PULMONARY ASPERGILLOSIS IN ASSOCIATION WITH TUBERCULOSIS AND HIV IN UGANDA

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy (PhD)
in the Faculty of Medical and Human Sciences.

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IAIN DUNSMUIR PAGE

SCHOOL OF MEDICINE / Institute of Inflammation and Repair
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ABBREVIATIONS

AAFB Acid and alcohol fast bacilli
ABPA Allergic bronchopulmonary aspergillosis
AIDS Acquired immunodeficiency syndrome
AU Arbitrary units
AUC Area under the curve
BAL Broncho-alveolar lavage
CCPA Chronic cavitary pulmonary aspergillosis
CD4 Cluster of differentiation 4
CF Cystic fibrosis
CFPA Chronic fibrosing pulmonary aspergillosis
CGD Chronic granulomatous disease
CI Confidence interval
CIE Counterimmunoelectrophoresis
COPD Chronic obstructive pulmonary disease
CNPA Chronic necrotizing pulmonary aspergillosis
CPA Chronic pulmonary aspergillosis
72
73 CT Computed tomography
74
75 CV Co-efficient of variation
76
77 CXR Chest X-ray
78
79 DD Double diffusion
80
81 DR Congo Democratic Republic of Congo
82
83 ELISA Enzyme-linked immunosorbent assay
84
85 EORTC European Organization for Research and Treatment of Cancer
86
87 ESCMID European Society of Clinical Microbiology and Infectious Diseases
88
89 FEIA Fluoroenzymeimmunoassay
90
91 GAFFI Global Action Fund for Fungal Infections
92
93 GM Galactomannan
94
95 GRRH Gulu Regional Referral Hospital
96
97 GVHD Graft versus host disease
98
99 HA Haemagglutination
100
101 HIV Human immunodeficiency virus
102
103 IA Invasive aspergillosis
104
<table>
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<tr>
<th>Page</th>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>105</td>
<td>IAV</td>
<td>Intra-assay variability</td>
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<td>106</td>
<td>IHA</td>
<td>Immunohaemagglutination</td>
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<td>107</td>
<td>ICU</td>
<td>Intensive care unit</td>
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<td>108</td>
<td>IDSA</td>
<td>Infectious Diseases Society of America</td>
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<td>109</td>
<td>IMMY</td>
<td>Immuno-Mycologics</td>
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<td>110</td>
<td>ISHAM</td>
<td>International Society for Human and Animal Mycology</td>
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<td>111</td>
<td>IRB</td>
<td>Institutional Review Board</td>
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<td>112</td>
<td>JCRRC</td>
<td>Joint Clinical and Research Centre</td>
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<td>113</td>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<td>114</td>
<td>LA</td>
<td>Latex agglutination</td>
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<td>115</td>
<td>LFD</td>
<td>Lateral flow device</td>
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<td>116</td>
<td>ManRAB</td>
<td>Manchester Respiratory and Allergy Biobank</td>
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<td>117</td>
<td>MIND-IHOP</td>
<td>Mulago Inpatient Noninvasive Diagnosis – International HIV Opportunistic Pneumonia</td>
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<td>118</td>
<td>MRI</td>
<td>Manchester Royal Infirmary</td>
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<td>119</td>
<td>MTA</td>
<td>Material Transfer Agreement</td>
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<td>120</td>
<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
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<td>138</td>
<td>MRC</td>
<td>Medical Research Council</td>
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<td>140</td>
<td>NAC</td>
<td>National Aspergillosis Centre</td>
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<td>142</td>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>144</td>
<td>RAST</td>
<td>Radioimmunoassay</td>
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<tr>
<td>146</td>
<td>ROC</td>
<td>Receiver operating characteristic</td>
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<tr>
<td>148</td>
<td>SAFS</td>
<td>Severe asthma with fungal sensitization</td>
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<td>150</td>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>152</td>
<td>TB</td>
<td>Tuberculosis</td>
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<td>156</td>
<td>TREGS</td>
<td>T-regulatory cells</td>
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<tr>
<td>158</td>
<td>UHSM</td>
<td>University Hospital of South Manchester</td>
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<td>160</td>
<td>UK</td>
<td>United Kingdom</td>
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<td>162</td>
<td>UNCST</td>
<td>Uganda National Council for Science and Technology</td>
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<td>164</td>
<td>USA</td>
<td>United States of America</td>
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<td>166</td>
<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Chronic pulmonary aspergillosis (CPA) is a serious disease that occurs secondary to tuberculosis and is estimated to affect 1.2 million persons globally. Pulmonary aspergillosis is found in 2-3% of all AIDS autopsies, but 90% of cases go undiagnosed ante-mortem. Here the sensitivity and specificity of optimal diagnostic thresholds for CPA have been defined in relation to six *Aspergillus*-specific IgG assays. The prevalence of CPA in an area of high tuberculosis prevalence has been measured.

Receiver operating characteristic (ROC) curves were used to compare results of testing with six *Aspergillus*-specific IgG assays in 241 patients with CPA and 100 healthy controls. ThermoFisher Scientific ImmunoCAP and Siemens Immulite had ROC area under curve (AUC) results of 0.995 and 0.991 respectively. Both were statistically significantly superior to all other assays. Both had a sensitivity of 96% and specificity of 98% using diagnostic cut offs of 20 mg/L and 10 mg/L respectively.

Eighty patients with allergic bronchopulmonary aspergillosis (ABPA) were also assessed. ROC AUC results were 0.959 for ImmunoCAP and 0.932 for Immulite. The new thresholds produced specificities of 98% for both assays and sensitivities of 78% and 81% respectively. Levels in ABPA patients were also compared to asthmatic controls.

398 patients with treated tuberculosis in Gulu, Uganda were assessed in a cross-sectional survey. CCPA diagnostic criteria were; 1 – Cough or haemoptysis for one month, 2 – Progressive cavitation on serial chest X-ray or either paracavitary fibrosis or aspergilloma on CT scan and 3 – Raised Siemens Immulite *Aspergillus*-specific IgG. All three were required for diagnosis. CCPA was present in 5.7% of patients and simple aspergilloma in 0.7% of patients. There was a non-significant trend to less frequent CCPA in HIV positive patients (p=0.18).

*Aspergillus*-specific IgG levels were measured in stored sera from two adult in patient groups at Mulago Hospital, Kampala, Uganda. 26% of 39 patients with HIV infection and subacute respiratory illness and no diagnosis after extensive investigation had raised levels. 47% of 57 patients with proven active pulmonary tuberculosis had raised levels.

The Immulite and ImmunoCAP assays both have good sensitivity and specificity for the diagnosis of CPA. New diagnostic thresholds improve the performance of all assays. CCPA has been shown to complicate pulmonary tuberculosis in Gulu, Uganda. Subacute invasive pulmonary aspergillosis is likely to affect many patients with AIDS and subacute respiratory illness. CPA may begin during active pulmonary tuberculosis infection. CPA associated with tuberculosis constitutes a significant unrecognized public health problem, which is probably being incorrectly identified as ‘smear-negative tuberculosis’ clinically and in public health data. Prospective studies are now needed to confirm the prevalence of CPA secondary to tuberculosis and define the optimal strategy for routine CPA screening, followed by studies to define optimal treatment regimes for use in research poor-settings, where most cases of CPA are likely to occur.
AUTHOR DECLARATION

The author has not submitted any portion of the work referred to in the thesis in support of an application for another degree or qualification of this or any other university or other institute of learning.

Results of Aspergillus-specific IgG from 100 healthy controls are compared to other groups throughout this thesis. These results from healthy controls were also used by Mr. Richard Kwizera as part of his 2014 MSc (Medical Mycology) thesis at The University of Manchester. In this work he compares results in healthy controls to patients with chronic obstructive pulmonary disease (COPD). While the author provided some assistance to Mr. Kwizera on this project, his role was peripheral and as a result it does not form part of this PhD thesis.
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DEDICATION

The author would like to thank his supervisors Profs David Denning, Malcolm Richardson and Angela Simpson for all their guidance and support throughout this project. Many thanks are due to Dr Julie Morris for her assistance with statistical planning and analysis throughout this work. I am also grateful to the staff of the Mycology Reference Centre at University Hospital of South Manchester and the pathology laboratory of Christie Hospital, Manchester for providing training and support throughout the laboratory aspects of this work.

Further thanks are due to the UHSM Academy charity and to the commercial companies Astellas Pharma, Siemens Immulite, Serion, Genesis, Dynamiker and OLM Medical, all of whom provided support to this work in the form of grants, donations of test kits or provision of accommodation and insurance. Without this support this study would not have been possible.

This study has required many collaborative efforts. Particular thanks are due to John Opwonya and the staff at the Gulu District Health Office, who played a critical role in patient recruitment for the main CPA prevalence study in Gulu. The reporting of chest X-rays was crucial to this study and required a substantial commitment by Dr Cyprian Opira (Senior Radiologist and Clinical Director, St. Mary's Hospital, Lacor, Uganda), Dr Sharath Hosmane (Specialty Radiology Registrar, University Hospital of South Manchester) and Dr Richard Sawyer (Senior Consultant Radiologist, University Hospital of South Manchester).

I am also indebted to Mr. Nathan Onyachi (Clinical Director, Gulu Hospital) for his assistance in planning the Gulu survey and to Drs. William Worodria, Alfred Andama, Irene Akaka and the MIND-IHOP study group at Mulago Hospital, Kampala. This substantial study was undertaken in collaboration with the University of California, San Francisco aiming to identify the range of conditions present in patients admitted with chronic cough. They were kind enough to provide me with stored sera from selected patients in this study, the analysis of which forms part of this thesis.
Above all I must dedicate this thesis to my wife Sarah, whom I married six weeks into this PhD study period. She has been a source of endless support and tolerance, especially when I moved to Uganda to commence clinical work just a few months into my recovery from a very severe illness.
Dr Iain Dunsmuir Page MBChB, BSc, MRCP, DTM&H

The author graduated from The University of Edinburgh Medical School in 2002. He was awarded an intercalated BSc in Virology with 2.1 Honours. He gained membership of the Royal College of Physicians of Edinburgh in 2006 and was awarded the Diploma of Tropical Medicine and Hygiene with Distinction by the University of Liverpool in 2007.

He worked as a junior doctor in Edinburgh, Glasgow and Leeds between before taking the post of Clinical Lecturer at the University of Malawi from 2007 to 2008. He then returned to the UK to become a Specialty Registrar in Infectious Diseases and General Medicine, working at Blackpool Victoria Hospital and North Manchester General Hospital. From 2012 he has been working as a Clinical Research Fellow at the University of Manchester, based at the National Aspergillosis Centre at University Hospital of South Manchester, with fieldwork in Gulu, Uganda.

The authors BSc included a research thesis entitled 'Expression of Human Herpes Virus 8 protein vOx-2', which was awarded 2.1 Honours. He has published four research articles, listed below, prior to his current post. He also authored the gastro-intestinal medicine section of the 2008 Malawian National Prescribing guidelines.

In addition to the contents of this thesis, the Dr Page also authored two conference abstracts relating to pulmonary aspergillosis. He was an invited speaker at the 6th Advances Against Aspergillosis conference (Madrid, February 2014) and the Global Action Fund for Fungal Infections Forum (GAFFI, Seattle, February 2015). He was an
invited panel member at the International Society for Human and Animal Mycology (ISHAM) expert group on azole resistance (Copenhagen, October 2013), which will be published in due course. He co-authored the UK National Aspergillosis Centre’s submission to the UK All-Party Parliamentary Group on Global Tuberculosis in 2014.

Significant difficulties were encountered in the course of this work. Fieldwork was delayed by several months due to outbreaks of Ebola fever in Uganda in 2012 and 2014. The author contracted leptospirosis while on honeymoon in the second month of the study period and was admitted to intensive care with multi-organ failure. While he was able to return to work and was granted a 3-month extension to his study period, he suffered persistent hepatitis and severe fatigue for a year after discharge from hospital. He has now fully recovered.

Prior Publications


Conference presentations – unpublished work not included in thesis


INTRODUCTION

Part 1 – Thesis structure

This thesis is submitted in the alternative format. This format was selected as the completed research takes the form of five separate studies. Each of these has been completed with positive results that will be submitted for publication in due course. The alternative format is therefore suitable. The introduction is based on a review article published in the journal Medical Mycology, although this article has been edited from its published format in response to corrections produced at the PhD viva examination. There is then a further review of the literature relating to the global prevalence of chronic pulmonary aspergillosis (CPA) in association with tuberculosis and human immunodeficiency virus (HIV) infection.

The primary goal of this thesis is to measure the prevalence of CPA secondary to tuberculosis. Additional studies provide evidence of the existence of pulmonary aspergillosis in adult patients admitted to an African hospital with acquired immunodeficiency syndrome (AIDS) and chronic cough. Before these tasks could be undertaken, it was first necessary to demonstrate the sensitivity and specificity of existing tests for the diagnosis of CPA. The key test in the diagnosis of CPA is measurement of Aspergillus-specific IgG. While many assays are available to measure this, the sensitivity and specificity of these assays for the diagnosis of CPA was barely described prior to this work.

The first paper in this thesis describes a comparison of six assays for the measurement of Aspergillus-specific IgG in cohorts of patients with known CPA and ABPA at the UK National Aspergillosis Centre plus healthy controls. This paper demonstrates that two of the available assays perform well in the diagnosis of CPA and ABPA. The Siemens Immulite assay was then selected for use in the prevalence studies as the manufacturer kindly offered to donate kits for this purpose.
The third and fourth papers in the thesis describe the results of a cross-sectional study to measure the prevalence of CCPA in patients with treated pulmonary tuberculosis in Gulu, Uganda. This required two surveys two years apart in order to detect progressive cavitation on chest X-ray. Clinical assessment was performed and \textit{Aspergillus}-specific IgG measured. CT scan was performed in patients with evidence of possible CPA from the first survey. The first study commenced in September 2012 and is described in paper two. The second survey was completed in February 2015 and is described in paper three.

In addition to these cross-sectional studies in Gulu, two collaborative studies were arranged with the MIND-IHOP study group at Mulago Hospital, Kampala. This forms the basis of papers four and five. This study group had been collecting sera from adult patients admitted to their hospital with chronic cough. They had performed extensive investigations to diagnose the underlying conditions in these patients, but did not have access to \textit{Aspergillus} serology. We therefore measured \textit{Aspergillus}-specific IgG in selected stored sera from the MIND-IHOP study. The first group was from patients with a clinical presentation that was consistent with subacute invasive pulmonary aspergillosis. This forms the basis of paper four. The second group was from patients with proven pulmonary tuberculosis. Here a positive test for \textit{Aspergillus}-specific IgG provides evidence of \textit{Aspergillus} co-infection. This forms the basis for paper five.

Overall this thesis provides definitive data on the sensitivity and specificity of various \textit{Aspergillus}-specific IgG assays. It demonstrates that two assays have statistically significantly superior performance for the diagnosis of CPA. It then uses one of these assays to measure the prevalence of CCPA in an area of high tuberculosis prevalence for the first time. Evidence is also provided of undiagnosed pulmonary aspergillosis in patients with AIDS and chronic cough and of \textit{Aspergillus} co-infection in patients with proven pulmonary tuberculosis.

It demonstrates CPA is a sufficiently common problem to be considered a public health issue in Uganda. For the first time it defines the radiological and serological characteristics of CPA in HIV-infected people, after they have recovered from...
tuberculosis. The work described in this thesis provides the first substantial evidence that CPA is a significant global public health issue.

Part 2 – Author contribution to enclosed papers

The author of this thesis will be the first author of all papers included in this alternative format thesis. In each case he took the lead role in all aspects of the studies including planning, data gathering, analysis and presentation as well as ensuring compliance with all regulatory requirements. All clinical aspects of this work took place in Uganda, where the author worked independently for 18 months of the study period.

The concepts for papers one and two were devised by the author’s lead supervisor (Prof David Denning) and discussed prior to commencement of the PhD. The concepts for papers three to five originated with the author and were developed after commencing the PhD.

The author was awarded funding for the Clinical Research Fellow post following an open application process. This post did not include research funding, but £26,000 funding was made available by UHSM Academy to complete the first CPA prevalence study in Uganda. The author then applied for and was awarded a further £24,000 by pharmaceutical firm Astellas Pharma in an open applications process.

The first paper was completed in collaboration with several commercial test manufacturers. The author contacted all known manufacturers of *Aspergillus*-specific IgG assays regarding the serology comparison studies. Test manufacturers Siemens Immulite (Germany), Serion (Germany), Genesis (UK), OLM Medical (UK) and Dynamiker (China) all agreed to donate kits for use in the comparison study. Dynamiker and Serion both also agreed to provide £2500 towards laboratory costs. The total value of funding and donations arranged by the author is around £100,000.

Once kits were donated the author performed around 95% of assays involving these kits, with the remainder performed by a laboratory assistant (Matthew Kneale) who was trained in their use.
The Ugandan prevalence studies were led by the author, who personally assessed around 95% of patients, with the remainder assessed by study assistant Matthew Kneale following training by the author. The author was assisted by two Ugandan employees who performed translation and venepuncture. The Ugandan prevalence studies required the collaboration of local health workers. The author visited Gulu for one week prior to the PhD with his supervisor Prof. David Denning and was introduced to most of the local collaborators. He then went on to develop these relationships over the next three years.

Gulu District Health team played a key role in identifying eligible patients by communicating with village health workers. Laboratory staff at the Joint Clinical and Research Centre (JCRC), Gulu laboratory performed serum separation and storage during the first Gulu survey as well as CD4 counts on HIV infected patients. During the second survey this role was performed by staff at the Gulu Regional Referral Hospital laboratory, who also performed GeneXpert tuberculosis PCR testing on sputum samples from patients with productive cough. Control samples from healthy blood donors were acquired by the Gulu Blood Transfusion service.

Chest X-rays were performed by staff at the radiology department of St. Mary’s Hospital, Lacor. CT scans were performed by staff at the Kampala Imaging Centre. While the author reported all chest X-rays and scans, results in paper two are based on reports by three blinded radiologists. This process is underway for chest X-rays and CT scans for paper three, but is not complete at the time of thesis submission. Radiological results in paper three are therefore based on the author’s own reports.

Papers four and five describe the results of opportunistic testing of stored samples from another study. The original MIND-IHOP study was a collaborative venture between Mulago Hospital, Kampala and the University of California, San Francisco. The author contacted the Kampala team after reading published results of their study. He then set up two new collaborative studies after it became clear that the initial MIND-IHOP study did not include adequate testing for pulmonary aspergillosis. The MIND-IHOP study took place in 2010-11. The author played no role in the design or conduct of this study.
After stored samples were identified the author arranged shipment to the UK and performed Aspergillus-specific IgG testing in the Manchester laboratory. He then analysed these results in relation to clinical data provided by the MIND-IHOP group and took a lead role in the subsequent presentation of these results.

**Part 3 – Publication plan**

The review article based on the PhD literature review has now been published in the journal Medical Mycology. Final data for each of the original research papers was only received from collaborators between January and March 2015. As a result none of these have been published yet. Paper one has been submitted for publication in a peer reviewed journal. All other studies refer to the diagnostic cut offs and sensitivity and specificity results from that paper. The other papers will be submitted for publication once paper one is in print. Interim data has, however been presented at academic conferences as listed below:-

*Review article publication*


*Interim results presented at conferences*

**Page ID**, Kwizera R, Richardson M, Denning D. Comparative efficacy of five Aspergillus-specific IgG ELISAs for the diagnosis of Chronic Pulmonary Aspergillosis (CPA) and Allergic Bronchopulmonary Aspergillosis (ABPA). 25th European Conference on Clinical Microbiology and Infectious Diseases, Copenhagen, 25th-28th April 2015.

**Page ID**, Onyachi N, Opwonya J, Opira C, Odongo-Aiginya E, Mockridge A, Byrne G, Richardson M, Denning DW. Chronic Pulmonary Aspergillosis (CPA) is likely to be a common complication of pulmonary tuberculosis: initial results of a cross-sectional survey. 25th European Conference on Clinical Microbiology and Infectious Diseases, Copenhagen, 25th-28th April 2015.


