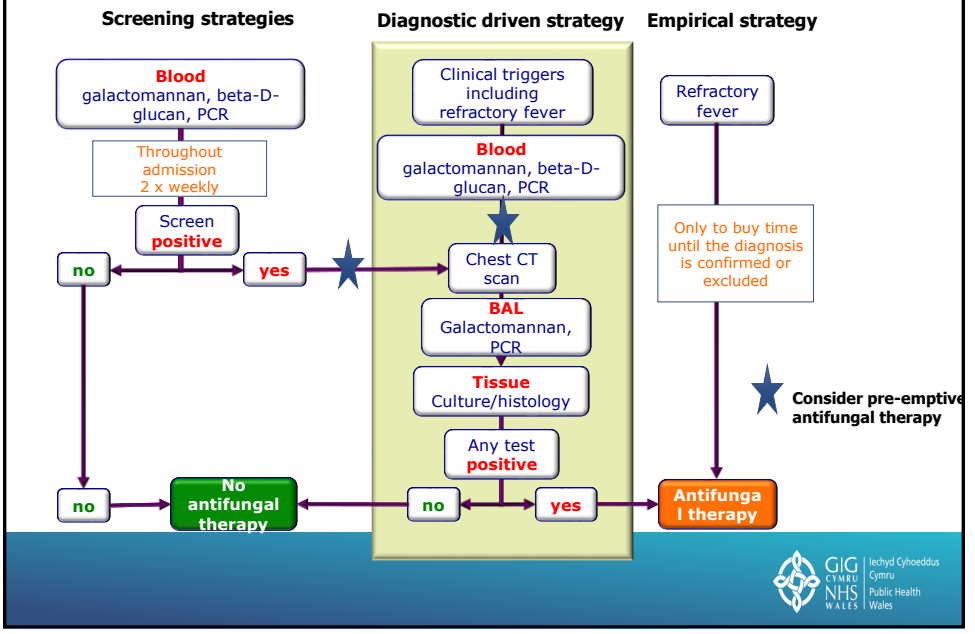
Combined molecular/serological testing for IA

	Aguado	Morrissey	Barnes
empirical therapy	↓	↓	↓
Earlier diagnosis	✓	✓	✓
antifungal usage	↓	↓	↓
Proven probable disease	↓	↑	→ ↓
Mortality	↓	→	→

GM/PCR Meta-analysis Se: 99% Sp: 98%

Aguado J M et al Clin Infect Dis. 2014
Morrissey CO et al Lancet 2013
Barnes RA J Infect 2013; Arvanitis et al 2015

Combination testing - Different strategies (IA)



Standardization of *Aspergillus* PCR

ISHAM WORKING GROUP EUROPEAN ASPERGILLUS PCR INITIATIVE EAPCRI

FPCRI FUNGAL PCR INITIATIVE ISHAM

ASTEC ASPERGILLUS TECHNOLOGY CONSORTIUM

IAAM INVASIVE ASPERGILLOSIS ANIMAL MODELS

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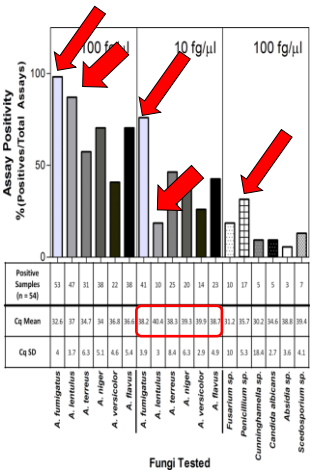
Analytical Specificity

- Current *Aspergillus* PCR assays are better suited for detecting *A. fumigatus*
- Inferior detection of other *Aspergillus* species.
  - Geographical variation is important when developing assays
- Genus sp assays increase detection but compromise analytical specificity
- Current strategies for IA are better suited to excluding disease – assay sensitivity is paramount
- The use of an *Aspergillus* genus specific PCR assay targeting the rRNA genes is preferential.