Part 4 – Published review article - Antibody testing in aspergillosis – quo vadis?

Iain D Page\textsuperscript{a,b,c}#, Malcolm Richardson\textsuperscript{a,b,c}, David W Denning\textsuperscript{a,b,c}

Institute of Inflammation and Repair, The University of Manchester, UK\textsuperscript{a}, Manchester Academic Health Science Centre, UK\textsuperscript{b}, National Aspergillosis Center and Mycology Reference Centre, University Hospital of South Manchester, UK\textsuperscript{c}.

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Humans are constantly exposed to airborne Aspergillus spores. Most develop Aspergillus-specific antibodies by adulthood. Persons with chronic lung disease or Aspergillus airway colonization often have raised levels of Aspergillus-specific IgG. It is not known whether this signifies an increased risk of future aspergillosis.

Chronic and allergic forms of pulmonary aspergillosis are estimated to affect over three million people worldwide. Antibody testing is central to diagnosis of these conditions, with raised Aspergillus-specific IgG in chronic pulmonary aspergillosis and raised Aspergillus-specific IgE in allergic aspergillosis. Antibody levels are also used to monitor treatment response in these syndromes. Acute invasive disease is less common. There is a more limited role for antibody testing in this setting as immunosuppression often results in delayed or absent antibody response.

Many methods exist to detect Aspergillus-specific antibodies, but there are limited published data regarding comparative performance and reproducibility. We discuss the merits of the available tests in the various clinical settings and their suitability for use in resource-poor settings, where the majority of cases of aspergillosis are thought to occur. We summarise the gaps in existing knowledge and opportunities for further study that could allow optimal use of antibody testing in this field.
INTRODUCTION

Aspergillus is a mould that causes disease in humans. Infection can lead to a spectrum of clinical syndromes, ranging from rapidly fatal acute invasive infection to chronic debilitating pulmonary disease. The latter can normally be characterized as either allergic airways disorders closely associated with asthma or chronic lung infection that can be complicated by progressive fibrosis and massive haemoptysis. Understanding of these conditions has improved significantly over the course of several decades, with associated changes in the case definitions and terminology used to describe disease.

It is likely that chronic and allergic forms of pulmonary aspergillosis are sufficiently common to be considered a public health issue on a global scale. The most common form of aspergillosis is undoubtedly chronic pulmonary aspergillosis secondary to treated tuberculosis. It is therefore likely that most patients with pulmonary aspergillosis will be living in the resource-poor settings where tuberculosis is most common.

Treatment with antifungal medication is associated with clinical and radiological stabilization or improvement in all common forms of aspergillosis. It can be successfully delivered in resource poor settings. Surgery can cure chronic pulmonary aspergillosis in selected patients with localized disease and can also be performed in resource poor settings.

Diagnosis of aspergillosis is challenging. Unfortunately the clinical presentation of chronic and allergic aspergillosis overlaps considerably with other, better-recognised conditions and it is likely that the vast majority of cases go undiagnosed. The development of assays to detect antigenaemia has led to improved ability to diagnose invasive infections promptly and the interpretation and performance of these antigen detection assays have been reviewed extensively. Chronic and allergic forms of aspergillosis are much more common than invasive disease, but have been relatively neglected. Antibody testing is central to the diagnosis of these conditions.

It is the goal of this article to describe the antibody response that occurs in Aspergillus infection and its role in the diagnosis and management of aspergillosis. The strengths and limitations of the various techniques available to measure Aspergillus-
specific antibodies will be described, together with a review of the evidence of their comparative efficacies.

**CLINICAL SYNDROMES DUE TO **Aspergillus** infection**

It is likely that human exposure to **Aspergillus** spp. is near universal, as **Aspergillus** spp. are consistently recovered from air samples in urban and rural areas throughout the year. Human disease due to **Aspergillus** spp. has also been recorded worldwide. The vast majority of patients with aspergillosis have one or more underlying disorders and the presentation of aspergillosis varies in line with the underlying disorder. While there can be a significant degree of overlap between syndromes it is nonetheless useful to summarise the commonly observed syndromes. The antibody response to **Aspergillus** and thus the role of antibody measurement in diagnosis and management varies greatly from one syndrome to another.

**Superficial aspergillosis**

Cutaneous aspergillosis is uncommon as the physical barrier provided by the epidermis prevents **Aspergillus** inoculation. **Aspergillus** spp. do cause keratitis, otitis externa and onychomycosis in immunocompetent persons, but antibody response is not normally seen in these conditions and diagnosis relies on microscopy and culture.

**Aspergillus bronchitis**

**Aspergillus** can grow in the human respiratory tract. This can occur in asymptomatic patients and in these circumstances is termed colonisation. However in some patients with no significant immune deficit, **Aspergillus** growth in the respiratory tract occurs and is associated with cough and recurrent chest infections, but without radiological evidence of pulmonary aspergillosis. These patients are considered to have **Aspergillus** bronchitis. This is well described in persons with cystic fibrosis, but is not restricted to this group. Evidence of **Aspergillus** growth is provided by either
recurrent culture growth from respiratory samples or raised levels of *Aspergillus*-specific antibodies.

**Acute invasive aspergillosis**

Acute invasive disease can occur in immunocompromised persons and is termed invasive pulmonary aspergillosis, invasive rhinosinusitis, invasive tracheo-bronchial aspergillosis or disseminated aspergillosis depending on the site of the invasive infection\(^\text{41–43}\). These conditions are mostly associated with severe neutropaenia, but can also be seen in association with a large range of conditions including corticosteroid use, intensive care unit (ICU) admission, diabetes, liver failure, tuberculosis, chronic obstructive pulmonary disease (COPD), chronic granulomatous disease (CGD), graft versus host disease (GVHD), solid organ transplantation and acquired immunodeficiency syndrome (AIDS)\(^\text{42–47}\).

Pneumonia is the most common initial presentation, but lesions involving the kidneys, cardiac valves, brain and skin have been documented\(^\text{42,44,46}\). Clear diagnostic guidelines have been published by the European Organization for Research and Treatment of Cancer (EORTC)\(^\text{48}\). Measurement of *Aspergillus*-specific antibodies do not form part of these criteria, with diagnosis resting on biopsy evidence for proven disease or a combination of risk factors, radiological change and microbiological evidence in the form of culture growth or antigen detection for probable disease.

**Subacute invasive pulmonary aspergillosis**

In addition to this well-recognised acute presentation of invasive disease, there can also be a more indolent presentation with progressive destruction of the lung over several weeks or months. This has been frequently referred to as chronic necrotising pulmonary aspergillosis or semi-invasive aspergillosis in the past\(^\text{6,49}\), but the term subacute invasive pulmonary aspergillosis has been adopted more recently\(^\text{50}\) and will be used throughout this article. The condition is normally seen in patients with mild immunosuppression due to diabetes, steroid use, alcoholism, COPD, tuberculosis or
AIDS. A similar condition occurs in the sinuses, where is termed chronic invasive fungal rhinosinusitis.

Diagnosis of subacute invasive aspergillosis is based on a combination of symptoms, radiological changes and laboratory tests, including antibody and antigen tests or culture.

There is a large degree of overlap between subacute invasive pulmonary aspergillosis and chronic pulmonary aspergillosis. The duration of symptoms is the main difference, over one month of symptoms considered appropriate for subacute invasive aspergillosis. In the absence of treatment, death from progressive lung destruction and massive haemoptysis is common. Those who survive subacute invasive pulmonary aspergillosis can go on to develop chronic pulmonary aspergillosis.

**Chronic pulmonary aspergillosis**

The term aspergilloma refers to a fungal ball in a lung cavity. The cavity may be pre-existing or be created by *Aspergillus* as an aspergilloma forms. This can be an incidental radiological finding in an asymptomatic person and is termed simple aspergilloma in these cases. Fungal balls are also well described in the sinuses.

Formation of new cavities and fibrosis of surrounding lung tissues often occurs in response to chronic *Aspergillus* infection. This process has been referred to as complex aspergilloma, but is now preferably referred to as chronic pulmonary aspergillosis (CPA). It can be subdivided into chronic cavitary pulmonary aspergillosis (CCPA) and chronic fibrosing pulmonary aspergillosis (CFPA). CPA occurs in patients with underlying lung conditions, including treated tuberculosis, atypical mycobacterial infection, sarcoidosis, COPD, pneumothorax, prior lung surgery, rheumatoid arthritis or lung cancer. CPA can also complicate subacute invasive pulmonary aspergillosis or allergic bronchopulmonary aspergillosis. Progressive lung destruction due to fibrosis and cavitation occurs, with massive life-threatening haemoptysis complicating advanced disease. CPA is estimated to affect 3 million people worldwide. The five-year mortality of CPA is up to 85%.
Diagnosis is based on a combination of chronic symptoms, radiological changes and laboratory tests. Unfortunately the symptoms of cough and breathlessness can overlap greatly with the underlying lung diseases. Radiological changes of cavitation, fibrosis and pleural thickening can also overlap greatly with underlying conditions, with distinctive aspergilloma detected only in a minority of patients. Laboratory testing is therefore crucial in differentiating patients with CPA from those with underlying lung disease alone. Serum galactomannan has been documented in up to 50% of CPA cases, but may not have any diagnostic value due to a high false positive rate. Culture of sputum is positive in up to 44% of CPA cases, but raised levels of *Aspergillus*-specific immunoglobulin G (IgG) antibodies are almost always present and are central to diagnosis.

**Allergic aspergillosis**

Sensitisation to *Aspergillus* can occur in asthmatics and such patients are more likely to have severe asthma with life-threatening complications. This is referred to as severe asthma with fungal sensitization (SAFS). Allergy to *Aspergillus* can also result in rhinosinusitis. Diagnosis of sensitization can be achieved by skin testing or by detection of raised levels of *Aspergillus*-specific immunoglobulin E (IgE) antibodies.

Allergic bronchopulmonary aspergillosis (ABPA) complicates 1-4% of adult asthma cases, many of whom have otherwise healthy lungs and no immunocompromise. ABPA can also complicate cystic fibrosis and occasional cases are also seen in persons with neither condition. ABPA is characterized by recurrent exacerbations resulting in cough and breathlessness with lung infiltrates on chest X-ray and can be complicated by the development of bronchiectasis or CPA. In contrast to other forms of aspergillosis, steroids are the main treatment, with antifungals used as steroid sparing agents in some cases.

The International Society for Human and Animal Mycology (ISHAM) has recently revised the diagnostic criteria for ABPA. Diagnosis requires the presence of cystic fibrosis or asthma plus a total serum IgE of > 1000 IU/ml and evidence of *Aspergillus* sensitivity from either skin prick testing or raised *Aspergillus*-specific IgE antibodies. Two of the three following minor criteria must also be present; radiographic changes
consistent with ABPA, raised eosinophil count or raised levels of *Aspergillus*-specific precipitating or IgG antibodies.

The diagnostic criteria for different clinical syndromes in aspergillosis are summarised in table 1. Figure 1 is a visual representation of the number of patients with each clinical syndrome overlaid with the bars showing the total number of patients where each test is diagnostic.

**ANTIBODY RESPONSE TO ASPERGILLUS**

**Asymptomatic persons**

While human airways are constantly exposed to *Aspergillus* spores present in the air\textsuperscript{30,31}, these spores are rendered immunologically inert by the presence of surface hydrophobin\textsuperscript{68}. In healthy persons the innate immune system ensures that most spores are promptly destroyed\textsuperscript{69}. Those that germinate into hyphae are normally recognised and killed by neutrophils before they can invade host tissue\textsuperscript{70}.

Nonetheless, antibodies to *Aspergillus* are formed in healthy persons, with mean levels increasing from childhood into adulthood\textsuperscript{71,72}. In accordance with this, tests for *Aspergillus*-specific antibodies are normally positive using sensitive methods such as enzyme-linked immunosorbent assay (ELISA), with abnormal results defined as a raised level above a cut-off related to the range of antibody levels seen in healthy persons\textsuperscript{38,73,74}.

Asymptomatic persons with *Aspergillus* airway colonisation may develop raised levels of *Aspergillus*-specific antibodies and the correct interpretation of this is not clear. Some authors classify raised levels in this population as false positives\textsuperscript{38}. However to our knowledge there are no published studies describing the long-term outcome in colonised patients. It is therefore not clear whether patients described in the literature as colonised are at higher risk of developing pulmonary aspergillosis in the future or not. If this were the case then raised levels of *Aspergillus*-specific antibodies in asymptomatic persons might be an indication of pre-clinical disease.

Aspergillosis normally develops in patients with underlying diseases\textsuperscript{4,14}. The range of levels of *Aspergillus*-specific antibodies in persons with these diseases may not be the same as healthy persons. Up to 20% of patients with treated tuberculosis have
positive tests for *Aspergillus*-specific antibodies \(^{75}\), this rises to 25% when lung cavities are present \(^{76}\) and 36% in those with haemoptysis \(^{77}\). Raised *Aspergillus*-specific IgG levels are also seen in 13% of Indian asthmatics \(^{62}\), 24% of British cystic fibrosis patients \(^{78}\), 25% of Indian children with thalassemia and human immunodeficiency virus (HIV) infection \(^{79}\) and 8% of all patients attending a Brazilian tertiary respiratory clinic \(^{80}\).

These surveys did not include further tests to diagnose pulmonary aspergillosis and some of these patients with raised antibody levels may therefore have undiagnosed aspergillosis. Nonetheless these results suggest that the range of *Aspergillus*-specific antibody levels in patients at risk of developing aspergillosis may be different from the ranges described in healthy individuals. Indeed the mean level of *Aspergillus*-specific IgG in cystic fibrosis patients without ABPA is higher than the manufacturers upper limit of normal \(^{78}\).

Further work is needed to define the range of *Aspergillus*-specific antibody levels in other patient groups who are at risk of, but have not developed aspergillosis, as this is the population that is most likely to undergo testing for CPA or ABPA. It may be that existing diagnostic cut-offs for *Aspergillus*-specific antibody levels, which were defined using healthy persons as a control, are not appropriate for those at most risk of developing aspergillosis.

**Aspergillus bronchitis**

Seventy one percent of patients with symptomatic *Aspergillus* bronchitis have raised *Aspergillus*-specific IgG and 29% have positive precipitins \(^{39}\). While raised levels of *Aspergillus*-specific IgE are not typical of *Aspergillus* bronchitis, there is considerable overlap between the clinical presentation of *Aspergillus* bronchitis and that of ABPA. Measurement of total and *Aspergillus*-specific IgE would therefore be appropriate in patients with symptoms of *Aspergillus* bronchitis, with the aim of identifying cases of serological ABPA \(^{4}\).
Acute invasive aspergillosis

Acute invasive aspergillosis normally occurs in patients with profound immune dysfunction, meaning that antibody production may not occur in response to infection. However, Aspergillus-specific IgG antibodies are detectable by ELISA in 29-100% of patients during the course of acute invasive aspergillosis. Sensitivity is higher in non-neutropaenic patients (48%) than neutropaenic patients (6%).

When antibodies do develop in acute illness, they take a mean of 10.8 days to appear and historically a majority of patients with invasive aspergillosis died without producing antibodies. This greatly reduces their utility for diagnosis of acute disease as early treatment is crucial for survival. Nonetheless when a patient with suspected invasive aspergillosis does develop newly raised Aspergillus-specific IgG antibodies this finding does provide evidence of acute infection.

There may be other uses for antibody testing in invasive aspergillosis other than diagnosis of acute disease. A retrospective survey described an increase in all-cause mortality in Aspergillus colonised lung transplant patients, with a hazard ratio of 2.2. Another similar study failed to show this association, but this cohort was complicated by the fact that colonised patients considered high risk for development to invasive aspergillosis were not included. This suggests that patients colonised with Aspergillus might then benefit from antifungal prophylaxis or early empirical antifungal treatment when immunosuppressed. Screening patients for raised Aspergillus-specific IgG antibodies prior to initiation of immunosuppressive therapy might be a convenient method of identifying such patients.

There can also be a role for serial measurement of Aspergillus-specific IgG antibodies after commencing treatment for presumed invasive aspergillosis. In this situation a fall in Aspergillus-specific IgG levels is a bad prognostic marker. A rise in Aspergillus-specific IgG antibodies can retrospectively confirm the diagnosis in those who recover following empirical treatment for suspected invasive aspergillosis. This knowledge might affect decisions about whether to forgo further immunosuppressive therapy or to provide antifungal prophylaxis with it.
Subacute invasive aspergillosis

Raised levels of *Aspergillus*-specific IgG antibodies are more likely to occur and thus are of greater use for diagnosis in this group than in acute disease\textsuperscript{6,53}. In lung transplant recipients, invasive aspergillosis often develops months after transplantation and can evolve slowly. A rise in *Aspergillus*-specific IgG titres preceded radiological changes by 1-2 weeks and diagnosis of invasive aspergillosis by 2-20 weeks in this group\textsuperscript{95}. Raised levels of *Aspergillus*-specific IgG antibodies were detected in 93\% of 43 Korean patients\textsuperscript{6} and 77\% of 45 Japanese patients with subacute invasive pulmonary aspergillosis\textsuperscript{53}. Sensitivities of serum (1,3)-\(\beta\)-D glucan and galactomannan testing in the Japanese patients were 60\% and 64\% respectively.

The sensitivity of galactomannan antigen testing is much lower when *Aspergillus*-specific antibodies are present than when they are absent\textsuperscript{96}. This effect may be due to direct binding of anti-*Aspergillus* antibodies to the galactomannan antigen\textsuperscript{97}. It is therefore possible that both antigen and antibody testing will both needed to achieve acceptable sensitivity for the diagnosis of subacute invasive aspergillosis in mildly immunosuppressed patients.

Chronic pulmonary aspergillosis

Raised levels of *Aspergillus*-specific IgG antibodies are almost always found in CPA\textsuperscript{5,8,98}. Production of specific Immunoglobulin M (IgM) is also noted in up to 50\% of CPA cases\textsuperscript{72,88,99–102}. This might be considered unusual in a chronic disease, as raised levels of specific IgM are typically associated with the acute phase of an infection.

Ongoing growth of *Aspergillus* produces numerous different antigens at different stages in its growth cycle that interact with the immune system in different ways\textsuperscript{103}. IgM might therefore be repeatedly re-stimulated as an immune response develops to each new, individual *Aspergillus* antigen over time. An assay that detects IgM antibodies to a wide range of *Aspergillus* antigens could therefore remain positive for some time. The specificity of *Aspergillus*-specific IgM testing is poor, limiting its utility\textsuperscript{72,89,100}.

Persistently raised levels of specific Immunoglobulin A (IgA) are found in up to 76\% of CPA cases\textsuperscript{72,88,99–102}. This immunoglobulin type is normally associated with
mucosal immunity and it may be persistently raised as the mucosa is constantly exposed to fungal growth. *Aspergillus*-specific IgE levels are also sometimes raised in CPA cases and may indicate the presence of underlying ABPA when present as the mucosa is constantly exposed to fungal growth. Aspergillus-specific IgE levels are also sometimes raised in CPA cases and may indicate the presence of underlying ABPA when present. Measurement of *Aspergillus*-specific IgG antibodies had a higher sensitivity than either IgM, IgA or IgE in all these studies and it should therefore be considered the most appropriate test for screening. However small numbers of cases of CPA have been identified which have normal *Aspergillus*-specific IgG, but raised *Aspergillus*-specific IgA or IgM. This may be explained by the fact that *Aspergillus*-specific IgA and IgM can bind different *Aspergillus* antigens than *Aspergillus*-specific IgG. Overall *Aspergillus*-specific IgM probably has little to offer due to poor specificity, but there may be a role for *Aspergillus*-specific IgA and IgE testing, in patients with symptoms and/or radiological changes of CPA, but normal *Aspergillus*-specific IgG levels.

Measurement of *Aspergillus*-specific IgG has additional uses beyond initial diagnosis of CPA. Precipitins titres fall following surgical resection of aspergilloma and rise in correlation with clinical treatment failure. *Aspergillus*-specific IgG levels have been successfully used to monitor response of CPA to medical therapy.

**Allergic aspergillosis**

In this context the patient may initially have healthy lungs and an intact immune function. However an exaggerated inflammatory response develops in response to fungal allergen exposure. This is characterised by over-expression of T helper (Th) 2 and Th17 CD4+ cells and down-regulation of T-regulatory cells (TREGs). This results in the high levels of both total and *Aspergillus*-specific IgE in serum in patients with SAFS and ABPA. Raised total and *Aspergillus*-specific IgE in serum are also noted in patients with allergic fungal rhinosinusitis caused by *Aspergillus*. In this patient group raised levels of *Aspergillus*-specific IgE can also be found in the ‘allergic mucin’ extracted from the sinuses themselves.

Raised *Aspergillus*-specific IgG has been described as an exclusion criteria for the diagnosis of SAFS on the grounds it implies more complex disease and airways infection. It should be noted though, that 10% of all asthmatics have raised *Aspergillus*-specific IgG or precipitins. There are therefore likely to be some cases of...
asthma with *Aspergillus* sensitization where *Aspergillus*-specific IgG is raised in addition to IgE, but all diagnostic criteria for more severe conditions such as ABPA are not met.

Precipitating *Aspergillus*-specific antibodies were frequently found in ABPA cases in early studies\(^\text{113,114}\). They were then considered a mandatory diagnostic criteria for ABPA by some authors\(^\text{115,116}\), whereas others regarded it only as a supporting criteria\(^\text{110}\). Reports on the frequency of raised *Aspergillus*-specific IgG or precipitins in ABPA will of course be heavily dependent on whether or not it is considered a mandatory diagnostic criteria, but 14% of ABPA cases have recently been reported as having a negative precipitins test\(^\text{67}\).

ABPA can be complicated by the development of CPA, which is characterized by raised levels of *Aspergillus*-specific IgG. However elevated *Aspergillus*-specific IgG is much more common in ABPA than is the development of CPA\(^\text{117,118}\). Levels of *Aspergillus*-specific IgG are generally higher in CPA than ABPA. Unusually high levels in patients with ABPA may therefore suggest that CPA has developed and should prompt further investigation\(^\text{4,5}\). Raised *Aspergillus*-specific IgA has also been noted in ABPA\(^\text{119}\), but it occurs only in a minority of patients and is of limited diagnostic value.

In patients with underlying cystic fibrosis (CF) quantitative measurement of *Aspergillus*-specific IgG has been suggested as a means to differentiate CF with ABPA from CF without APBA\(^\text{78}\). It was found that the mean *Aspergillus*-specific IgG concentration in CF patients without ABPA was 51.1 mg/L, compared to 132.5 mg/L in CF patients with ABPA. The authors of this study suggested an *Aspergillus*-specific IgG cut off of 90 mg/L to differentiate the two patient groups with a sensitivity of 91% and specificity of 88%.

Latent class analysis is a statistical technique used to find groups or subtypes of cases in multivariate categorical data. A recent publication used this technique to identify different disease groups in relation to *Aspergillus* infection in CF\(^\text{66}\). Four disease groups were identified; 1 – patients with no evidence of *Aspergillus* disease, 2 – patients with serological ABPA, 3 – patients sensitized to *Aspergillus* and 4 – patients with *Aspergillus* bronchitis.

*Aspergillus*-specific IgG could be used to differentiate between *Aspergillus* sensitization and serological ABPA with a sensitivity of 96% and a specificity of 90% when a cut off of 75 mg/L was used. *Aspergillus*-specific IgE could differentiate between *Aspergillus* bronchitis and serological ABPA with 100% sensitivity and specificity using
a cut off 3.7 kUA/L. Patients with no *Aspergillus* disease could be differentiated from patients with *Aspergillus* sensitization using *Aspergillus*-specific IgE with a cut-off of 2 kUA/L. To our knowledge the performance of the diagnostic cut-offs suggested by these two studies have not been confirmed in populations other than the ones used to define the cut-offs.

Total IgE falls with effective treatment of ABPA\textsuperscript{110,115,120–122}. *Aspergillus*-specific IgE can also fall with treatment\textsuperscript{110}, but this effect was noted later than the fall in total IgE in this study and was not reported in the majority of other treatment studies. *Aspergillus*-specific IgG has also been noted to fall in line with treatment\textsuperscript{116}, but this occurred at the same time as a fall in total IgE and provided no additional information. It therefore appears that total IgE is currently the most appropriate test for monitoring response to treatment in ABPA.

**LABORATORY METHODS FOR DETECTION OF *ASPERGILLUS*-SPECIFIC ANTIBODIES**

Multiple techniques are available to measure levels of *Aspergillus*-specific antibodies in human serum in the laboratory in different ways. Since raised *Aspergillus*-specific IgG, IgE, IgA and IgM all have different interpretations in different clinical scenarios it is important to understand which assays measure which antibody types when interpreting results.

**Precipitation in gels.**

Detection of *Aspergillus*-specific antibodies in serum was first achieved by precipitation of antibody-antigen complexes in gels\textsuperscript{123,124}. This method has also been referred to as double diffusion, immunodiffusion or the precipitins test. Antigens and antibodies are placed in separate wells within the gel and are allowed to diffuse towards one another. The presence of multiple binding sites on antibodies such as IgM\textsuperscript{125} allows the formation of immune complexes that ‘precipitate’ when they become too large to pass through the gel. These ‘precipitin bands’ are visible to the naked eye with non-specific staining.

All antibody classes precipitate, but IgG predominates. The method takes around five days to perform, is labour intensive and relies on human interpretation of results.
No complex equipment is needed. Commercial preparations of *A. fumigatus* antigens for use in precipitin tests are available from Microgen (UK), Bio-Rad Laboratories (France) and Immuno-Mycologics (IMMY) Inc. (USA).

**Counterimmunoelectrophoresis.**

An improvement on the precipitation method was described with the development of counterimmunoelectrophoresis (CIE). Movement through the gel is accelerated by application of an electric current and precipitation occurs within a few hours.

Figure 2 is a picture of a CIE gel with visible precipitin bands.

**Haemagglutination.**

Haemagglutination tests use erythrocytes pre-coated with antigens. These erythrocytes clump together when antibodies cross-react with antigens on more than one cell. The resulting ‘plaque’ prevents erythrocytes from settling at the bottom of the test well. The difference in appearance between positive and negative wells is visible to the human eye. This method produces a result in around two hours and does not require complex equipment, but does rely on human interpretation of results. It is commercially produced by ELITech Group (France). Antibody levels are considered raised if a positive reaction takes place at a titer greater than the manufacturers stated cut off level.

Figure 3 is a picture of a haemagglutination plate showing positive and negative results.

**Complement Fixation.**

Complement fixation tests rely on the fact that human complement will both react with antibody-antigen complexes and also lyse sheep erythrocytes that are pre-bound to anti-sheep erythrocyte antibodies. Complement is removed from human serum by heating. *Aspergillus* antigens, complement and sheep erythrocytes, pre-bound to anti-sheep erythrocyte antibodies are added in steps. In the absence of *Aspergillus*-specific antibodies a reaction takes place that results in lysis of the erythrocytes and thus colour
change visible to the naked eye\textsuperscript{84}. The method is fairly labour intensive and relies on human interpretation of results. Kits are produced by and Serion/Virion (Germany) and IMMY (USA).

All of the above techniques can produce semi-quantitative results by following serial dilutions of serum.

**ELISA.**

This well-described technique allows the detection of individual types of antibody (IgG, IgM, IgA etc.). Antibodies from patient sera bind to antigens and are then detected by anti-human antibodies. Enzyme reactions produce a colour change that is measured with a spectrophotometer. ELISA has been used in diagnosing aspergillosis for decades\textsuperscript{130,131}. It can be fully automated, which reduces labour costs and can produce results within two hours. The reaction can also be performed manually. ELISA produces a positive result in most sera, with a cut-off provided by the manufacturer to differentiate raised levels from normal ones.

Commercial *Aspergillus*-specific IgG plate ELISA tests are currently produced by Serion, (Germany), IBL (Germany/USA), Dynamiker/Bio-Enoche, (China), Bio-Rad (France), Bordier (Switzerland) and Genesis (UK). Siemens Immulite (Germany) supply an automated *Aspergillus*-specific IgG ELISA system (Immulite) and ThermoFisher Scientific/Phadia (multi-national) supply an automated *Aspergillus*-specific IgG fluoroenzymeimmunoassay (FEIA) system (ThermoFisher Scientific ImmunoCAP), which is an ELISA variant. The Serion and Bio-Rad *Aspergillus*-specific IgG assays can also be automated. Siemens Immulite and ThermoFisher Scientific both produce automated *Aspergillus*-specific IgE ELISA/FEIA tests. Serion and IBL produce commercial *Aspergillus*-specific IgA and IgM ELISA tests. The units of measurement often differ from one assay to another.
Immunoblot.

Gel electrophoresis is used to separate *Aspergillus* antigens by molecular weight. Antigens are then transferred to a membrane to which human serum is added. An identical series of reactions to ELISA is then performed, producing a colour change visible to the naked eye at the location of the antigen on the membrane when positive. It does not require complex equipment but is fairly labour intensive. A commercial *A. fumigatus* immunoblot was released in 2012 by LDBIO diagnostics (France).

The attributes of a selection of commonly used methods for detection of *Aspergillus* antibodies are summarised in table 2.

**SOURCES OF ANTIGENS FOR USE IN ANTIBODY DETECTION ASSAYS**

**Extraction of antigens from fungal cultures**

The traditional methods of antigen preparation for use in tests involves growth of *Aspergillus* culture in the laboratory, followed by either mechanical disintegration of intact cells to provide somatic antigens or culture filtration to provide extra-cellular antigens. The latter have often been referred to as ‘metabolic’ antigens in the literature and product information sheets. This terminology is, however inaccurate as many of the antigens are not metabolites. These crude processes produce mixtures of many of the different antigens produced by *Aspergillus*. Up to 52 separate precipitins bands have been identified on double diffusion testing using this type of antigen preparation and electrophoresis of culture extracts has identified up to 200 bands, each representing a potential antigen that might react with human sera.

While the extraction of antigens from *Aspergillus* cultures has been taking place for decades there have been several difficulties encountered in attempts to provide consistent and reliable antigens for use in tests. It is clear that different laboratory strains of *Aspergillus fumigatus* produce different groups of antigens. Even when a single strain is used somatic and culture filtrate methods produce different groups of antigens, which can produce different results when tested against patient sera. Various factors such as the culture medium used, pH, and culture temperature...
Have all been noted to affect the nature of antigens produced by cultures\textsuperscript{140}. Antigens also vary with the age of the culture\textsuperscript{98,135}.

Even when identical methods are used, batch to batch variation from a single strain processed in the same lab has been noted\textsuperscript{141}. In addition to antigens, culture extracts also contain enzymes and toxins, which might interfere with test performance\textsuperscript{142}. When the same antigen extracts are used in different test formats they can produce widely variable results\textsuperscript{143}. The antigen mixtures produced from culture extracts have also been shown to cross-react with antibodies produced against other fungi and bacteria\textsuperscript{144,145}.

As these traditional antigen extraction techniques can be performed in any mycology laboratory, reference laboratories often produce their own internally manufactured antigens for use in assays\textsuperscript{59,86,96}. However the extensive difficulties noted above mean that quality control in \textit{Aspergillus} antigen production is exceedingly challenging and by their nature internally manufactured assays in reference laboratories are not amenable to validation in inter-laboratory studies. In contrast commercially manufactured assays can be performed and assessed across multiple laboratories and can also be compared to other assays under identical conditions in a single laboratory.

\textbf{Measurement of antibodies in non-\textit{fumigatus} aspergillosis}

All the tests described above are designed to detect \textit{A. fumigatus}. However, in India the most prevalent \textit{Aspergillus} species causing fungal sinusitis is \textit{A. flavus}\textsuperscript{10}. This species also accounts for 38\% of \textit{Aspergillus} cultures from patients with chronic lung diseases in India\textsuperscript{146}. In Brazil \textit{Aspergillus niger} is a common cause of chronic pulmonary aspergillosis\textsuperscript{147}. The frequency of growth of different \textit{Aspergillus} species in association with human disease in selected countries is shown in table 3.

Evidence on the performance of antibody detection assays in these cases is extremely limited. Culture filtrate antigens from \textit{A. fumigatus} are positive in around 50\% of cases with aspergilloma caused by \textit{A. flavus} or \textit{A. niger}\textsuperscript{148}. \textit{A. niger}-specific precipitins were positive in 78\% of 23 patients with CPA due to \textit{A. niger} in Brazil\textsuperscript{147}. Other species-specific precipitins tests are available and might prove effective, but have been tested on very few patients\textsuperscript{98}. Siemens Immulite produce ELISA tests for IgG
specific to *Aspergillus niger, nidulans, terreus and flavus*, but to our knowledge there are no published data on the performance of these assays.

**Detection of antibodies specific to individual *Aspergillus* antigens**

Early experience with precipitins testing demonstrated that precipitin bands of consistent molecular weight appeared in many patients with aspergillosis and corresponded to enzymes associated with the fungus. Individual antigens were identified, which had variable sensitivity and specificity for the diagnosis of aspergillosis. Many specific antigens reacting with human IgG and IgE have since been identified and the genes relating to these antigens have been sequenced. This has allowed the production of recombinant antigens by expressing these genes in genetically modified bacteria or fungi, which then produce pure extracts of single antigen.

Mitogillin-specific IgG is positive in 100% of aspergilloma cases, 64% of invasive pulmonary aspergillosis cases and only 1.3% of healthy volunteers in a single study. Antibodies to purified recombinant Afmp1p, an *Aspergillus* cell wall galactomannanprotein, are positive in 100% of patients with aspergilloma and 33% of patients with invasive aspergillosis. To our knowledge the performance of these assays has not been confirmed in other laboratories and the assays have not been released commercially.

Testing for IgG specific to recombinant catalase, ribonuclease and dipeptidylpeptidase V showed sensitivity of 77%, 81% and 79% respectively for aspergilloma. This increased to 95% by using all three antigens together. Bio-Rad (France) released a commercial recombinant assay following this study. It has shown good agreement with Serion culture filtrate ELISA in a retrospective survey. Bio-Rad has not revealed which antigens are used in their commercialized test.

The Dynamiker *Aspergillus*-specific IgG ELISA assay utilizes purified galactomannan as its sole antigen. No data has yet been published on the sensitivity and specificity of this test for the diagnosis of aspergillosis, but an earlier study detected antibodies to galactomannan in only 26% of aspergilloma cases.

Many efforts have been made to identify individual antigens against which specific IgE is formed in allergic aspergillosis. These antigens are commonly
referred to as allergens in this context. To date 23 Aspergillus-specific allergens have
been recognised by the International Union of Immunology Societies\textsuperscript{158}. This is likely to
be an under-representation of the true number of Aspergillus-specific allergens as 81
IgE binding Aspergillus proteins have been identified using a highly sensitive phage
display detection method\textsuperscript{159}.

Attempts have been made to develop individual allergen-specific IgE assays for
use in allergic aspergillosis and to use them to differentiate between different diseases.
IgE to allergen Asp f1 is found in 60-85\% of ABPA cases\textsuperscript{160,161}, but has also been
detected in the sera of Aspergillus sensitized asthmatics without ABPA\textsuperscript{156}. Genomic
studies have demonstrated that sensitization to this allergen is produced only by a small
number of fungi\textsuperscript{162}, suggesting that there is likely to be limited cross-reactivity with this
recombinant protein.

One study found IgE specific to allergens Asp f2/3/6 were all raised in both
asthma and ABPA, but not in other forms of pulmonary aspergillosis. However another
study found that IgE specific to allergens Asp f1/2/3/4/6 were all present at
significantly higher concentrations in ABPA than asthma\textsuperscript{151}.

In patients with underlying cystic fibrosis, one study showed mean IgE to Asp f1
was ten times higher in those with ABPA than those without\textsuperscript{163}, but another study failed
to show this differentiation\textsuperscript{164}. IgE to Asp f4 and Asp f6 were found to differentiate CF
with ABPA from CF without ABPA in this second study. A similar result was later found
when these same antigens were used in skin prick testing\textsuperscript{165}.

A more recent study showed that no single allergen was absolutely effective in
differentiating CF patients with and without ABPA\textsuperscript{166}. IgE to Asp f1 showed non-specific
binding with ABPA cases and controls, IgE to Asp f2 was consistently present in the sera
of CF patients with ABPA, but was frequently also present in CF patients without ABPA.
IgE to Asp f3 was highly specific for ABPA in CF but had poor sensitivity. Aspergillus-
specific IgG subtypes and IgA were also analyzed and found not to differentiate CF with
ABPA from CF without ABPA.

Attempts have also been made to identify single antigen-specific antibodies for
the diagnosis of acute invasive aspergillosis\textsuperscript{83,167}, but to our knowledge no commercial
assays have been released for this purpose and detection of serum antigenemia is
preferred in this patient group due to its superior sensitivity\textsuperscript{48}. 
Overall, while the detection of antibodies specific to individual antigens might eventually result in more accurate and reproducible diagnosis of aspergillosis, existing study results are mostly inconsistent or unconfirmed. No individual antigen or group of antigens has been consistently shown to be more efficacious than traditional methods of antigen extraction for the diagnosis of any form of aspergillosis.

**COMPARATIVE PERFORMANCE OF DIFFERENT LABORATORY METHODS**

**Invasive aspergillosis**

Antibody measurement plays a peripheral role in the diagnosis of invasive aspergillosis and data on the comparative performance of different techniques is limited\(^2^3\). *Aspergillus*-specific IgG ELISA was more sensitive than precipitins or CIE in two studies with a total of 18 patients\(^8^2, ^8^7\). Comparison of haemagglutination and *Aspergillus*-specific IgG ELISA showed superior sensitivity for haemagglutination in one study with 14 patients\(^8^4\), but superior sensitivity for ELISA in another study with 26 patients\(^8^6\). To our knowledge there are no comparisons of currently commercially produced *Aspergillus* antibody assays in this patient group, although the Serion *Aspergillus*-specific IgG ELISA formed part of a mix of methods for antibody detection that were less sensitive than galactomannan antigen test in one comparison\(^9^0\).

**Chronic pulmonary aspergillosis**

The original reports of precipitins tests for diagnosis of aspergilloma reported sensitivity of 98% against patients with definite histological or radiological diagnosis of aspergillosis, with no positive results in healthy controls\(^9^8\). However it should be noted that the radiological methods available at the time did not include computed tomography (CT) scanning and would thus only have detected cases with fairly advanced disease.

Since then precipitins detection has been used as part of the diagnostic criteria for chronic forms of aspergillosis. The lack of a clear gold standard creates a difficulty in
subsequent studies. Sensitivity is normally measured against clinical diagnosis recorded in the patients’ medical records. Precipitins will often have formed part of the diagnostic criteria. It is therefore difficult to prove that other tests are more sensitive than precipitins in study populations defined in this way.

In more recent studies the interpretation of reported sensitivity rates against diagnoses of CPA taken from case notes might be further complicated by the fact that many patients will be on antifungal treatment, which leads to reduction in Aspergillus specific IgG levels. It is not known whether this would affect all tests equally or bias results in favor of one technique. Prospective studies comparing performance of tests in patients not yet diagnosed with aspergillosis would resolve these issues, but are difficult to conduct due to the low frequency of new diagnoses. One such study recently demonstrated that Aspergillus-specific IgG is more sensitive than serum galactomannan antigen for the diagnosis, but did not compare different Aspergillus-specific IgG assays.

Many retrospective studies have shown equally excellent sensitivity when precipitins testing is compared to CIE or ELISA in cases of aspergilloma. Precipitins were even reported as being more sensitive than other methods in one comparison. However not all cases of aspergilloma or CPA are precipitins positive. Negative precipitins results might occur as not all antibody-antigen complexes precipitate in gels.