Morton et al. Medical Mycology 2016

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Extraction recommendations

ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY | WORKING GROUP EUROPEAN ASPERGILLUS PCR INITIATIVE EAPCRI

"Nucleic Acid extraction is the critical step"

	Serum/Plasma Testing	Whole Blood testing
Sample type	N/A	EDTA only
Sample volume	≥0.5ml	≥3.0ml
Potential target	Free circulating DNA	Phagocytosed fungal fragments
Extraction requirements	Commercial extraction kits	Red and White cell lysis, Bead-beating, commercial extraction kits.
Elution volume	Elute in <100µl,	
Special requirements	Screen all reagents for contamination with fungal DNA. Positive and negative extraction controls recommended. PCR testing in duplicate. Perform an IC PCR (Ct typical of Aspergillus PCR +tve, not human DNA, can be incorporated into extraction process)	

FPCRI FUNGAL PCR INITIATIVE ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

BAL testing – Finalizing analysis

White *et al* 2010a, 2010b, 2011 and Loeffler et al. 2015

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Dynamics of release

- IAAM - Guinea pig inhalation IA model
  - Euthanized: 1 hour, 5, 7 and 9 days
- P(
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- Combination testing optimal

Assays	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC
WB PCR/GM/BDG	**100**/67/14	78/96/**100**	89/82/59	**0.95**
Serum PCR/GM/BDG	**100**/48/33	61/96/**100**	80/73/68	0.88
WB/Serum PCR/BDG	90/71/10	65/**100**/**100**	77/86/57	0.90
WB/Serum PCR/GM	**100**/81/14	57/91/**100**	77/86/59	0.92
All four assays	**100**/91/44/10	57/91/**100**/**100**	77/91/73/57	**0.95**

White et al. JCM 2016

The impact of standardization

- EAPCRI recommendations improved PCR performance
  - Independent analysis
- Sensitivity: 87% vs 82%
- Specificity: 98% vs 85%
- Not total compliance with EAPCRI recommendations (1 deviation)

Arvanitis M, et al. *J Clin Microbiol*. 2014;52:3731–42

On-going work

- International PCR standard
  - Independent International Calibrator[a]
  - Compare performance of PCR systems
  - Develop international standard for PCR
  - Negates the need for an individual test?
    - Multiple PCR assays for other infections exist
    - HIV/HEP C/CMV – international standard
- Source material: Plasma

- Evaluation of Standardized PCR protocol in two RCT
  - Ambiguard
  - EORTC 65091 – 0693 RCT
    Empirical Versus Pre-emptive (Diagnostic-driven) AFT of Haem/Allo SCT

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[a] Lyon *et al.* 2013

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EORTC-MSG Definitions for IFD – 2nd revision

- *Aspergillus* PCR
  - Continued efforts to standardise
    - EAPCRI
    - Commercial Assays

- There is sufficient evidence, that is at least equivalent to that used for galactomannan-EIA and β-D-Glucan testing, that PCR is now mature enough for inclusion in the EORTC/MSG definitions.
  - BAL/Blood (serum/plasma)
  - ≥2 positive results
  - Combined biomarkers?

FPCRI FUNGAL PCR INITIATIVE ISHAM

ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY WORKING GROUP EUROPEAN ASPERGILLUS PCR INITIATIVE EAPCRI

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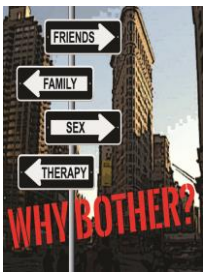
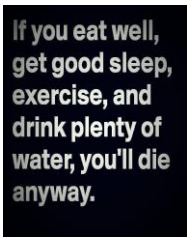
Diagnosis of IA in ICU

Why bother?