The one prospective study comparing precipitins to CIE showed that CIE is more sensitive than traditional precipitins for detection of Aspergillus-specific antibodies. However CIE has also been reported as producing more false positive results than precipitins. A recent retrospective study suggested that the sensitivity of ELISA for the diagnosis of CPA was 30% higher than precipitins.

Unlike ‘home-brew’ assays using internally manufactured Aspergillus antigens, commercially available assays can be compared to one another in head-to-head comparisons. Commercial ELISAs with published performance data for the diagnosis of CPA include ThermoFisher Scientific Aspergillus-specific IgG FEIA, Serion culture filtrate Aspergillus-specific IgG ELISA and Bio-Rad recombinant Aspergillus-specific IgG ELISA. Each showed good correlation with precipitins test results and superior reproducibility when automated. The Siemens Immulite and ThermoFisher Scientific assays have good head-to-head correlation, but the Siemens Immulite assay produces a higher absolute result with a mean ratio of 1.78. This study also demonstrated
acceptable inter-laboratory reproducibility for the ThermoFisher Scientific with a coefficient of variation of 7.3 - 18.1%.

ThermoFisher Scientific ImmunoCAP Aspergillus-specific IgG FEIA, Bio-Rad recombinant Aspergillus-specific IgG ELISA and CIE using Microgen antigens were compared in 116 patients with CPA, who were mostly on antifungal treatment. Sensitivity was 86% for ThermoFisher Scientific ImmunoCAP, 85% for Bio-Rad and 56% for CIE. However 4% of cases were positive on precipitins testing only. This may be due to the ability of precipitins to detect IgM and IgA in addition to IgG. In the case of the Bio-Rad recombinant antigens assay, false negative results may also occur in patients who do not have antibodies to the selected antigens within their spectrum of anti-Aspergillus antibodies. These results suggest that while these ELISAs are more sensitive than precipitins testing for first line screening, there may be a role for precipitins testing in patients suspected of CPA with unexpectedly negative ELISA results.

The Bio-Rad test has also been directly compared to Serion ELISA in 51 cases with CPA. Sensitivities of 94% and 92% respectively were noted. Specificity in healthy controls was 100% for Bio-Rad and 96% for Serion.

The published comparisons of the sensitivity of different methods of Aspergillus-specific antibody detection in patients with CPA are summarised in table 4.

**Allergic pulmonary aspergillosis**

A recent review compared the performance of different diagnostic tests for identifying new cases of ABPA in Indian asthmatics using latent class analysis. Aspergillus skin prick testing was 95% sensitive and 80% specific, total IgE of >1000 IU/ml was 97% sensitive but only 40% specific, raised Aspergillus specific IgE was 100% sensitive and 70% specific, whereas Aspergillus precipitins testing was only 43% sensitive, but 97% specific.

These results suggest that Aspergillus-specific IgE testing is the most appropriate screening test for ABPA and can be used in place of skin prick testing where available. However the high specificity of precipitins testing means that the diagnosis of ABPA can be made with high confidence in asthmatic patients with both raised Aspergillus-specific
IgE and positive *Aspergillus* precipitins. Unfortunately most patients with ABPA in this study did not meet all of these criteria.

CIE has been reported as more sensitive than precipitins for the detection of precipitating antibodies in cases of allergic aspergillosis\textsuperscript{181}. There are no published direct comparisons of the performance of the commercially available *Aspergillus*-specific IgE assays, but it should be noted that marked variation has been noted between *Aspergillus*-specific IgE levels and skin prick test results, with concordance of only 14-56\%\textsuperscript{65,182,183}. There is also marked variation between the Siemens Immulite and ThermoFisher Scientific assays in tests for peanut-specific IgE\textsuperscript{184}. The Siemens Immulite system produces *Aspergillus*-specific IgG results roughly 2 fold higher than the ThermoFisher Scientific ImmunoCAP system\textsuperscript{180}. Results of *Aspergillus*-specific IgE assays from different commercial assays should therefore be compared with caution.

The published comparisons of the sensitivity of different *Aspergillus*-specific antibody assays are summarised in table 5.

**SUITABILITY OF AVAILABLE LABORATORY TECHNIQUES FOR RESOURCE-POOR SETTINGS**

As noted earlier the majority of patients suffering from pulmonary aspergillosis are likely to be located in resource poor settings. We would suggest that many commonly used assays are not ideal for use in such settings. Automated ELISAs require equipment, which is expensive to purchase and requires both a reliable electricity supply and regular maintenance. Manual ELISAs might be suitable, but still require a properly maintained spectrophotometer that may not be available in many resource poor settings. Such manual ELISAs have been described as having much poorer reproducibility than automated systems\textsuperscript{74}.

Precipitation in gels requires less high-tech equipment than ELISA, but is time consuming, requires significant operator training and produces subjective results. Complement fixation and immunoblot have similar difficulties. We consider haemagglutination assays a potentially attractive option as no complex equipment is required, but to our knowledge there are no published data describing the sensitivity and specificity of the sole commercially available haemagglutination test (ELITech).
The lateral flow device (LFD) is well known for its use in point-of-care pregnancy tests. This format is also widely used for the diagnosis of HIV and malaria in resource poor settings. To our knowledge no LFD for the detection of *Aspergillus*-specific antibodies exists at this time. An LFD that detects an *Aspergillus* antigen has recently been developed and seems to perform well using serum for the diagnosis of acute invasive aspergillosis in mostly neutropaenic patients. It is also effective using BAL fluid to diagnose invasive aspergillosis in non-neutropaenic patients with underlying lung disease. However to our knowledge there is no published evidence regarding its sensitivity and specificity for the diagnosis of CPA. It is possible that in this context the sensitivity of this LFD will be low, as the alternative galactomannan antigen assay has poor sensitivity in this patient group.

Figure 4 shows examples of tests that are unsuitable and potentially suitable for use in resource-poor settings.

**CONCLUSIONS**

Aspergillosis has been estimated to affect millions of persons worldwide, with CPA as the most common clinical syndrome. Many of these patients are likely to reside in resource-poor countries, given the current and previous prevalence of tuberculosis in these locations. Improved diagnosis of CPA is a critical need in the battle to improve CPA outcomes and expanding access to *Aspergillus*-specific IgG testing in areas of high tuberculosis prevalence is key to achieving this goal.

Expanding the diagnosis of aspergillosis presents many challenges. The clinical and radiological signs of aspergillosis often overlap significantly with associated underlying diseases and so cannot be relied upon to diagnose aspergillosis. Culture can be helpful, but the sensitivity of culture for the diagnosis of aspergillosis is sub-optimal and access to reliable fungal culture is frequently challenging or even non-existent in poorly resourced countries.

Serological testing is therefore of crucial importance. For acute invasive aspergillosis this mostly means antigen testing, which has been reviewed extensively elsewhere. However there may be a secondary role for antibody testing in this setting for retrospective diagnosis of recovering patients. The screening of patients for
Evidence of *Aspergillus* colonisation prior to immunosuppressive therapy may also be useful. Outside of this setting the interpretation of raised levels of *Aspergillus*-specific antibodies in asymptomatic colonised patients is not well described and follow up studies of such patients that describe their risk of developing symptomatic forms of aspergillosis would be welcome.

In chronic and allergic aspergillosis measurement of *Aspergillus*-specific antibodies is central to diagnosis, with raised *Aspergillus*-specific IgG found mostly in chronic disease and raised total and *Aspergillus*-specific IgE found mostly in allergic disease. It is important to note, though that there is a degree of overlap between these clinical syndromes and many patients will have clinical and serological features of both.

Similarly subacute invasive aspergillosis occurs in mildly immunosuppressed patients with a presentation that overlaps acute invasive disease and CPA. Here patients may have positive antigen tests, raised *Aspergillus*-specific IgG or both simultaneously. As a result it is possible that this group of patients will need to be tested for both *Aspergillus*-specific IgG and *Aspergillus* antigens to achieve early diagnosis with good sensitivity.

Measurement of antibodies can also be used to monitor response to treatment. A falling *Aspergillus*-specific IgG indicates poor prognosis in acute invasive aspergillosis, but a good response to therapy in CPA. For allergic aspergillosis, total IgE remains the best method for monitoring treatment response, although it is far from optimal.

Many methods exist for the measurement of *Aspergillus*-specific antibodies, with differing performance characteristics. It is thus unfortunate that they are frequently mislabeled in the literature with the term ‘precipitins’ often used to refer to *Aspergillus*-specific IgG ELISA rather than precipitation in a gel and ‘RAST’ often used to refer to *Aspergillus*-specific IgE ELISA rather than the older radioimmunoassay.

Evidence of sensitivity and specificity of different methods is sparse, but *Aspergillus*-specific IgG ELISA is likely to be more sensitive than precipitation in gels. However there are some patients with CPA with normal *Aspergillus*-specific IgG ELISA results and positive precipitins tests or raised levels of *Aspergillus*-specific IgA. Performing these assays in patients suspected of CPA with negative *Aspergillus*-specific IgG ELISA would therefore probably result in better overall sensitivity.

*Aspergillus*-specific IgM ELISA is probably not useful for diagnosis of CPA due to poor specificity, although it should be noted that the specificity data comes from studies.
of ‘home-brew’ assays. The commercially produced *Aspergillus*-specific IgM assays might have different performance characteristics, but to our knowledge there are no published data on this topic.

The product inserts of most commercial ELISAs report good specificity at the manufacturers diagnostic cut-offs, but the evidence for these statements is often not published in peer-reviewed journals. It should be noted that these cut-offs are normally calculated against the range of antibody levels found in a cohort of healthy volunteers. This is probably an appropriate comparator for most invasive aspergillosis patients. However healthy volunteers may not be the ideal comparator for CPA or ABPA, as these conditions almost always occur in persons with underlying chronic lung disease or chronic immune dysfunction. Unfortunately, to our knowledge there is no published data on the distribution of *Aspergillus*-specific IgG levels in patients with these chronic underlying conditions, with the exception of cystic fibrosis. Our research team is undertaking a study measuring *Aspergillus*-specific IgG levels in patients with treated tuberculosis, COPD and asthma using several assays. The diagnostic cut-offs for CPA and ABPA may need to be changed in response to this data.

Global standardization of assays has proved difficult, with many laboratories using assays derived from antigens manufactured ‘in-house’. By their nature these assays are impossible to validate in other laboratories. Many commercially produced *Aspergillus*-specific IgG and IgE tests exist, but to our knowledge only one (ThermoFisher Scientific / ThermoFisher Scientific ImmunoCAP) has published inter-laboratory variability data. The Bio-Rad recombinant *Aspergillus*-specific IgG has been tested against a reasonable number of persons with CPA at more than one centre with good sensitivity reported. The IBL and ThermoFisher *Aspergillus*-specific IgG assays have been tested in reasonable numbers of patients with CPA at single sites. Most patients in all of these studies will have been on treatment and it is not known how this may have biased the results. Many other assays have no published performance data at all.

The publication of data from studies demonstrating the reliability of available assays both in terms of sensitivity and specificity in untreated patients and in terms inter-assay and inter-laboratory reliability is a pre-requisite for their use in the large scale screening that will be necessary to achieve diagnosis of the predicted number of
cases. Our unit is currently undertaking a single centre study with this goal, but studies across multiple laboratories will be needed to determine inter-laboratory variability.

Many attempts have been made to develop ELISAs for the detection of antibodies specific to one or more individual *Aspergillus* antigens and commercially produced tests based on this principle do exist. In theory this should allow production of a reliable test and resolve the many problems that exist with traditional antigen extraction techniques. However, to our knowledge there is no published evidence that these assays are consistently either more reliable or efficacious than traditional techniques for the diagnosis of either allergic or chronic aspergillosis. Assays based on culture filtrate or somatic antigens remain in common usage.

As the majority of patients with pulmonary aspergillosis are predicted to live in resource-poor settings it will be necessary to identify a reliable test that is suitable for widespread use in such settings if such patients are to be diagnosed and treated. The haemagglutination assay may be suitable for use in this setting, but requires further validation. The *Aspergillus* antigen LFD is in the ideal test format, but is likely to have poor sensitivity for the diagnosis of CPA. An LFD that detects *Aspergillus*-specific IgG may need to be developed to allow widespread access to testing in resource poor settings.
Table 1 – Abbreviated diagnostic criteria for acute pulmonary IA, sub acute pulmonary IA, CCPA and *Aspergillus* bronchitis

<table>
<thead>
<tr>
<th></th>
<th>Proven Invasive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Probable Invasive&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sub Acute Invasive (aka CPA)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CCPA&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Aspergillus bronchitis&lt;sup&gt;f&lt;/sup&gt;</th>
<th>ABPA&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical criteria</strong></td>
<td>not required</td>
<td>neutropenia OR stem cell transplant OR high dose corticosteroids for &gt;3 weeks OR Immune-suppressant drugs OR CGD OR SCID</td>
<td>&gt; 1 MONTH SYMPTOMS; weight loss OR productive cough OR haemoptysis AND absence of host factors for acute invasive disease</td>
<td>3 MONTHS SYMPTOMS; weight loss OR productive cough OR haemoptysis AND absence of host factors for invasive disease</td>
<td>persistent productive cough OR recurrent chest infections AND does not meet diagnostic criteria for chronic or allergic aspergillosis</td>
<td>arthritis OR cystic fibrosis</td>
</tr>
<tr>
<td><strong>Radiological criteria</strong></td>
<td>not required</td>
<td>dense lesions +/-halo sign OR air-crescent sign OR one or more cavities</td>
<td>new cavitation OR expanding cavity OR paracavitary infiltrates</td>
<td>new cavitation OR expanding cavity OR paracavitary infiltrates</td>
<td>absence of changes consistent with CPA or ABPA</td>
<td>transient opacifications or permanent evidence of bronchiectasis of pleuropulmonary fibrosis (see other criteria below)</td>
</tr>
<tr>
<td><strong>Laboratory criteria</strong></td>
<td>culture from a sample from a normally sterile site OR histology</td>
<td>culture from sputum or BAL OR GM in blood or BAL OR β(1,3)-D-glucan in blood</td>
<td>culture from sputum or BAL OR GM in blood or BAL OR β(1,3)-D-glucan in blood OR raised <em>Aspergillus</em>-specific IgG OR histology</td>
<td>raised <em>Aspergillus</em>-specific IgG OR culture from sputum or BAL OR GM in blood or BAL* OR β(1,3)-D-glucan in blood*</td>
<td>raised <em>Aspergillus</em>-specific IgG AND EITHER recurrent culture growth from sputum or BAL OR persistently positive PCR from sputum or BAL</td>
<td>Obligatory criteria total IgE &gt; 1000 IU/ml AND raised <em>Aspergillus</em>-specific IgE (or positive skin prick test) OR Other criteria (2 of 3 needed) raised eosinophil count OR raised <em>Aspergillus</em>-specific IgG / precipitins OR radiological changes as above</td>
</tr>
</tbody>
</table>

CNPA = chronic necrotising pulmonary aspergillosis, CCPA = chronic pulmonary aspergillosis, ABPA = allergic bronchopulmonary aspergillosis, CGD = chronic granulomatous disease, SCID = severe combined immunodeficiency, CXR = chest X-ray, CT = computed tomography, BAL = bronchoalveolar lavage, GM = galactomannan antigen test, IgG = immunoglobulin g, IgE = immunoglobulin e, PCR = polymerase chain reaction. **Unless stated otherwise patients must meet all 3 criteria for diagnosis of each condition**. *GM and β(1,3)-D-glucan are less sensitive than *Aspergillus* serology in CPA and so not included in all published case definitions, but are consistent with CPA when present together with appropriate clinical and radiological features.
### Table 2 - Comparison of the features of selected commercial *Aspergillus* antibody assays

<table>
<thead>
<tr>
<th>Test</th>
<th>CIE</th>
<th>Thermo Fisher Scientific IgG FEIA</th>
<th>Siemens IgG ELISA</th>
<th>Bio-Rad IgG ELISA</th>
<th>Serion IgG ELISA</th>
<th>Dynamiker IgG ELISA</th>
<th>ELITech HA</th>
<th>LDBIO Immuno blot</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigen type</strong></td>
<td>fungal extract</td>
<td>fungal extract</td>
<td>fungal extract</td>
<td>unspecified recombinant antigen</td>
<td>fungal extract</td>
<td>galacto-mannan</td>
<td>fungal extract</td>
<td>fungal extract</td>
</tr>
<tr>
<td><strong>Volume (µL)</strong></td>
<td>10</td>
<td>140 (dead volume = 100)</td>
<td>255 (dead volume = 250)</td>
<td>10</td>
<td>10</td>
<td>1</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td><strong>Dilutions</strong></td>
<td>titres as required</td>
<td>1 if result &gt; 200 mg/L</td>
<td>1 if result &gt; 200 mg/L</td>
<td>1 pre-test and second in samples with high result</td>
<td>2 pre-test and second in samples with high result</td>
<td>1 pre-test and second in samples with high result</td>
<td>titres as required</td>
<td>none</td>
</tr>
<tr>
<td><strong>Units</strong></td>
<td>mg/L</td>
<td>mg/L</td>
<td>AU/ml</td>
<td>AU/ml</td>
<td>AU/ml</td>
<td>AU/ml</td>
<td>dilution titres</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>No samples tested per batch</strong></td>
<td>30 + 2 controls*</td>
<td>continuous testing</td>
<td>continuous testing</td>
<td>92 + 4 controls</td>
<td>92 + 4 controls</td>
<td>92 + 6 controls</td>
<td>94 + 2 controls*</td>
<td>1</td>
</tr>
<tr>
<td><strong>Equipment needed</strong></td>
<td>gels, antigens, Coomassie blue stain, de-stain and washing solution</td>
<td>CIE tank</td>
<td>Siemens Immulite analyzer and antigen packs, test tubes, barcode labels</td>
<td>kit pipettes, test tubes, incubator, spectrophotometer, OR automated analyzer</td>
<td>kit pipettes, test tubes, moist chamber incubator, distilled water, spectrophotometer, OR automated analyzer</td>
<td>kit pipettes, test tubes, incubator, distilled water, spectrophotometer</td>
<td>kit pipettes, tweezers, rocking tray</td>
<td></td>
</tr>
<tr>
<td><strong>Suitable for a resource poor laboratory?</strong></td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
<td>YES (if manual)</td>
<td>YES (if manual)</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td><strong>Total batch time</strong></td>
<td>2 days</td>
<td>3 hours</td>
<td>2 hours</td>
<td>4 hours</td>
<td>4 hours</td>
<td>4 hours</td>
<td>2 ½ hours</td>
<td>3 hours</td>
</tr>
<tr>
<td><strong>Hands on time - approx</strong></td>
<td>4 hours</td>
<td>30 mins</td>
<td>30 mins</td>
<td>2 hours</td>
<td>2 hours</td>
<td>2 hours</td>
<td>30 mins</td>
<td>1 hour</td>
</tr>
</tbody>
</table>

CIE = counterimmunoelectrophoresis, IgG = immunoglobulin g, FEIA = fluoroenzyme immunoassay, ELISA = enzyme immunoassay, HA = haemagglutination, AU = arbitrary units. *Represents total number of sera wells per test. Can perform this many screening tests in one batch or use 1 well for each serial dilution if dilutional titres are required.
### Table 3 – Frequency of different *Aspergillus* species grown in different respiratory conditions