- Incidence of IFD in ICU
  - FIRE Study [a] – 61K UK ICU Admissions
  - 0.6% (94% Candida)
- No need to use diagnostics?
  - Classical methods inadequate
  - Incidence is under-estimated
- Why use prophylaxis or empirical therapy?
  - What fungi does the strategy cover?
  - Over use of AFT drives resistance
    - Environmental azole resistance

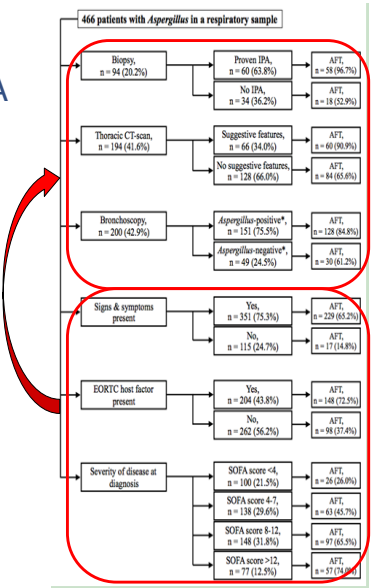




[a] Harrison et al. 2013

Does diagnosis matter?

- Low Pre-test probability of IA
- Limited sensitivity
- Poor LR -tive
- Low diagnostic uptake:
  - BAL/CT: <50%
  - GM: 13%
  - PCR/BDG: 0.6%
- No difference in Mortality
  - AFT v No AFT in colonized
  - AFT v No AFT in IA
- Incorporate advances in diagnosis of IPA



Paiva et al. 2017

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B-D-Glucan versus GM in ICU

- 49 Immunosuppressed pts with Respiratory failure/Mech Vent
  - 13pts with probable IA
  - BAL and CT
  - 64% Haem, 16% autoimmune, 12% SOT, 8% Solid Ca
- BDG Fungitell (serum) vs GM-EIA (serum and BAL)
  - Serum GM Se: 31% Mean GMI: 4.2
  - BAL GM Se: 92% Mean GMI: 3.7
  - BDG Se: 88% Mean: 375pg/ml
- CRP: IA: 26.2 No IA: 19.4 PCT: IA: 22.4 No IA: 10.9
- Prognostic Marker in 47 cases of IA – Twice weekly GM/BDG Reduction within 2 weeks AFT or Persistent Negativity in first two weeks = improved survival rates at 6 and 12 weeks

Lahmer et al J Crit Care 2016;
Neofytos et al Plos One 2015

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Molecular Diagnosis in ICU

- Commercial assays
- BAL testing [a,b]
  - MycAssay Aspergillus [a] (n=158) (Incl 90 ICU pts)
  - PCR — Se: 94% (100% for ICU) Sp: 99% LR+:67 LR-: 0.06
  - Micro/Cult — Se: 41% (25-50% for ICU)
  - MycAssay Aspergillus [b] — Se: 100% Sp: 100%
- Molecular tests are the only non-culture methods capable of determining resistance[c]
  - Pathonostics Aspergenius Assay
  - BAL 8/11 azole profiles were directly determined

[a] Torelli et al. 2011

[b] Orsi et al. 2012, [c] Chong et al 2015

Paediatrics - IA

- Limited data
- Variable performance
  - GM Meta-analysis Serum — Se: 68% Sp: 91% (Screening); Se: 89% Sp: 85% (Diagnostic)
  - GM BAL — Few studies – Invasive sampling
  - BDG — Se: 50-83% Sp: 29-82%
  - PCR (Blood) — Se: 0-100% Sp: 36-85%
- Performance may differ from adults
  - False positive GM – *Bifidobacterium*
  - High background BDG in healthy children
- Biomarkers not recommended in recent guidelines
- A comprehensive/international effort is needed
- Non-Invasive Diagnosis of Pediatric Pulmonary Invasive Mold Infections
  - PI: Bill Steinbach
  - 2018-2023

Lehrnbecher et al 2016, 2017, 2018

Chronic Pulmonary Aspergillosis

ERS and ESCMID guideline 2015

TABLE 6 Antibody diagnosis of chronic pulmonary aspergillosis (CPA)

Population	Intention	Intervention	SoR	QoE	Ref.	Comment
Cavitary or nodular pulmonary infiltrate in non-immunocompromised patients	Diagnosis or exclusion of CPA	*Aspergillus* IgG antibody	A	II	[42–44]	IgG and precipitins test standardisation incomplete
		Aspergillus precipitins	A	II	[29, 43, 45–47]	Mostly in-house tests and poorly validated; uncertain sensitivity is the major problem