<table>
<thead>
<tr>
<th>Paper</th>
<th>Country</th>
<th>Disease</th>
<th>No of cases</th>
<th><em>A. fumigatus</em> (%)</th>
<th><em>A. niger</em> (%)</th>
<th><em>A. flavus</em> (%)</th>
<th><em>A. terreus</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baddley 2009</strong>&lt;sup&gt;191&lt;/sup&gt;</td>
<td>USA</td>
<td>invasive aspergillosis</td>
<td>274 isolates</td>
<td>66</td>
<td>10</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td><strong>Herbrecht 2002</strong>&lt;sup&gt;20&lt;/sup&gt;</td>
<td>International</td>
<td>invasive aspergillosis</td>
<td>110</td>
<td>77</td>
<td>8</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td><strong>Denning 2003</strong>&lt;sup&gt;5&lt;/sup&gt;</td>
<td>UK</td>
<td>CPA</td>
<td>10</td>
<td>100</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td><strong>Baxter 2013</strong>&lt;sup&gt;6&lt;/sup&gt;</td>
<td>UK</td>
<td>cystic fibrosis</td>
<td>39</td>
<td>100</td>
<td>none</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td><strong>Camuset 2007</strong>&lt;sup&gt;88&lt;/sup&gt;</td>
<td>France</td>
<td>CPA</td>
<td>21</td>
<td>95</td>
<td>none</td>
<td>5</td>
<td>none</td>
</tr>
<tr>
<td><strong>Nam 2010</strong>&lt;sup&gt;6&lt;/sup&gt;</td>
<td>South Korea</td>
<td>subacute invasive aspergilosis + CPA</td>
<td>34</td>
<td>91</td>
<td>9</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td><strong>Jhun 2013</strong>&lt;sup&gt;8&lt;/sup&gt;</td>
<td>South Korea</td>
<td>CPA</td>
<td>18</td>
<td>78</td>
<td>22</td>
<td>17</td>
<td>none</td>
</tr>
<tr>
<td><strong>Ohba 2012</strong>&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Japan</td>
<td>CPA</td>
<td>75</td>
<td>68</td>
<td>15</td>
<td>4</td>
<td>none</td>
</tr>
<tr>
<td><strong>Kurhade 2002</strong>&lt;sup&gt;92&lt;/sup&gt;</td>
<td>India</td>
<td>treated tuberculosis</td>
<td>14</td>
<td>79</td>
<td>14</td>
<td>7</td>
<td>none</td>
</tr>
<tr>
<td><strong>Shahid 2001</strong>&lt;sup&gt;46&lt;/sup&gt;</td>
<td>India</td>
<td>'chronic lung diseases'</td>
<td>12</td>
<td>67</td>
<td>33</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td><strong>Michael 2008</strong>&lt;sup&gt;93&lt;/sup&gt;</td>
<td>India</td>
<td>allergic <em>Aspergillus rhinosinusitis</em></td>
<td>125</td>
<td>11</td>
<td>3</td>
<td>79</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>invasive <em>Aspergillus rhinosinusitis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prateek 2013</strong>&lt;sup&gt;94&lt;/sup&gt;</td>
<td>India</td>
<td><em>Aspergillus rhinosinusitis</em></td>
<td>16</td>
<td>19</td>
<td>none</td>
<td>75</td>
<td>6</td>
</tr>
</tbody>
</table>

CPA = chronic pulmonary aspergillosis. Note multiple species identified in some cases.
Table 4 – Direct comparisons of sensitivity of antibody tests in proven CPA / aspergilloma

<table>
<thead>
<tr>
<th>Paper</th>
<th>No of patients</th>
<th>DD (%)</th>
<th>CIE (%)</th>
<th>HA (%)</th>
<th>Culture filtrate IgG ELISA (%)</th>
<th>ThermoFisher Scientific ImmunoCAP FEIA (%)</th>
<th>Bio-Rad recombinant IgG ELISA (%)</th>
<th>Bio-Rad galactomannan antigen test (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dee 1975168</td>
<td>9</td>
<td>89</td>
<td>89</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Warnock 1977171</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kurup 1978170</td>
<td>23</td>
<td>87</td>
<td>91</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kauffman 1983169</td>
<td>13</td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mishra 198387</td>
<td>17</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>100</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gugnani 1990173</td>
<td>5</td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Faux 1992172</td>
<td>11</td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kitasato 200960</td>
<td>28</td>
<td>89</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Guitard 201238</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>92 (Serion)</td>
<td>94</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Baxter 201374</td>
<td>116</td>
<td>56</td>
<td>-</td>
<td>-</td>
<td>86</td>
<td>85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jhun 20138</td>
<td>47</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>99 (IBL)</td>
<td>-</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>Shin 201459</td>
<td>168</td>
<td>98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>23</td>
<td>-</td>
</tr>
</tbody>
</table>

CPA = chronic pulmonary aspergillosis, DD = double diffusion (precipitins), CIE = counterimmunoelectrophoresis, HA = haemagglutination, IgG = immunoglobulin g, ELISA = enzyme immunoassay, FEIA = fluoroenzyme immunoassay. *galactomannan positive when index ≥0.5
Table 5 – Direct comparisons of sensitivity of antibody and antigen tests in invasive aspergillosis

<table>
<thead>
<tr>
<th>Paper</th>
<th>Clinical group</th>
<th>No. of patients</th>
<th>DD (%)</th>
<th>CIE (%)</th>
<th>HA (%)</th>
<th>IgG ELISA (%)*</th>
<th>Serum GM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holmberg 1980</td>
<td>autopsy proven IA</td>
<td>10</td>
<td>-</td>
<td>70</td>
<td>-</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>Mishra 1983</td>
<td>IA</td>
<td>8</td>
<td>37</td>
<td>50</td>
<td>-</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Manso 1994</td>
<td>mixed proven and probable IA</td>
<td>18</td>
<td>55</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>38 (LA)</td>
</tr>
<tr>
<td>Kappe 1996</td>
<td>biopsy proven IA</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LD - 29 Roche - 36</td>
<td>-</td>
</tr>
<tr>
<td>Kappe 2004</td>
<td>biopsy proven IA</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Herbrecht 2002</td>
<td>definite IA</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td>68</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>probable IA</td>
<td>67</td>
<td></td>
<td></td>
<td></td>
<td>58</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>possible IA</td>
<td>55</td>
<td></td>
<td></td>
<td></td>
<td>70</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>all IA</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
<td>64</td>
<td>29</td>
</tr>
<tr>
<td>Cornillet 2006</td>
<td>neutropaenic IA</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td>6.25 (mix of DD, CIE and Serion ELISA)</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>non-neutropaenic IA</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td>48 (mix of DD, CIE and Serion ELISA)</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>all IA patients</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
<td>30 (mix of DD, CIE and Serion ELISA)</td>
<td>65</td>
</tr>
</tbody>
</table>

DD = double diffusion (precipitins), CIE = counterimmunoelectrophoresis, HA = haemagglutination, IgG = immunoglobulin g, ELISA = enzyme immunoassay, GM = galactomannan antigen test (ELISA unless stated otherwise), LA = latex agglutination, IA = invasive aspergillosis. *IgG ELISA tests are internally manufactured by the research laboratory unless stated otherwise.
FIGURES

1 – Visual representation of the number of patients with each condition and the number of patients where each test is diagnostic

Note – IA = invasive aspergillosis, CPA = chronic pulmonary aspergillosis, ABPA = allergic bronchopulmonary aspergillosis. The size of each circle is relative to the estimated European population affected by the disease, with prevalence used for the chronic conditions ABPA (887,000 cases) and CPA (240,000 cases) and incidence for the acute condition IA (63,000 cases)\(^\text{29}\).

The length of the bars represents the total number of patients where each test is diagnostic by combining frequency of positive results annually for each condition. *Aspergillus*-specific IgE is raised in almost all cases of ABPA\(^\text{4,22}\) and up to 66% of CPA cases\(^\text{5}\). *Aspergillus*-specific IgG is raised in 65% of ABPA (Smith and Denning, unpublished data), up to 100% of CPA\(^\text{5,8}\) and up to 65% of cases of IA\(^\text{96}\). *Aspergillus* antigen tests are positive in around 62% of cases of IA in adults\(^\text{24}\) and 23% of cases of CPA\(^\text{8}\).
Blue-stained precipitin lines are formed where antigens and antibodies meet and precipitation of antibody-antigen complexes occurs. They represent a positive result. Sera in the left hand column produced no lines and are negative.
3 – Haemagglutination assay

The ELITech haemagglutination assay can be performed with no equipment other than a pipette. Results are visible to the naked eye. In the image above each row is a test sample with dilutional titres increasing from left to right. Result is the last titre at which a ‘plaque’ is still visible as shown.
The automated ELISA machine shown is of little use in settings with no regular electricity, whereas lateral flow devices, such as the *Aspergillus* antigen LFD above, are ideal.
Part 5 – CPA as Global Public Health issue

The presence of a fungal ball in a human was first documented by Plaignaud in the 18th century\textsuperscript{195}, with fungal growth in human tubercular lung cavities described half a century later by Bennett\textsuperscript{196}. The first survey of the frequency of pulmonary aspergillosis secondary to tuberculosis was undertaken by the British Medical Research Council (MRC) in 1970\textsuperscript{76,197} and the modern syndrome of CPA was defined in 2003\textsuperscript{5}. CPA occurs in patients with underlying structural lung disease. Underlying conditions include COPD, sarcoidosis, and cystic fibrosis, but the most common underlying condition in the UK is treated pulmonary tuberculosis\textsuperscript{14}. Large CPA case series have now been reported in Europe, India, China, Korea and Japan and the large majority of CPA cases described are secondary to tuberculosis\textsuperscript{7,8,14,15,18,108,198}.

Aspergillosis has been known to exist in Africa for some time. \textit{Aspergillus} sinusitis has been well recognized in Sudan since the 1960s\textsuperscript{199} and \textit{Aspergillus} has documented as the most common cause of otomycosis in Nigeria\textsuperscript{200}. The first documented case of pulmonary aspergillosis in Africa was that of an aspergilloma in a South African farmer in 1965\textsuperscript{201}. Over 170 cases of CPA have since been reported throughout Africa, including South Africa, Nigeria, Ivory Coast, Senegal, Central African Republic, Djibouti, Ethiopia, Tanzania and Uganda\textsuperscript{16,201–212}. Over 90% of these cases occurred in persons previously treated for pulmonary tuberculosis. Cases of co-infection with \textit{Aspergillus} and active pulmonary tuberculosis have also been documented in Tunisia and Egypt\textsuperscript{213,214}.

An estimated 9 million people developed tuberculosis in 2013\textsuperscript{215}. It was associated with 1.5 million deaths\textsuperscript{14}, of which only 210,000 are estimated to be due to multidrug resistant strains of \textit{M. tuberculosis}. Many of these deaths in patients with initially drug-susceptible disease will be due to lack of diagnosis, poor access to treatment or inadequate compliance, given that they mostly occur in resource-poor countries with weak health infrastructure. However some may be due to undiagnosed CPA.

CPA presents with progressive pulmonary cavitation associated with weight loss, persistent cough and haemoptysis\textsuperscript{5,7,8}. This presentation is near identical to that of
pulmonary tuberculosis itself. Chest X-ray often cannot distinguish the two conditions because cavities, pleural thickening and fibrosis are characteristic of both tuberculosis and pulmonary aspergillosis. Aspergilloma is distinctive, but it is present in only 36% of cases of CPA and probably represents a late manifestation of infection. Raised levels of *Aspergillus*-specific IgG are present in almost all cases of CPA, but this test is essentially unavailable in Africa.

The UK Medical Research Council (MRC) study, performed between 1968 and 1970, remains the only published measure of the prevalence of CPA secondary to pulmonary tuberculosis. It investigated 544 patients with residual lung cavities after tuberculosis treatment. Precipitating antibodies to *Aspergillus fumigatus* were present in 34% of patients, of whom 63% went on to develop an aspergilloma within the 2-year follow-up period. Haemoptysis occurred in 42% of those with aspergilloma. This study has not been replicated, but positive *Aspergillus*-specific antibodies have been found in 20-27% of patients previously treated for pulmonary tuberculosis in Japan, India and Brazil.

The MRC study had a number of limitations. The radiological criteria used to define 'aspergilloma' are not well described. It was performed before the invention of the CT scan and therefore probably underestimates the frequency of aspergilloma and other characteristic features of CPA. It also used precipitation-in-gel technique to detect *Aspergillus*-specific antibodies, which probably has inferior sensitivity in comparison to modern ELISA techniques. Furthermore it was restricted to those with visible cavitation on chest X-ray after tuberculosis. Aspergillosis might also develop in cavities smaller than those detected on chest X-ray. These cases would not be detected with this study design.

The global prevalence of CPA secondary to tuberculosis was nonetheless calculated on the basis of the 1968-70 MRC study results, together with current prevalence data for pulmonary tuberculosis and residual cavitation following pulmonary tuberculosis. The rate of post-tuberculous cavitation was assumed to be 22% everywhere except Europe where it was estimated to be an arbitrary 12%. As CPA may occur in those without visible cavitation, an arbitrary 2% of post-tuberculous patients without visible...
cavitation were also included in the estimates. The 5 year period prevalence of CPA was estimated to be 1,173,881 million people secondary to tuberculosis\(^{11}\). The 5-year point prevalence of CPA secondary to tuberculosis in DR Congo was estimated to be 43 per 100,000 population\(^{11}\). However any inaccuracies in the original 1968-70 study would be reflected in this prediction. Multiple other underlying diseases are associated with CPA in addition to tuberculosis\(^{14}\). The total global prevalence of CPA is estimated at around 3 million cases when these are included\(^{11-13}\).

There has never been a survey measuring the prevalence of CPA secondary to tuberculosis in a current area of high tuberculosis prevalence. CPA prevalence in these areas might differ from the UK in 1968-70. Rates of *Aspergillus* rhinitis and keratitis are higher in countries with warm climates and many subsistence farmers\(^{10}\). The same pattern might apply to CPA. Frequent exposure to wood smoke is common in areas of high tuberculosis prevalence and is associated with increased frequency of respiratory diseases\(^{222}\). CPA rates might also be higher in this group. Most importantly HIV co-infection might alter the rate of CPA, either increasing it due to immunosuppression\(^{52,223,224}\) or decreasing it by reducing the rate of residual cavitation after tuberculosis treatment\(^{225-227}\).

Fungal infections are a well-documented aspect of AIDS. Oral candidosis affects 90% of AIDS patients and more than 10% develop oesophageal candidiasis\(^{228}\). *Cryptococcus neoformans* can cause both pulmonary infection\(^{229}\) and meningitis\(^{230}\). *Pneumocystis jirovecii*, *Blastomyces dermatitidis*, *Coccidioidomyces imitis* and *Paracoccidioidomyces brasiliensis* are all known to cause pneumonia in patients with AIDS\(^{231-234}\).

*Aspergillus* tracheo-bronchitis is perhaps the most well known form of invasive aspergillosis associated with AIDS\(^{43}\). However, there is also a documented association between pulmonary aspergillosis and AIDS, in the absence of pulmonary tuberculosis\(^{52,223,224,235,236}\). Many of these cases occurred in patients with drug-induced neutropenia, but in 44% of cases neutrophil counts were normal\(^{235}\). Advanced AIDS is, however associated with impaired neutrophil function\(^{237}\) and almost all of these patients had CD4 count below 100 cells/μL\(^{235}\). There is therefore a plausible mechanism by which AIDS could directly place the patient at risk of invasive aspergillosis.
Most cases of pulmonary aspergillosis in AIDS are best described as subacute invasive disease, rather than CPA. Indeed AIDS has been considered an exclusion criteria for CPA on the grounds that marked immunosuppression is likely to result in more rapid disease progression. Chronic presentations have, however, also been described in AIDS. In reality there is probably a spectrum of disease related to the severity of AIDS related immunosuppression. The precise nature of the clinical syndrome is less important than the outcome. Autopsy studies from Italy, India and Uganda have demonstrated that aspergillosis is associated with 3-11% of all AIDS related deaths, with only 10% of these cases diagnosed during life.

In resource-poor settings pulmonary tuberculosis is often diagnosed on the basis of World Health Organization (WHO) approved ‘smear-negative’ criteria, with microbiological proof of tuberculosis infection not required. These clinical diagnosis protocols are used extensively in areas of high tuberculosis and HIV prevalence. In Uganda 54% of HIV positive out-patients commencing tuberculosis therapy are diagnosed in this manner, but only 35% of patients in the ‘smear-negative’ group who submit sputum for culture ultimately grow mycobacterium tuberculosis. In Ugandan HIV positive in-patients there is essentially no correlation between the results of the WHO smear-negative diagnostic protocol and eventual confirmation of tuberculosis.

Comparing smear-negative pulmonary tuberculosis cases to smear-positive pulmonary tuberculosis cases, the hazard ratio is 1.49 for 2-month mortality in DR Congo and 2.2 for 7-year mortality in Malawi. One potential explanation for the excess mortality in the smear-negative group is that some cases of ‘smear-negative tuberculosis’ are in fact undiagnosed and untreated cases of pulmonary aspergillosis.

Treatment greatly reduces mortality in pulmonary aspergillosis. In invasive aspergillosis 12-week survival rates of 71% and 57% have been achieved with voriconazole and amphotericin, in comparison to almost 100% mortality without treatment. Amphotericin is already used effectively to treat cryptococcal meningitis in resource-poor settings. CPA is also treatable. Oral itraconazole has been shown to prevent clinical and radiological progression and is available in low-cost generic preparations. Voriconazole and posaconazole have also been associated
with positive outcomes\textsuperscript{58,108,198}. Surgery is curative in selected patients with localized

disease\textsuperscript{15,21} and has been safely delivered in resource-poor settings\textsuperscript{16,54,212}. Identifying

and treating patients with CPA could therefore result in a reduction of the mortality rate
currently ascribed to pulmonary tuberculosis.

The existing evidence therefore raises a real possibility that pulmonary aspergillosis

commonly occurs in association with both tuberculosis and AIDS. The situation is

complicated by the fact that CPA both complicates pulmonary tuberculosis and mimics

its clinical and radiological presentation\textsuperscript{14,217,218}. It therefore might well be frequently

misdiagnosed as recurrent ‘smear-negative tuberculosis’ in those with previous treated

pulmonary tuberculosis\textsuperscript{215}. Pulmonary aspergillosis can also occur as a direct

consequence of AIDS\textsuperscript{52,223,236}. In this circumstance patients are also at high risk of

pulmonary tuberculosis and the presentation of the two conditions would be near

identical.

The central goal of the work contained in this thesis is to determine the frequency of

pulmonary aspergillosis in association with tuberculosis and AIDS. A prerequisite of this

is to define the performance characteristics of \textit{Aspergillus}-specific IgG assays in a well-
defined CPA population. The UK National Aspergillosis Centre has the world’s largest

collection of stored sera from known CPA cases, which have been used to define the

sensitivity and specificity of various assays. The best available assay is then used in

surveys to measure the prevalence CPA complicating treated pulmonary tuberculosis.

This study includes both HIV infected and uninfected persons to determine the impact

of HIV infection on CPA prevalence. Two additional Ugandan cohorts are also assessed

for evidence of primary pulmonary aspergillosis; those with proven pulmonary

tuberculosis and those with AIDS and ‘smear-negative pulmonary tuberculosis’, but no

microbiological proof of diagnosis.
METHODOLOGY

Paper 1 – Performance of six *Aspergillus*-specific IgG assays for the diagnosis of chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA)

Patients

241 patients with CPA and 80 patients with ABPA were identified at the UK National Aspergillosis Centre (NAC) who had a stored sample of serum taken at a time when they were either off treatment, or had only started treatment in the last 3 months. Samples were stored between 2004 and 2014. Diagnosis of CPA or ABPA was taken from clinical notes.

All patients with ABPA at our centre are routinely screened for the development of CPA. Any patient with CPA complicating ABPA was classified as CPA. The ImmunoCAP *Aspergillus*-specific IgG assay (ThermoFisher Scientific, multi-national) was used for clinical diagnosis at our laboratory during this period. However the NAC accepted referrals from all over the UK and those that fulfilled the microbiological diagnostic criteria with culture growth or positive result with an alternative *Aspergillus* antibody assay used by the referring hospital were accepted as cases regardless of their ThermoFisher Scientific ImmunoCAP result.

CPA diagnostic criteria

CPA is diagnosed at the NAC on the basis of criteria proposed by Denning et al in 2003\(^5\) and later endorsed by the Infectious Diseases Society of America (IDSA)\(^{250}\) and European Society of Clinical Microbiology and Infectious Diseases (ESCMID)\(^{251}\). It required the presence of all of the following: 1 - underlying disease, 2 - symptoms, 3 - radiological changes and 4 - microbiological evidence. The latter could take the form of biopsy, repeated *Aspergillus* culture or PCR from sputum or broncho-alveolar lavage fluid. However in the vast majority of cases microbiological evidence was provided by
raised levels of Aspergillus-specific IgG. As the NAC accepts patents from the whole UK a variety of Aspergillus-IgG assays will have been used to make the original diagnosis at referring hospitals.

Antibody positivity with either the ThermoFisher Scientific ImmunoCAP (cut-off 40mg/L) or two different precipitins assays (positive at any dilution) is the commonest microbiological means of making the diagnosis at the NAC, but histological evidence from biopsy or resection, positive culture from sputum or bronchoscopy samples, Aspergillus PCR on sputum and galactomannan on bronchoalveolar lavage fluid are all also accepted as ‘microbiological evidence’ of CPA. Most patients had several positive samples, at the referring hospital and at the NAC.

ABPA diagnostic criteria

ABPA is diagnosed at the NAC in line with International Society for Human and Animal Mycology (ISHAM) diagnostic criteria. These require the presence of raised total IgE and raised Aspergillus-specific IgE (or positive skin prick testing) for diagnosis. Positive precipitins or Aspergillus-specific IgG is one of three additional features, along with raised eosinophil count and radiological features, of which two out of three are also required to confirm the diagnosis.

Control samples were collected from 100 healthy Ugandan students aged 16-18 who were donating blood and were negative for HIV. Samples were tested for Aspergillus-IgG by all methods. Diseased controls for comparison to ABPA sera were taken from 100 asthmatic patients under the care of the North West Lung Centre at UHSM. These samples were stored as part of the ManRAB biobank project. Ethical clearance was granted by the ManRAB committee prior to their use in this study.

Assays

Tests were performed between January and July 2014. Aspergillus-specific IgG levels were measured by the author on all stored samples using the Immulite 2000 (Siemens, Germany) performed at Christie Hospital, Manchester UK. Manual plate ELISAs were
performed by the author using kits supplied by Serion (Germany), Genesis (UK) and Dynamiker (China) at the Mycology Reference Centre, University Hospital of South Manchester, UK.

Each manual plate ELISA kit followed a similar process that first required serum to be diluted using a diluent supplied by the manufacturer. A fixed volume of diluted serum was then added to wells that were pre-coated with *Aspergillus* antigens, for a fixed period of time. The Serion and Dynamiker kits required this incubation to take place in an incubator at 37°C, while the Genesis kit required incubation at room temperature. After washing with a washing solution provided with the kit a fixed volume of conjugate was added and incubated for a fixed period of time. After further washing a fixed volume of substrate was added and incubated for a fixed period of time. Finally a fixed volume of stopping solution was added.

Optical density was measured within five minutes of test completion on a PolarStar Omega spectrophotometer (BMG Labtech, UK), with settings for each individual kits utilized in line with manufacturers’ instructions. For the Genesis and Dynamiker assays a formula was installed on the spectrophotometer software to convert optical density to arbitrary units in line with the manufactures’ guidelines. For Serion the optical density results were entered onto an Excell database supplied by the manufacturer with embedded formulae to convert optical density to arbitrary units. Where a result was greater than a threshold specified by the manufacturer a 1 in 10 dilution was performed and the assay was repeated.

Results were rejected if the manufacturers’ stated quality control criteria were not met for an individual test plate. If this occurred the tests were re-run on a fresh plate. The exception was the Dynamiker assay where all kits failed the same one of the three stated quality controls criteria. This was discussed with the manufacturer. After reviewing our data in detail the manufacturer concluded that they would revise the quality control guidance for this kit in line with our findings. A decision was made with the manufacturer to proceed with the use of their kits in the study, as long as the other quality control criteria were met for each plate. A summary sheet comparing the exact processes for each manufacturer is shown in appendix 1.
The ImmunoCAP Aspergillus-specific IgG assay is used for routine testing at the National Aspergillosis Centre. Results of tests from CPA and ABPA patients, performed as part of routine clinical care, from the same sample that was subsequently stored, are used in the analysis. 100 control samples were sent for ThermoFisher Scientific ImmunoCAP Aspergillus-specific IgG testing at the Manchester Royal Infirmary immunology laboratory. These tests were performed by the staff at this laboratory.

Precipitation in gel (precipitins) testing was also performed by the author using the counterimmunoelectrophoresis (CIE) technique. 10ml agarose was melted and poured onto a hydrophobic gel bond film (GE Healthcare, USA). Three mm diameter test wells were cut once the gel had set. Twenty µL sera were placed in one row of wells with 20 µL antigens (Microgen, UK) at 2 mg/ml placed in the adjacent row. The gel was placed above a CIE tank filled with veronal buffer and blotting paper wicks were used to connect either end of the gel to the buffer tanks before applying 34V for 90mins. The gel was then placed in sodium chloride tri-sodium citrate buffer overnight before being dried with a hair dryer. After drying the gel was placed in a Coomassie Blue stain for 15 minutes, followed by 2 serial de-stains using methanol and acetic acid. Each de-stain step lasted 10 minutes. After further drying by hair dryer the gels were read on a light box with the assistance of a magnifying glass. The presence of any precipitins bands was reported as a positive result. Neat serum was tested for all samples. Where samples were positive serial dilution to a maximum 1 in 32 dilution was produced to provide dilutional titres.

Where a sample produced the same result (positive or negative) on a single test by all test methods this result was accepted. Where a sample produced divergent results on different assays it was repeated twice. If the 2 new tests resulted in a different outcome (positive or negative) to the first test then the mean of these 2 new tests replaced the first test. Final results after any repeat testing are reported.

Statistics

Intra-assay variability (IAV) was measured for all assays except ThermoFisher Scientific ImmunoCAP. Two samples were selected for each assay, one that produced a low result
and one that produced a high result. Each assay was repeated 20 times on each sample. When results were complete the study team (the author and his two supervisors) identified outliers. These were defined as results that were markedly different to the other tests, with a likely explanation for an error. These outliers were removed from the final analysis. Result range, mean, standard deviation and co-efficient of variation (CV) are reported for each assay. CV was calculated as (standard deviation / mean) x 100.

Statistical analyses were performed using SPSS version 20 (IBM, USA) under license to the University of Manchester, UK. Descriptive statistics are reported for each test, including the range, mean and median results in each patient group. Receiver operating characteristic (ROC) curve analysis was performed for each test. The performance of each test is compared using the Area Under the Curve (AUC) for ROC analysis with 95% confidence intervals (95% CI). Wald statistic is used to compare the ROC AUC results from different assays. ROC curve analysis is not performed for precipitins as the semi-quantitative nature of precipitins results are not compatible with this analysis.

CPA and ABPA patient results were both compared directly to healthy controls for each test. ABPA results were also compared to asthmatic diseased control sera. As CPA can develop as a complication of ABPA the results of tests in these groups are compared by ROC analysis to determine the performance of each assay for the diagnosis of CPA in patients with underlying ABPA.

An ideal diagnostic threshold for the best performing test was selected. This threshold had a specificity of 98% and sensitivity of 96% for the diagnosis of CPA. For ease of comparison we then selected diagnostic thresholds for the other tests that also had 98% specificity. The sensitivity and specificity for each test is reported using both the manufacturer’s suggested diagnostic threshold and the new suggested thresholds.

The statistical analysis of all aspects of this thesis was discussed with Julie Morris, statistician based at UHSM Academy.
Recognition of the existence of the disease CPA

The work contained in this thesis is based around the syndrome chronic pulmonary aspergillosis. While the existence of pulmonary aspergilloma has been documented for centuries\(^{195,196}\) and complex aspergilloma recognised as a complication of cavitation lung disease for decades\(^{252}\) the existence of CPA in the form described above was only described in 2003\(^5\). Since that time there have been major cohort descriptions published in Japan\(^{7,59,60}\) and Korea\(^8\) and treatment studies described in Japan\(^{253}\), India\(^{18}\) and France\(^{108,254,255}\) in addition to further work published by colleagues at the UK National Aspergillosis Centre\(^{14,21,58,198,256,257}\). The total number of CPA cases recorded in these papers is 763.

Published evidence in the field of CPA has been summarized in recent review articles\(^{37,50,258}\). The Infectious Diseases Society of America has published guidelines for the diagnosis and management of CPA\(^{250}\) and the European Society for Clinical Microbiology and Infectious Diseases is in the process of drafting its own guidelines\(^{251}\).

The existence of CPA is therefore now widely accepted.

Use of composite gold diagnostic standard for CPA

All these publications and professional bodies accept that CPA is diagnosed using a combination of symptoms, radiological findings and microbiological evidence. There is no single gold standard test for this condition. For many fungal infections including invasive aspergillosis biopsy is the accepted gold standard\(^{48}\). In the case of invasive fungal aspergillosis the presence of fungal hyphae invading lung tissue is the accepted definition of ‘proven’ disease.

Unfortunately biopsy cannot be used as a gold standard in CPA as there is no invasion of healthy tissue in this condition. Histological examination can demonstrate the presence of aspergilloma or *Aspergillus* nodules in resected lung tissue\(^{21}\). This sometimes represents the first diagnostic evidence of CPA, particularly in patients where the
diagnosis of lung cancer was suspected based on CT scan appearance and resection was deemed necessary. This is clearly not an ethically acceptable method for use in an epidemiological survey, as the risk of death or morbidity secondary to resection means it cannot be performed in patients with no clear medical indication for undergoing the procedure.

In paper one, the sensitivity and specificity of various Aspergillus-specific IgG assays is described. Histological examination of resected lung tissue was not used as a gold standard to compare the assays against. This was firstly on the grounds that only a handful of cases treated by the NAC were diagnosed in this way. Given that the NAC is the largest treatment centre for CPA in the world, it is unlikely that a sufficiently large cohort of CPA patients diagnosed in this manner exists anywhere.

Also, patients diagnosed as a result of histological examination of resected tissue may not be representative of the CPA population as a whole. Surgery is often performed on patients with clear aspergilloma and haemoptysis. As aspergilloma is present only in a minority of patients and regarded as a late complication of CPA. Conversely, Aspergillus nodules may mimic the appearance of cancer and be resected on those grounds, but while the natural history of Aspergillus nodule disease in patients without gross immunosuppression is not entirely clear, it probably represents a less severe form of CPA. Overall the surgical population is probably not representative of the CPA population as a whole.

For these reasons it was clear that the best possible standard to measure the various Aspergillus-specific IgG against was the composite gold standard used to diagnose clinical cases of CPA at the NAC. The diagnosis of CPA in this cohort is never based on the presence of raised Aspergillus-specific IgG alone. Rather, the combination of symptoms, radiological change and microbiological evidence of infection is required. As noted above this diagnostic standard is widely accepted. It is also important to note that the cohort of patients with CPA at the NAC includes patients with microbiological evidence of Aspergillus infection from various sources. Many have raised Aspergillus-specific IgG, but patients diagnosed on the basis of antigen tests such galactomannan,
repeatedly positive culture from respiratory samples and histological findings from resection or biopsy are also included, regardless of their *Aspergillus*-specific IgG levels.

While this represents the best gold standard option available, there are difficulties with this method. It is not possible to provide positive and negative predictive values for the *Aspergillus*-specific IgG assays studied here. These figures cannot be calculated from the case control study design used in paper one. They could theoretically be calculated from the cross-sectional survey such as the one described in paper three. Unfortunately raised levels of *Aspergillus*-specific IgG are a mandatory criterion in the composite gold standard used to define cases of CPA in this study. It is not therefore possible to calculate positive and negative predictive values, as it would be impossible to have a false negative result for *Aspergillus*-specific IgG in relation to this study design.

Potential for bias due to use of *Aspergillus*-specific IgG in composite diagnostic criteria for CPA

It is possible that the NAC cohort is biased in favour of patients with raised *Aspergillus*-specific IgG. The majority of cases in the NAC CPA cohort have raised *Aspergillus*-specific IgG\textsuperscript{74}, whereas culture is positive only in a minority of patients using standard techniques\textsuperscript{260}. Cases of CPA occur with positive culture or biopsy, but negative *Aspergillus*-specific IgG\textsuperscript{5,7,8,59}. However measuring *Aspergillus*-specific IgG is much more convenient than other potential sources of microbiological evidence. It is therefore likely that patients with raised *Aspergillus*-specific IgG are diagnosed more frequently than ‘antibody-negative’ cases in the course of clinical practice. As a result the sensitivity of any *Aspergillus*-specific IgG for the diagnosis of CPA may be exaggerated by this method.

The ThermoFisher Scientific ImmunoCAP assay is used as the standard method to measure *Aspergillus*-specific IgG at the NAC. While patients are referred from all over the country and results of other assays at other hospitals are accepted, the large majority probably had *Aspergillus*-specific IgG measured using the ThermoFisher Scientific ImmunoCAP assay. By comparison the manufacturers of the Siemens
Immulite, Serion, Genesis and Dynamiker kits advise us that their products are not in regular clinical use anywhere in the UK.

While these difficulties could not be entirely negated, the composite gold standard that was used was the best option available and the resulting study does represent the most informative assessment of the sensitivity and specificity of the various available Aspergillus-specific IgG assays. It is also worth noting that any potential bias in favour of ThermoFisher Scientific ImmunoCAP did not prevent the Siemens Immulite system from demonstrating equivalent sensitivity and specificity to the ThermoFisher Scientific ImmunoCAP assay.

Use of results from fresh and stored sera for different assays

The study was intended to be performed solely on stored sera. The length of time the sera had been stored for varied from patient to patient, but was up to ten years.

Unfortunately ThermoFisher Scientific ImmunoCAP declined to donate kits for use in the study. Grant applications for pay for the purchase of ThermoFisher Scientific ImmunoCAP kits were not successful. ThermoFisher Scientific ImmunoCAP is the most commonly used test in the UK. The study would have been much less valuable if it was excluded from the comparison. The results of ThermoFisher Scientific ImmunoCAP testing performed on fresh samples as part of routine clinical care were therefore used in the comparison, as they were the only available source of ThermoFisher Scientific ImmunoCAP results.

It is conceivable that this would introduce bias. There is no published data regarding the repeatability of Aspergillus-specific IgG testing after prolonged storage of frozen samples. There is some evidence that galactomannan antigen testing results are altered by prolonged storage, with lower results noted in stored samples\textsuperscript{261}. If this were also the case for Aspergillus-specific IgG testing as well then the study would be significantly biased in favour of ThermoFisher Scientific ImmunoCAP. There is, however evidence that antibody levels are unaffected by prolonged serum storage\textsuperscript{262,263}. There is no reason to believe that Aspergillus-specific IgG would behave any differently to other
antibodies in this respect. The use of stored sera for some assays and historical results from fresh sera for other assays is therefore unlikely to have introduced bias.

Number of tests performed on each serum sample

Ideally all the assays used in this study would have been performed in triplicate in every sample to ensure that any human error in an individual test was identified and removed from the analysis. This was not possible due to lack of time and insufficient supply of kits. The study was dependent on the kind donation of kits by the manufacturers. Many manufacturers declined to donate any kits and the ones that did donate were limited in the number of kits they could provide. There was also insufficient volume of stored sera available for such a study design in many cases.

It was, however possible to compare the results from each individual sample across the six assays used. While the results produced by different kits could not be directly compared as they did not all use the same units, it was possible to see if the results were reproduced across the kits in terms of positivity or negativity. Where a sample produced the same result on all seven kits this was unlikely to be the result of the same human error occurring in each separate test. These results could therefore legitimately be accepted on the basis of a single test for each kit. Where different kits produced divergent results for an individual sample then triplicate testing was performed. This method should be almost as reliable as performing triplicate testing in every case.

Use of single operator to perform assays

The author performed the almost all of tests included in the serology comparison papers himself. This has the major advantage of consistency between tests, but as a result this work cannot comment on inter-operator variability. The study required the performance of over 5000 individual assays, meaning the author became highly experienced in the use of these assays. The author received training on the performance of these assays by laboratory technicians experienced in their use prior to commencing the study. Assay runs used in the final analysis only went ahead after establishing that consistent results were obtained from selected test sera over several training runs.
Selection of control groups

The main analysis in paper one compares results of *Aspergillus*-specific IgG testing in known cases of CPA and ABPA to healthy controls. These healthy control sera are taken from Ugandan blood donors. These may not be the ideal comparator group. Ugandan control sera were selected because the primary goal of this study was to define diagnostic cut-offs for assays that might subsequently be used in a prevalence study in Uganda.

It is conceivable that Ugandans might form different levels of antibodies than Britons, perhaps due to different levels of environmental exposure to *Aspergillus* spp. If this was the case then the diagnostic thresholds suggested in this study might be less accurate outside Uganda. There is, however no published evidence that the levels of *Aspergillus*-specific IgG in healthy controls varies from country to country.

The age matching of controls and cases was sub-optimal. Mean age was 19 years for controls, but is 59 years for CPA cases at the NAC. *Aspergillus*-specific antibody levels rise throughout childhood and appear to stabilize at adult levels. Adolescents may still have antibody levels below that of healthy adults. Despite these potential flaws the use of control samples from healthy blood donors is common practice in research as these are the most practical healthy control samples to access. No other samples were available for use in these studies.

In the case of ABPA two analyses were performed in the serology comparison detailed in paper one. First the results of patients with ABPA with were compared with healthy controls and then the results of patients with ABPA were compared to asthmatic diseased controls. The reason for doing so was that ABPA very rarely occurs in non-asthmatics. One exception is ABPA occurring secondary to cystic fibrosis, but this has been investigated elsewhere. If asthmatic patients have different levels of *Aspergillus*-specific IgG to healthy persons then a diagnostic cut off defined in a healthy population might not be applicable to the asthmatic population where the test will actually be used.
To take account of this possibility sera from asthmatic diseased controls were also tested. These came from asthmatic patients enrolled into the ManRAB biobank at the North West Lung Centre, where the NAC is based. A benefit of selecting this particular group of asthmatics is that it is that screening for ABPA is probably more likely to have occurred in a unit that specializes in aspergillosis care than might be the case in other units. On the other hand patients recruited to a biobank at a regional tertiary specialist care centre are probably not representative of the asthmatic population as a whole.

The ideal control group would probably consist of persons with well-defined asthma in a primary care setting. Unfortunately such patients are harder to access. No biobank of sera from such a group was available for use in this project and no resources were available to recruit such patients. In the absence of an unbiased asthmatic control population we must consider the possibility that healthy persons might represent a better control group than severe asthmatics referred to a tertiary hospital when considering the diagnosis of ABPA in persons with mild asthma treated in the primary care system. Diagnostic thresholds for use in ABPA have therefore been calculated against both potential control groups.

The same difficulty was present when the diagnostic threshold for the use of Aspergillus-specific IgG in the diagnosis of CPA was defined. In this case Aspergillus-specific IgG levels in CPA cases were compared to healthy controls in paper one. However CPA occurs almost exclusively in patients with underlying diseases\(^1\). Unfortunately very little published data exists describing the levels of Aspergillus-specific IgG found in groups of patients with these underlying diseases. It was not possible to perform these assessments within the time and financial constraints of this PhD.

It should be noted that Aspergillus-specific IgG is only one aspect of the diagnostic process for CPA. It is therefore reasonable that the threshold reached by comparison of CPA cases to healthy controls be used to define ‘abnormally high levels of Aspergillus-specific IgG’, which form a single part of this diagnostic process. Patients with an Aspergillus-specific IgG above this threshold should undergo further investigation, with CPA confirmed only when all diagnostic criteria are met\(^5\).
Other limitations

Finally it should be noted that the serology comparison study reported here is incomplete in several respects. Further *Aspergillus*-specific IgG kits are produced by Bio-Rad (France), IBL (Germany), IMMY (USA) and Bordier (Switzerland). These companies did not donate kits for use in this study and no resources were available to buy them. Precipitins were performed only with Microgen (UK) *Aspergillus* antigens. It is possible that the precipitins technique might produce different results if antigens from another supplier were used.