IDSA guideline for aspergillosis 2016

more underlying pulmonary disorders. The *Aspergillus* IgG antibody test is the most sensitive microbiological test (*strong recommendation; moderate-quality evidence*). Sputum *Asper-*

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Comparison of *Aspergillus* IgG Test

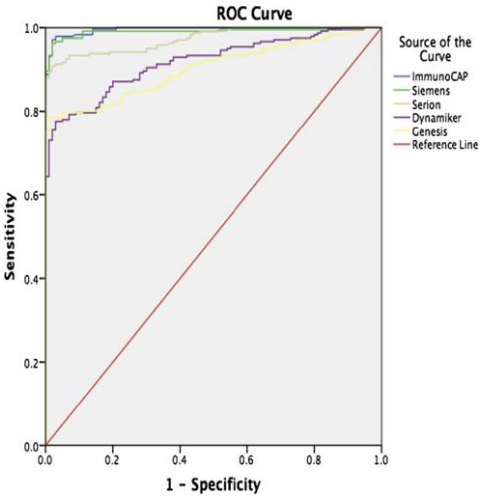

Table 4 Performance of potential diagnostic cut offs.

Assay (Unit)	Diagnostic cut-off	Sensitivity	Specificity	Youden's J statistic	AUC:
ImmunoCAP (mg/L)	10	100%	86%	0.86	0.99
	20	96%	98%	0.94	
	30	91%	99%	0.9	
	40	88%	100%	0.87	
Immulite (mg/L)	5	99%	78%	0.77	0.99
	10	96%	98%	0.94	
	15	94%	98%	0.92	
	20	93%	99%	0.92	
	30	91%	99%	0.9	
Serion (U/ml)	30	91%	95%	0.86	0.97
	35	90%	98%	0.88	
	40	88%	99%	0.87	
	45	85%	100%	0.85	
Dynamiker (AU/ml)	60	78%	94%	0.72	0.92
	65	77%	97%	0.74	
	70	75%	98%	0.73	
	75	73%	99%	0.72	
Genesis (U/ml)	10	86%	65%	0.51	0.90
	15	79%	95%	0.74	
	20	75%	99%	0.74	
	25	71%	100%	0.71	
Precipitins	–	59%	100%	0.59	

Optimal diagnostic cut-offs are highlighted in bold.

Page et al J Infect 2016

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Allergic Bronchopulmonary Aspergillosis – ABPA

CF – Consensus guidelines

Total serum IgE, IU/mL	Pulmonary symptoms and/or worsening PFT results	New infiltrates on CR or CT	Positive serology[a]	Treatment recommendation(s)
>1000 or >2-fold rise from baseline	Yes	Yes	Yes	Treat for ABPA
>1000 or >2-fold rise from baseline	No	No	Yes	No treatment; monitor IgE, CR, PFT
>1000 or >2-fold rise from baseline	No	Yes	Yes	Treat for CF-related infection; consider treatment for ABPA if no response
>1000 or >2-fold rise from baseline	Yes	No	Yes	Consider treatment for ABPA, CF-related infection, and/or asthma
>500 in the past; no change from baseline	Yes	Yes	Yes	Treat for CF-related infection; consider treatment for ABPA or asthma if no response
500–1000	Yes	Yes	Yes	Treat for ABPA

NOTE. CR, chest radiography; PFT, pulmonary function testing.

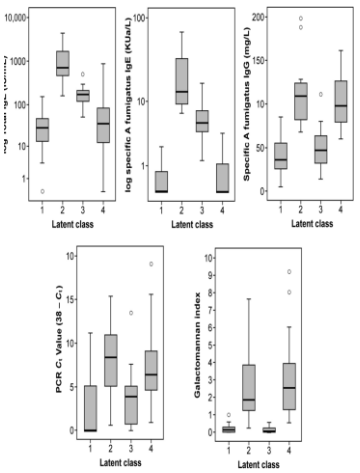
[a] Aspergillus-specific IgG or IgE or presence of precipitins to *Aspergillus fumigatus*. Because these test results may not be available quickly, they are not required for initiation of therapy but should be obtained.

- What about the other combinations?
- What about the other disease manifestations?