A single operator in a single laboratory performed all assays reported here. It therefore provides no information regarding inter-operator or inter-laboratory variability, which is crucial information that must be obtained before a test could be rolled out as part of a global screening program. Good inter-laboratory variation results have been published elsewhere for ThermoFisher Scientific ImmunoCAP\textsuperscript{180}, but no such comparisons exist for the other assays. The study also uses tests from a single batch. Batch-to-batch variation is described in the manufacture *Aspergillus* antigens\textsuperscript{141} and the results of this study would not be valid if it was present in the assays used in this study.

Nonetheless this study represents the largest comparison of different methods of measuring *Aspergillus*-specific IgG for the diagnosis of CPA and ABPA and is the first study conducted without the bias of long-term antifungal therapy. As such is a major contribution to the field.
Paper 2 – *Aspergillus*-specific IgG levels in patients previously treated for pulmonary tuberculosis in Gulu, Uganda

Ethical approval

Ethical approval was granted by the following bodies before the study was commenced;

- The University of Manchester Research Ethics Committee 1 on 7th June 2012 (reference 11424).
- Gulu University Faculty of Medicine Institutional Review Board (IRB) on 4th July 2012 (reference GU/IRC/04/07/12)
- Uganda National Council for Science and Technology (UNCST) on 20th September 2012 (reference HS 1253).

Recruitment criteria

Recruitment of patients took place in Gulu, Uganda from October 2012 until January 2013. Criteria for recruiting patients to the study were as follows:-

- Patients must be aged 16 years or over.
- Patients must be able to give informed consent.
- Patients must have completed a full course of treatment for pulmonary tuberculosis with a treatment start date of 1st January 2005 or later.

A diagnosis of treated pulmonary tuberculosis (TB) was considered valid if the patient had a positive sputum smear test for acid and alcohol fast bacilli (AAFB), culture growth of mycobacterium tuberculosis species (MTB) from sputum or a positive GeneXpert polymerase chain reaction (PCR) test for MTB. A diagnosis of smear negative TB was considered acceptable only if the patient reported complete resolution of symptoms at the end of treatment.
Patients had to have documentary evidence of tuberculosis. The following was accepted as documentary evidence of TB:

- A completion of treatment certificate from the Uganda tuberculosis treatment program.
- A tuberculosis treatment record from the Uganda tuberculosis treatment program.
- Medical notes from the treating hospital documentating tuberculosis treatment.
- Documentation of tuberculosis treatment on a HIV treatment card.
- Documentation of the patient’s tuberculosis treatment in the central record book kept at the regional or district tuberculosis centre.

Recruitment targets were:

- 200 HIV positive patients with previously treated TB.
- 200 HIV negative patients with previously treated TB.
- 100 healthy controls

Ugandan TB treatment protocols require HIV testing to be offered to all patients at the time of TB diagnosis. Testing is routinely performed using two different rapid test kits. If they produce different results a third brand of test kit with high sensitivity is used as a tiebreaker. The brands of rapid testing kit may have varied from site to site. The following was accepted as documentary evidence of HIV co-infection:

- HIV antibody test result form.
- HIV antibody test result recorded on Uganda tuberculosis treatment program treatment card.
- HIV antibody test result recorded in the tuberculosis record book at the regional or district tuberculosis centre.
- HIV antibody test result documented in patients medical notes.
- Treatment card stating that the patient is being treated or monitored at a HIV clinic.
Where patients presented without any of the above evidence they were offered HIV testing. Those who accepted were tested by certified laboratory assistants working with the study team and were enrolled into the study. Those who declined testing were not eligible to enter the study, although in practice no patient declined testing.

Recruitment strategy

Recruitment of patients initially took place within the Infectious Diseases clinic of Gulu Regional Referral Hospital (GRRH). Sequential recruitment of any patient attending the clinic who met the above criteria was planned. Unfortunately the patients recruited from this clinic were almost all HIV positive, as HIV-negative patients who have completed tuberculosis therapy are routinely discharged from active follow up. In order to recruit HIV negative patients a convenience sampling method was adopted, where patients were recruited directly from their villages with the assistance of the Gulu District Health team.

District health centers where patients had been treated for tuberculosis were identified from the records at the District Health team headquarters in Gulu. Recruitment was restricted to centres within a 1-hour drive of Gulu to allow time to transport patients to and from Gulu in addition to the time needed to perform clinical assessment and chest X-ray.

A study vehicle was sent to one district health centre daily. The centre was contacted in advance by telephone and asked to mobilise the patients with previously treated tuberculosis within their community. This was done by word of mouth with the assistance of village health workers. Radio announcements were also made inviting any patient with previous tuberculosis to present to the appropriate health centre for recruitment. A staff member (one of the two persons responsible for administering the tuberculosis program in this area) from the district health team travelled to the district health centre daily.

Potential recruits who brought written documentation of tuberculosis treatment were admitted to the study. Those without documentation had their names checked against
the district health office tuberculosis record book by the staff member and were recruited to the study if they were recorded as a case of treated tuberculosis. HIV status was also copied from the district health book if the patients did not bring documentation with them. A recruitment fee of around £2 per patient was paid to the district health team.

Patients were transported to Gulu Regional Referral Hospital HIV clinic for assessment and venepuncture and then transported to St. Mary’s Hospital, Lacor for chest X-ray, after which they were taken home. Patients were provided with expenses of around £2 each to buy food. They were provided with written information (in Acholi or English as preferred by the patient) and signed a consent form prior to recruitment. Copies of these documents are contained in Appendices 2 and 3. If patients could not read the information on the patient information sheet was communicated to them verbally, in Acholi, by a member of the recruitment team. Where patients could not write the consent form was stamped after discussion and verbal consent.

Healthy controls recruitment strategy

Control samples were gathered with the assistance of the Gulu district blood transfusion service. Donors gave verbal consent to having some of their blood used in the study. The majority of donors were healthy adolescents attending boarding school. Venepuncture was performed by blood transfusion service phlebotomists. Blood was stored in cool boxes and fridges for up to 24 hours before serum was separated and stored at minus 80 degrees then shipped alongside the patient samples. Details of donors’ age, gender and results of testing for HIV, syphilis and hepatitis B and C were provided by the blood transfusion service. Samples were anonymised by the blood transfusion service before being transferred to the study group. Test results were not fed back to control sample donors as the meaning of an unexpected positive test in an asymptomatic control patient is not clear.
Clinical assessment process

During clinical assessment demographic data was recorded including patient name, age, gender, village, and telephone number together with the name and telephone number of a nominated guardian. Details of previous TB treatment were taken including; date when treatment was commenced, smear status at diagnosis, culture results, GeneXpert PCR results and whether the patients symptoms resolved completely with treatment. The TB reference number was noted. HIV status was recorded together with date of diagnosis. Where available, baseline and current CD4 count were recorded.

The presence and duration of symptoms including; cough, haemoptysis, fatigue, breathlessness, fevers, night sweats, and chest pain were recorded. MRC Dyspnoea scale score was recorded. The MRC dyspnoea scale is described fully in Appendix 4. Patients were provided with an Acholi translation of the scale if they could not read English.

Ability to work normally or not was recorded. Lung percussion and auscultation were performed and the results recorded together with oxygen saturations.

Potential CPA risk factors were recorded, including living in a traditional ‘grass-thatch’ house in comparison to a modern brick dwelling with metal or slate roof. Other factors recorded were the reported presence of visible dampness in the patient’s house, regular exposure to wood smoke though cooking, farming or handling farm products and cigarette smoking.

Aspergillus-specific IgG testing process

Venepuncture was performed by trained laboratory technicians. Samples were stored in a cool box for up to 4 hours. At the end of the recruitment period they were transported to Joint Clinical Research Centre (JCRC) laboratory. Here serum was separated by trained and accredited JCRC laboratory staff. Serum was placed in labeled cryotubes and frozen in a minus 80 freezer with both diesel generator and battery back up in the event of mains power failure. The freezer had a temperature alarm system and the building was occupied at all times to ensure power failures were corrected without sample thawing.
Samples were shipped on dry ice to the Manchester University laboratory at University Hospital of South Manchester (UHSM) at the end of the study with the assistance of DHL couriers. Shipping authorization was provided in the form of a Material Transfer Agreement (MTA) between UHSM and JCRC, plus specific written authorization from the Gulu University IRB and UNCST.

CD4 count testing process

Where HIV positive patients had a recorded CD4 count result from within the last 12 months this was accepted as a current CD4 count. Where no such result was available CD4 count was performed at JCRC laboratory, Gulu.

Radiology process

All chest X-rays were performed at Lacor hospital radiology department by a qualified radiographer. Patients were transferred to and from Lacor hospital for chest X-ray after completing the clinical assessment and venipuncture.

Radiology reporting strategy

Chest X-ray results were reported by two radiologists. Dr Cyprian Opira is the senior radiologist at St. Mary's Hospital, Lacor. He viewed plain films, visualized on a light box in a darkened X-ray reporting room with curtains. Dr Sharath Hosmane is a specialty registrar in radiology, working at UHSM. Films were photographed at St. Mary’s Hospital using a Nikon digital camera. The images were sent to Dr Hosmane by email. Where the two radiologists produced discordant reports, a decisive third report was provided by Dr Richard Sawyer, senior consultant respiratory radiologist at the North West Lung Centre, UHSM.
Discussion

Limited nature of existing prevalence data for CPA

This work takes place in a neglected field. The only existing published survey measuring the prevalence of pulmonary aspergillosis in patients with treated pulmonary tuberculosis is from 1968-70\textsuperscript{16,197}. This study took place in the UK, where pulmonary tuberculosis is now much less common than it was at that time\textsuperscript{215}. No study measuring CPA prevalence has been performed in an area with currently high pulmonary tuberculosis prevalence. While the limited extent of prior research in this field results in opportunities to perform landmark original research, it also presents many challenges, which require discussion and explanation.

Cross-sectional survey design

Paper 2 describes a cross-sectional survey of the prevalence of CPA in patients with treated pulmonary tuberculosis in Gulu, Uganda. This study design was the only option available that could be completed within the three-year timeframe of the PhD. The total budget for this study was £50,000, which is limited and resulted in a number of constraints. It is, however the first and only survey of the prevalence of CPA in an area of current high tuberculosis prevalence. The primary goal of the study is simply to confirm that CPA exists at a measurable level in this population. This goal was achieved. As this study is unique it is appropriate to extract as much information as possible, but the interpretation of the data must take account of the restraints imposed by the study design.

Use of convenience sampling

The study used convenience sampling, with patients recruited via the village health worker network and radio announcements. This method may have introduced bias. Healthier patients may have been more likely to be recruited, as sick patients may have been unable to make the journey to the recruitment sites. Conversely motivation to enter the study might have been higher in symptomatic patients.
Most importantly this study design takes no account of patients who might have died after completing tuberculosis treatment without entering the study. The study accepted anyone who completed tuberculosis treatment within the last seven years, a duration selected on practical grounds as this was the period after the war with the Lord’s Resistance Army ended and for which good records were available.

The five year mortality of CPA is up to 85%. Many patients with tuberculosis treated in Gulu within the last seven years may therefore have developed CPA and died of it without ever entering the study. This might be especially true in HIV positive patients where pulmonary aspergillosis occurs in a subacute manner and so progresses to death quicker than CPA.

This cross-sectional recruitment method was the only practical option as patients with tuberculosis are discharged after completing treatment in Uganda. Equal opportunity to enroll was maximized by comprehensively contacting all health centres in the study zone and using radio announcements broadcast to the whole region. Sending the study vehicle to every health centre in the study zone minimized the barrier to recruitment that patients might experience due to transport difficulties. It was not, however possible to send a vehicle to every village as many were not accessible by road. Where patients travelled to the health centre by motorcycle taxi we re-imbursed their fares, but inability to pay up front may have limited the ability of some poorer patients in remote villages to join the study.

The study design allows us to state that CPA is present in this population, but the frequency of disease obtained must be interpreted in light of the cross-sectional design and convenience sampling. The ideal design would be a prospective study that recruited all patients completing treatment for pulmonary tuberculosis and then followed them up to see if they develop CPA. Such a study was not possible within the time and financial constraints of this PhD. The author is, however part of a team that has designed such a study in collaboration with the Kenya Medical Research Institute (KEMRI). Grant proposals have been submitted to the Japanese government to fund this study. Preliminary results from the work described in this thesis form a key part of that application.
Ethical approval

Ethical approval for this re-survey was granted by the University of Manchester ethics board, Gulu University IRB and UN CST. In each case the approval took the form of an extension and amendment to the approvals granted for paper two. New patients were not eligible.

CCPA diagnostic criteria

In this resurvey CCPA is diagnosed in patients where all of the following are present; 1 – cough or haemoptysis for one month or longer, 2 – either new or progressive cavitation on serial chest X-ray OR the presence of paracavitary fibrosis or aspergilloma on CT scan, 3 – raised Siemens Immulite Aspergillus-specific IgG, 4 – Absence of evidence of current pulmonary tuberculosis. Chronic fibrosing pulmonary aspergillosis (CFPA) is additionally diagnosed in patients who meet the diagnostic criteria for CCPA and also have extensive lung fibrosis that progressed between the 2 chest X-rays. Simple aspergilloma is diagnosed in patients with aspergilloma on CT scan and raised Aspergillus-specific IgG, but with no chronic cough or haemoptysis. Unspecified fungal ball is diagnosed in patients with apparent aspergilloma on CT scan, but with normal Aspergillus-specific IgG levels.

The frequency of raised Aspergillus-specific IgG in the absence of other signs of pulmonary aspergillosis is also reported and the frequency of symptoms and radiological changes compared between this group and those with normal Aspergillus-specific IgG.

Recruitment criteria

Only patients recruited to the first study in 2012 were eligible for recruitment to the re-survey.
Recruitment strategy

Mobile phone numbers were taken during the first study from all patients who had access to one. Each patient was telephoned by a member of the study team. Around two thirds of patients were reached by telephone, but the remainder either had no phone number available or did not respond when called. Radio messages were used to contact these outstanding patients. In addition the study team contacted local health workers in all areas where recruitment to the original study took place. Patients were then traced by personal visits from village health workers. Recruitment days were scheduled at each of the health centres that took part on the original survey. Patients were informed of the results from the first study.

Each patient was provided with expenses of around £2 to cover food costs. Any transport costs incurred by the patient to reach the study centre were re-imbursed.

Clinical assessment process

An identical clinical assessment to the first study was performed.

Aspergillus-specific IgG testing process

Venepuncture was performed by the same study assistants employed in the original study using the same methods. For this study, however serum samples were separated, labeled and stored at GRRH laboratory. The two study assistants, who are both trained laboratory technicians, performed sample processing. The laboratory was not considered suitable for storage at the time of the original survey due to inadequate back up power, but new battery and diesel generator back up was installed between the two surveys.

Serum was shipped to the University of Manchester laboratory at UHSM after the signing of an MTA by GRRH and UHSM and the provision of written clearance to ship by Gulu IRB and UNCST. These samples will be tested for Aspergillus-specific IgG on the Siemens Immulite 2000 machine at Christie Hospital, Manchester. However, these
results are not available at the time of thesis submission. Results of *Aspergillus*-specific IgG testing from the first survey are therefore used in the analysis contained in this thesis.

**Sputum GeneXpert tuberculosis PCR testing process**

Where patients had a productive cough and were able to produce a sputum sample this sample underwent GeneXpert PCR testing for *Mycobacterium tuberculosis* (Cepheid, USA) at the GRRH lab. When the GRRH GeneXpert IV machine malfunctioned samples were taken to St. Mary's Hospital, Lacor for testing on the GeneXpert IV machine at that laboratory. These were the only two GeneXpert machines in the region. On one occasion when both were malfunctioning sputum smear testing was performed at GRRH laboratory in place of PCR testing. All samples were tested within 72 hours of submission.

**Radiology processes**

When clinical assessment was complete patients were then transferred to St. Mary’s Hospital for chest X-ray. Trained radiographers performed the X-rays using the same equipment as the first study.

Patients who had either raised *Aspergillus*-specific IgG from samples taken during the first survey or suspected aspergilloma on chest X-ray from the first survey were invited to undergo CT thorax. A new patient information sheet was given to patients eligible for this test and is shown in appendix 5. Consent was then taken from those undergoing CT scan using the consent form shown in appendix 6. As in the first study these forms were made available to the patients in English or Acholi. Where patients were illiterate the contents of the sheets were read and explained to the patients by Acholi-speaking study staff. Where patients could not sign the form verbal consent was taken.

Patients who consented to CT scan were transported to Kampala and underwent CT scan at the Kampala Imaging Centre. This took place at the end of the study. A qualified radiographer employed by Kampala Imaging Centre performed the scan. Patients were
transported by private bus in groups of 30. The journey to Kampala took 10 hours each way. Hotel accommodation was provided for patients for one night in Kampala. They also received expenses of around £10, plus re-imbursement of any transport costs they incurred to reach the pick-up point.

Radiological reporting strategy

Chest X-rays from 2014 will be compared to chest X-rays from the original 2012 survey. Progressive cavitation will be noted if present. As with the first survey all X-rays will be reported by two radiologists, with a deciding report produced by a third radiologist in the event of divergent reports. Radiologist reports comparing X-rays from the two surveys are still ongoing at the time of thesis submission. These final reports will be used in the eventual publication. The author’s reports of new or progressive cavitation are used in data analysis for this thesis.

CT scans will be reported by three radiologists in the same manner as the chest X-rays. Drs Hosmane and Sawyer will both take part, in addition to Dr Rosemary Byanyima, the senior consultant radiologist at Kampala Imaging Centre. However these reports will not be completed during the PhD period. The author’s reports are therefore used in the analysis contained in this thesis.

Full digital records of each CT scan are available to the author and all radiologists and were accessed using OsiriX software (Pixmeo SARL, Switzerland).

Statistical analysis

A CPA prevalence of 6% was predicted prior to the study based on published estimates of CPA prevalence after pulmonary tuberculosis\textsuperscript{11}. Power calculations prior to recruitment concluded that by recruiting 400 patients the prevalence of CPA following successful tuberculosis treatment could be determined with an accuracy of ± 2.3%.

The frequency of symptoms, test results and diagnoses is described for all patients recruited to the resurvey. Statistical analysis is performed using SPSS v20 (IBM, USA).
Before commencing the main analysis the characteristics of the population from the first study were compared to the population in the resurvey. The frequency of categorical variables is compared by chi-squared test. The frequency of continuous variables with normal distribution is compared with 2-sided t-test and the frequency of continuous variables with skewed distribution is compared by Mann Whitney U analysis. Where there is no significant difference in the characteristics of the two surveys it is concluded that the recruitment process for the resurvey did not introduce significant bias.

Descriptive statistics are provided for all test results. For continuous variables, mean results are reported where the results had a near-normal distribution and are compared in different groups using a 2-sided t-test. Where results had a skewed distribution median results are reported and the results compared using the Mann Whitney U test.

Rates of symptoms and diagnoses in various patient groups are compared using chi-squared test, except for comparisons with less than 5 patients in one group, where Fisher's exact test was used. Comparison of means in different groups was performed using 2-sided t-test. 95% confidence intervals for the prevalence of CPA and other conditions were calculated as the frequency +/- (standard error of the percentage X 1.96).