Stevens *et al*. Clin infect dis. (2003) 37 (suppl3): S225-S264

ABPA

Immunological Diagnosis



- Class I: Non-diseased
  - ±Asp in sputum
  - - GM/Immune response
- Class II: Serological ABPA
  - + PCR/GM/Immune response
- Class III: *Aspergillus* IgE-Sens
  - ±Asp in sputum
  - + IgE
  - - IgG/GM
- Class IV: *Aspergillus* bronchitis
  - - IgE
  - + IgG, PCR and GM

Baxter et al 2013

Further considerations

Lateral Flow devices

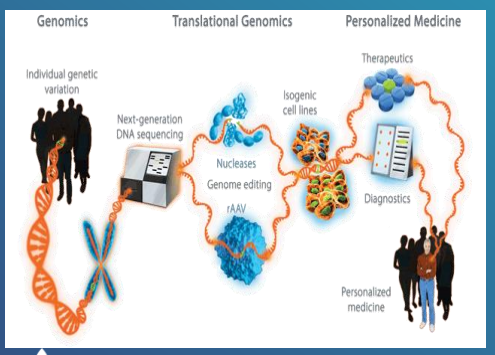
- Cheap/Simple/Quick
- Limited throughput/Qualitative (Semi-quant?)/Antigen titres
- Potential POCT
- Revolutionized the detection of CrAg
  - Meta-analysis [a] Serum Se: 98% Sp: 98%; CSF Se: 99% Sp: 99%
- 2017 CE marked *Aspergillus* LFD – JF5 Monoclonal AB
  - Meta-analysis [b] Serum Se: 68% Sp: 87%; BAL **Se: 86%** **Sp: 93%**
  - Serum - Combination testing with PCR[c]
    Se: 100% if either test was required positive
    Sp: 100% if both tests were positive
  - BAL – Combination testing with PCR [d]
    Se: 100% if either test was required positive

Urine LFD – MycoMed
GM LFD - IMMY

[a] Huang et al. PLOS One 2015; [b] Pan et al J Med Microbiol 2015
[c] White et al J Clin Microbiol 2013; [d] Johnson et al J Clin Microbiol 2015

Predicting the unpredictable

Individual risk stratification of patients at risk of IA



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Study population

- 322 haematology patients
- 54 patients with proven/probable IA
- 268 patients with NEF
- 225 patients with evidence not meeting EORTC/MSG criteria excluded

- Incidence of IA: 16.7%
- Combining clinical risk, with genetics and biomarkers

White et al J Clin Microbiol 2018

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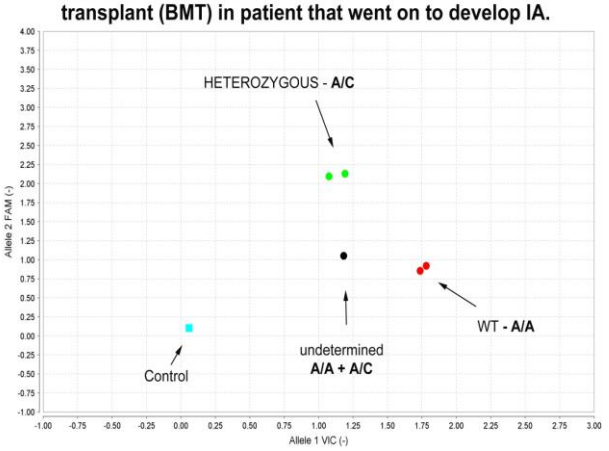
Genetic Screening

- C-type Lectin receptor genes
  - DC-SIGN
  - Dectin-1
  - Previously associated with IA
  - Commercially available real-time assays

Dectin-1 SNP (rs169) Genotyping:
Introduction of Polymorphism following bone marrow transplant (BMT) in patient that went on to develop IA.



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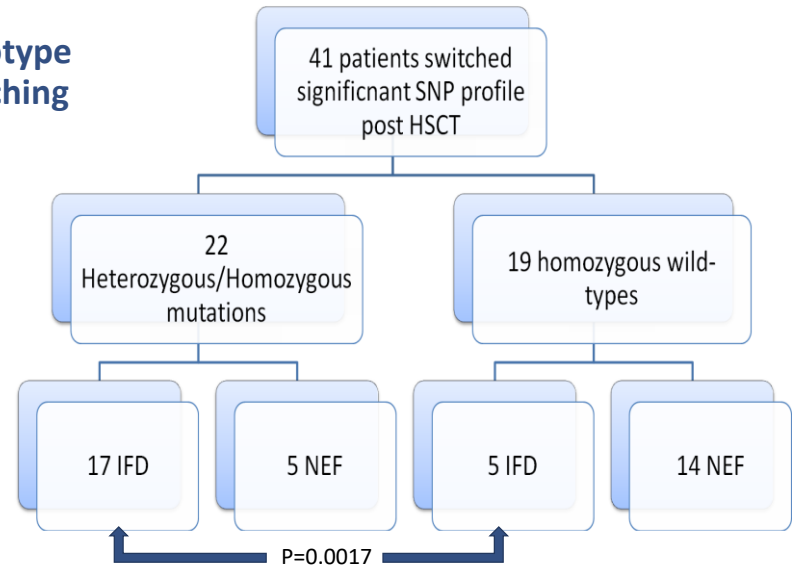
Genotype Switching



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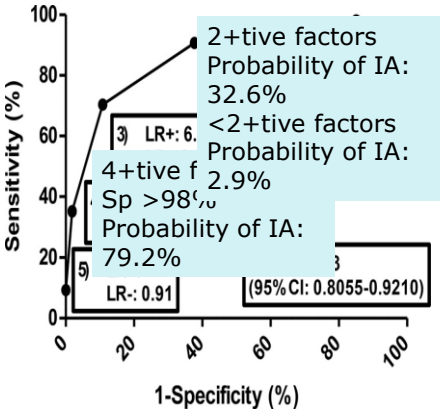
Final Predictive model



- Final Risk Variables:
  - Allogeneic SCT
  - SNP in Dectin 1 rs7309123
  - SNP in DC-SIGN Haplotype
  - Aspergillus PCR positivity
  - Respiratory virus infection

An optimal threshold 3 factors

Mean Se/Sp of: 70.4%/89.2%

Probability of IA: 56.7% (64% for BMT patients)

Probability of IA <3 factors: 6.3%

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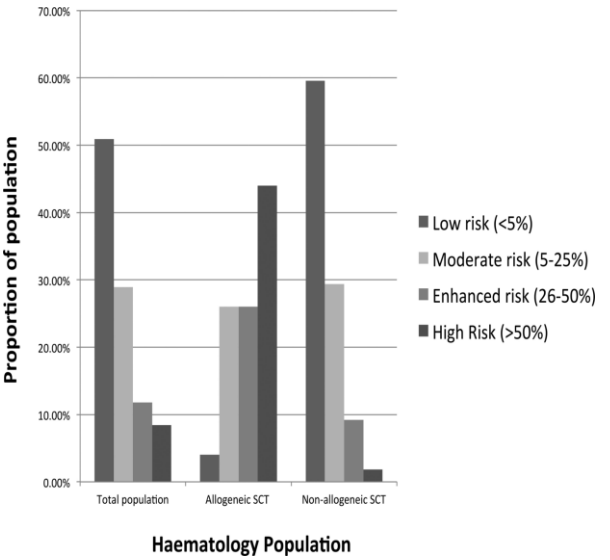
Breakdown of Risk

Threshold for pre-emptive therapy = 50%
8.3% of the total population treated

Increasing population receiving RIC-MUD tx



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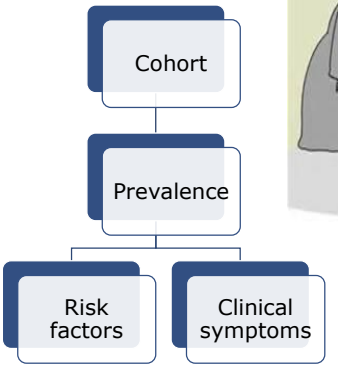
Concluding remarks

- Know your patient
- Understand your test
  - How best to use it
  - Will the result alter management
- Combination testing is optimal
- AFT Resistance is coming!



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