The frequency of CCPA in patients with and without various potential categorical risk factors is noted and compared using chi-squared test. Potential risk factors analyzed include; gender, HIV co-infection status, sputum smear status at time of original pulmonary tuberculosis, patient profession (subsistence farmer vs. paid employment), dwelling type (traditional grass-thatch dwelling vs. modern home), the presence of visible dampness inside the patients home (as reported by the patient), cigarette smoking status and frequent biomass smoke exposure status. The mean age, tuberculosis treatment date and CD4 count (in HIV positive patients) in CPA patients against other patients is compared by 2-sided t-test. The number of deaths in patients with and without CPA in the original survey is noted.
Discussion

Recruitment strategy issues

The resurvey attempted to recruit all patients recruited to the first study. Comprehensive efforts were made to contact all patients including personal telephone calls, telephone calls to patients relatives or guardians, radio announcements advising patients to self-present for review and direct personal contact from village health workers to request patients attend. 282 of 400 patients were successfully recruited. A further 18 died, 9 moved out of the region and 11 were contacted but declined to participate in the resurvey. 77 of 400 (19%) patients could not be contacted. This probably represents a reasonable contact rate for a resurvey that took place in a post-war region with variable mobile phone coverage where most patients lived in villages with no electricity.

Contacting patients was hampered by the fact that Gulu was still in a post-conflict situation at the time of the first survey. While most refugee camp residents had returned to their villages prior to the start of the first survey, some patients recruited to the first survey may still have been in temporary accommodation. The rate of house move was probably higher between the two surveys than might have occurred in other circumstances. Ugandans also frequently change their telephone provider and number to take advantage of lower call rates, which may have hampered attempts to contact patients by telephone. Phones may also been turned off due to lack of reliable mains electricity for charging.

Recruitment to the resurvey may have introduced bias if it was unintentionally selective in nature. The resurvey team was aware of the results from the first survey. There was a risk that they may have made a greater effort to contact those with raised Aspergillus-specific IgG. This should have been avoided, as the tracing plan was the same for all patients and the recruitment fee paid to District Health Team staff was the same for all patients. There was therefore no motive for them to concentrate on patients with positive results in the first survey. If bias was unintentionally introduced then this
would have been expected to result in a difference in the patient characteristics between the two cohorts. No such difference was detected.

**CCPA case definition**

The case definition of CCPA was first produced by the author's main supervisor in 2003 and has since been accepted in several publications. This definition was designed for use in trials relating to CPA in well-resourced health care settings such as the UK. It was necessary to adapt it in several ways for use in this study.

**Inclusion of HIV positive patients**

The original definition explicitly excludes patients with HIV infection as potential cases of CPA, on the grounds that gross HIV induced immunosuppression would put the patient at risk of invasive aspergillosis. This exclusion is important for the conduct of clinical trials in well-resourced settings, where an unbalanced inclusion of HIV infected persons could bias the results. It is not appropriate for use in Uganda, where a large proportion of patients with treated tuberculosis are co-infected with HIV. Gross immunosuppression is not present in the great majority of HIV positive persons enrolled in this study, who had controlled HIV on effective therapy with good CD4 counts. Indeed more recent CPA case definitions have only excluded patients with uncontrolled HIV infection.

As HIV co-infection is so common in African tuberculosis patients it was appropriate and necessary to include patients with HIV co-infection in this study in order to maintain a sample that was representative of the overall population at risk. Including patients with HIV co-infection allows the first measurement of CPA prevalence in a HIV co-infected population with treated tuberculosis and allows comparison of the serological and radiological findings of CPA in those with and without HIV co-infection.

To take account of HIV co-infection the duration of symptoms required to define a case was reduced from 3 months to 1 month. This is in line with the case definition of CNPA / subacute invasive aspergillosis proposed by other authors. This definition captures
all cases of CPA, but would also capture cases of subacute invasive pulmonary aspergillosis that might be seen in patients with AIDS\textsuperscript{52}. As this study is the first to be conducted in an area of high HIV and tuberculosis prevalence it was more important to measure the overall prevalence of pulmonary aspergillosis in this population than it was to accurately subdivide these cases into chronic and subacute pulmonary aspergillosis.

**Definition of cough in clinical assessment**

The original case definition of CPA\textsuperscript{5} includes productive cough as a case defining symptom. This was included in the original case definition for this survey. During the course of recruitment, however it became clear that many persons reporting productive cough did not produce sputum samples and many persons reporting non-productive cough did produce samples. In order to minimize the impact of language barrier on clinical assessment written patient information documents and questionnaires were produced in Acholi as well as English and distributed to all patients. However, most patients recruited to the study were illiterate and spoke no English.

Histories were taken through a translator as a result. This translator was a qualified laboratory assistant who spoke excellent English and native Acholi, but was not a clinician. No clinicians were available to assist with the study. It seemed likely that while the concept of cough was being translated well the differentiation into productive and non-productive was perhaps not being well communicated to the patients. Any patient who reported cough was therefore accepted as meeting the symptoms requirement for the case definition.

**Absence of weight loss from clinical survey**

The original case definition\textsuperscript{5} includes weight loss alongside cough and haemoptysis as a defining feature of CPA. It was not possible to include this in the first survey, which involved only a single assessment. Records of previous weight were not always available and where they were it was by no means certain that the scales used at local health centres were appropriately calibrated before use. It would have been possible for
us to include weight loss in the second survey, by measuring weight during both surveys. Unfortunately recruitment of patients to the first survey took place before it was clear that a second survey was possible. As a result weight measurement was not included in that study design.

Absence of inflammatory markers from case definition

The original 2003 case definition\(^5\) includes the presence of raised inflammatory markers. Some later cohort descriptions do not include this requirement\(^{108,264}\). Their inclusion in the case definition of CPA is therefore a matter of debate. In practice it was not possible to perform inflammatory markers as these tests were not available at Gulu Regional Referral Hospital and no funds were available to purchase them at other locations.

Definition of radiological features of CPA

Our case definition included aspergilloma as an accepted radiological feature of CPA. The original 2003 case definition\(^5\) did not include this. The definition in this study reflects the new CPA case definition that has been drafted by ESCMID\(^{251}\).

Another difficulty with the radiological definition of CPA is that while aspergilloma is a well-defined radiological finding\(^{218}\), paracavitary fibrosis is more subjective. Progressive cavitation is also subjective, as the degree of progression necessary to meet this criterion is not defined. These flaws are inherent in the accepted case definition\(^5\). A more precise and objective definition of the radiological features of CPA has not yet been proposed in the literature.

The impact of subjective reporting was minimized in this study by including the reports of multiple, independent radiologists who were blinded to clinical and serological information. This is a commonly used method in radiological surveys\(^{244,265,266}\) where a degree of human interpretation is often required. Unfortunately radiologist reports for the resurvey are not available at the time of submitting this thesis. The radiologists involved in this study all have demanding full time clinical jobs and took two years to
complete the reports of the first survey. Delaying the submission of the thesis to await these reports was not, therefore a good option. The resurvey results in this thesis are based on the author’s own reports. Reporting by multiple radiologists will be completed and the survey results re-analysed in the light of these reports prior to publication.

Exclusion of other conditions

The original 2003 CPA case definition\textsuperscript{5} includes the need for exclusion of other conditions before a diagnosis of CPA can be reached. Active pulmonary tuberculosis was excluded by performing GeneXpert PCR testing on sputum. However other conditions might also mimic CPA. Non-tuberculous mycobacteria are commonly present as co-infections in patients with CPA\textsuperscript{14}, but would not be detected by the GeneXpert system, however their presence does not exclude a diagnosis of CPA. The new ESCMID guidelines define some infections as acceptable co-infections, whereas others, such as coccidioidomycosis and histoplasmosis are differential diagnoses\textsuperscript{251}.

Other complications of tuberculosis, such as bronchiectasis, could produce similar symptoms to those of CPA. Raised levels of \textit{Aspergillus}-specific IgG are seen in cystic fibrosis\textsuperscript{66,78} and bronchiectasis from other sources, in the absence of CPA and may be due to \textit{Aspergillus} bronchitis in these settings\textsuperscript{39}. Indeed this might be the explanation for the patients identified in the study with raised \textit{Aspergillus}-specific IgG, but no CPA. The inclusion of CT scan in all patients with raised \textit{Aspergillus}-specific IgG allows the detection of aspergilloma or paracavitary fibrosis and so differentiates CPA from other causes of raised \textit{Aspergillus}-specific IgG.

Use of an \textit{Aspergillus fumigatus} specific serological technique

Perhaps the most relevant source of diagnostic uncertainty comes from the possibility of infection with fungi other than \textit{Aspergillus fumigatus}. Other species of \textit{Aspergillus} might be prevalent in Africa. \textit{A. flavus} is a more common cause of human disease than \textit{A. fumigatus} in India\textsuperscript{146,267} and \textit{A. niger} is common in Brazil\textsuperscript{147}. There is very little published information relating to the frequency of infection by various species of \textit{Aspergillus} in Africa. The sole study of the frequency of fungal co-infection in African
tuberculosis patients was performed in Egypt and showed two cases of *A. niger* and three cases of *Histoplasma capsulatum*. Histoplasmosis is known to exist in Uganda and blastomycosis elsewhere in Africa. The frequency of these other fungal infections that might mimic the clinical and radiological presentation of CPA in Africa is not known.

Cross reactivity between other *Aspergillus*-specific IgG assays and *Penicillium* antibodies has been noted. Cross-reaction with other fungi has not been studied. If cross-reaction did occur then infections by other fungi might be misdiagnosed as CPA in this study. It is also not known whether the Siemens Immulite *Aspergillus*-specific IgG assay detects non-*fumigatus* species with the same accuracy as *A. fumigatus*. The sensitivity of other assays based on *A. fumigatus* antigens for the detection of antibodies to other *Aspergillus* species has been poor. The sensitivity of the Siemens Immulite assay might therefore be poor if non-*fumigatus* species are common in Uganda and responsible for a significant proportion of CPA cases. This might explain the five cases in the study with apparent aspergilloma on CT scan, but negative serology. Sensitivity of *Aspergillus*-specific IgG assays can be improved by including extracts from multiple *Aspergillus* species in the antigen mixture.

There is therefore some legitimate doubt as to whether the study can measure the precise prevalence of CPA, as opposed to other chronic fungal lung diseases. Future studies, involving extended fungal and mycobacterial culture of sputum and broncho-alveolar lavage fluid, together with more extensive fungal serological testing will be needed to determine if fungi other than *A. fumigatus* are common in this patient group.

This does not diminish the value of the study however, as it is likely that all the patients classified as CPA in this study have chronic fungal lung disease. This is most likely due to *Aspergillus* infection, as Siemens Immulite *Aspergillus*-specific IgG assay is based on *A. fumigatus* antigens. As there has never been a survey of fungal lung disease complicating pulmonary tuberculosis in Africa this study this study represents a significant contribution to the field, even if it cannot be stated with absolute certainty that the cases detected here are CPA, as opposed to other forms of chronic fungal lung disease.
Issues relating to HIV infection

While the primary goal of the study was to measure the prevalence of CPA in all patients, an attempt was also made to compare the prevalence of CPA in patients with and without HIV infection. Ideally such a design would have included HIV testing on all patients at the time of admission to the study. Unfortunately funds were not available for this. The only source of information regarding HIV infection status was the patient notes. The vast majority of patients recruited to the study were tested for HIV at the time of entering the tuberculosis treatment program in line with national guidelines. These guidelines include the use of both screening and confirmatory point of care tests that should produce accurate results. This does not exclude the possibility that patients might have contracted HIV infection between commencement of tuberculosis treatment and recruitment to the study and some patients classified as HIV negative were indeed on HIV treatment at the time of the resurvey.

While the study design used was unavoidable due to funding restrictions it could be argued that HIV status at the time of active tuberculosis infection is the more appropriate risk factor to measure as HIV infection at that time is associated with reduced residual lung cavitation, which has itself been suggested as a determinant of the rate of CPA.

It was also necessary to include historical CD4 counts in the study analysis due to lack of funds to perform new CD4 counts on all patients. It is plausible that the historical CD4 count may no longer be representative in some patients. However given that almost all the HIV infected patients admitted to our study knew their status and were enrolled in treatment programs prior to recruitment it is more likely that their CD4 count will have risen with time rather than fallen. The key assumption that the median CD4 count in this cohort is high and that few patients had AIDS is likely to be valid.

The Aspergillus-specific IgG assay might be less effective in HIV infected patients, where antibody response might be impaired. It should be noted, however that the median CD4 count in our patients was 424 cells/µL and that acceptable antibody response to vaccination has been recorded in patients with similar CD4 counts. It
therefore seems likely that most patients in the study group would produce an antibody response to *Aspergillus* infection as most have normal or near normal CD4 counts.
Paper 4 - “Frequency of pulmonary aspergillosis in ‘smear-negative tuberculosis cases” and Paper 5 “Frequency of *Aspergillus* co-infection in patients admitted to a Ugandan hospital with pulmonary tuberculosis”

Ethical approval

The Mulago study team agreed to provide stored sera to the author for use in this study. An ethical amendment from Makerere University IRB was acquired and permission to ship samples to the UK granted by Makerere University IRB and UNCST. These sera were transported to the University of Manchester Mycology Reference Centre at University Hospital of South Manchester for further testing.

Recruitment Strategy

These two papers report the results of retrospective opportunistic testing of stored sera from a prior study undertaken by collaborators.

The Mulago Inpatient Noninvasive Diagnosis – International HIV Opportunistic Pneumonia (MIND-IHOP) study was undertaken by the respiratory research group at Makerere University, Kampala, Uganda in association with the University of California, San Francisco, USA. The author had no involvement in the design or conduct of the original study. The full MIND-IHOP study protocol is attached in appendix 7.

MIND-IHOP was a prospective cohort study. Recruitment of patients included in this thesis took place between March 2010 and March 2011. During this period all adults admitted to the casualty department of Mulago Hospital, Kampala on weekdays, with a cough of between 2 weeks and 6 months duration were offered the chance to be admitted to the study.
Sample selection criteria

Stored sera were available from around three quarters of patients originally recruited to the study. Sera were selected from patients meeting the following criteria; 1 – Patients with HIV infection, abnormal chest X-ray and no diagnosis, including no evidence of pulmonary tuberculosis, following the investigations detailed above, 2 – Patients with pulmonary tuberculosis proven by culture, GeneXpert PCR or smear testing. These two groups are the subjects of reports in the two separate papers.

Clinical assessment and diagnostic testing processes during MIND study

Clinical details were recorded for each patient. Sputum samples were taken for tuberculosis AAFB smear testing, culture and tuberculosis PCR testing with the GeneXpert automated nucleic acid amplification assay. Where patients could not produce sputum spontaneously induced sputum was acquired. Blood was taken for CD4 count (in HIV infected patients) and cryptococcal antigen testing. Bronchoscopy was offered to any HIV positive patient with persistent symptoms and negative sputum smear test. Bronchoalveolar lavage specimens underwent staining and culture for mycobacteria and fungi including staining for Pneumocystis jirovecii. Patients were reviewed at two months after recruitment and the mortality rate at this point was recorded.

Aspergillus-specific IgG testing process

Selected sera were shipped to Manchester University, UK on dry ice. Aspergillus-specific IgG levels were then measured in each sample using the Siemens Immulite 2000 assay, performed by the author at the laboratory at Christie Hospital, Manchester.

Statistical analysis

Results of these assays are reported in comparison to healthy Ugandan blood donor controls. This is the same control group described in papers 1 and 2. The mortality rate of patients with and without raised Aspergillus-specific IgG levels is described. Median
Aspergillus-specific IgG levels in patients and controls and CD4 counts in those with and without raised Aspergillus-specific IgG levels are compared with Mann-Whitney U test. Mean age in those with and without raised Aspergillus-specific IgG levels is compared by 2-sided t-test. Categorical variables are compared with Chi-squared test, except for comparison of number of positive Aspergillus-specific IgG tests in tuberculosis cases vs. healthy controls, where Fisher’s exact test is used.

Discussion

Interpretation of raised Aspergillus-specific IgG in these studies

Papers 4 and 5 describe opportunistic testing of stored sera in an attempt to estimate the prevalence of pulmonary aspergillosis in patients with presumed ‘smear negative pulmonary tuberculosis’ and proven pulmonary tuberculosis respectively. These studies do not claim to measure the prevalence of pulmonary aspergillosis. To achieve a measurement of the prevalence of ‘proven’ acute invasive pulmonary aspergillosis in line with EORTC guidelines one would need to perform biopsy on all patients. To define patients as having ‘probable’ acute invasive pulmonary aspergillosis would require CT scan evidence of progressive cavitation with paracavitary infiltrates or aspergilloma.

These investigations were not performed as part of the MIND-IHOP study. It is therefore not possible to claim that the investigations described here measure the prevalence of invasive pulmonary aspergillosis in this population and that claim is not made in the articles. However these definitions were designed to identify invasive aspergillosis in profoundly immunosuppressed patients. The subacute pulmonary aspergillosis seen in non-neutropaenic AIDS patients is probably closer the syndrome of chronic necrotizing pulmonary aspergillosis seen in patients with moderate immunosuppression secondary to diabetes or alcohol excess.

Over 200 stored sera were available for use. From these, 39 were selected from a group of patients whose presentation was as close as possible to the definition of subacute invasive pulmonary aspergillosis. These were HIV positive patients with chronic
cough with abnormal chest X-ray who did not have evidence of pulmonary tuberculosis after the most extensive investigation possible, including use of the highly sensitive Cepheid GeneXpert assay\textsuperscript{279}.

Such patients are at risk of subacute invasive pulmonary aspergillosis\textsuperscript{52,223,236} and as no other diagnosis was found it is reasonable to suggest that pulmonary aspergillosis is probable where raised \textit{Aspergillus}-specific IgG is present in this group. The fact that the diagnostic threshold for a positive test was defined in relation to Ugandan healthy controls support this claim. The 40% mortality rate noted in this group is potentially consistent with undiagnosed pulmonary aspergillosis occurring in this group.

\textbf{Limitations in case definition for ‘smear negative tuberculosis’}

There are, however several potential limitations to this approach. The duration of symptoms required for the accepted case definition of subacute invasive aspergillosis is $\geq 1$ month\textsuperscript{6,264}. The MIND-IHOP study accepted patients with cough duration of 2 weeks or more\textsuperscript{276}. As a result many will have had bacterial pneumonia and improved with antibiotics. Such patients were, however excluded by restricting the analysis to patients with ‘unknown’ final diagnosis. Any patients whose symptoms resolved with antibiotics were classified as probable ‘bacterial pneumonia’. Those who presented with less than 1 month of symptoms, but had raised \textit{Aspergillus}-specific IgG and did not improve with antibiotics are likely to have had acute invasive aspergillosis\textsuperscript{48}, which is also often associated with raised \textit{Aspergillus}-specific IgG\textsuperscript{90,96}. The study design cannot accurately differentiate acute from subacute invasive pulmonary aspergillosis, but this differentiation is less important than simply estimating the overall prevalence of likely pulmonary aspergillosis in this group for the first time.

The diagnosis of subacute invasive pulmonary aspergillosis also normally requires the presence of progressive cavitation or paracavitary infiltrates\textsuperscript{6,264}. These were defined on CT scan in the published cohorts of this condition. The MIND-IHOP study did not include CT scan\textsuperscript{276}. As the interpretation of chest X-rays is challenging in the context of subacute respiratory disease\textsuperscript{265} and the features of invasive pulmonary aspergillosis are often non-specific\textsuperscript{218,280}, any patient with abnormal chest X-ray was included in this analysis if
other criteria were met. It is unlikely that all patients in this group would have met the diagnostic criteria for subacute invasive pulmonary aspergillosis\textsuperscript{6,48,264} if CT scan had been performed.

\textbf{Limitations of serological testing in patients with AIDS}

The original design of this study included testing for galactomannan, which has a higher sensitivity than \textit{Aspergillus}-specific IgG for the diagnosis of acute invasive pulmonary aspergillosis\textsuperscript{90}. Unfortunately most patients in the study received Ceftriaxone prior to sampling. This can cause false positive galactomannan results\textsuperscript{281}. Galactomannan levels can also fall significantly with frozen storage\textsuperscript{261}. As a result of these major flaws galactomannan was dropped from the study design, leaving \textit{Aspergillus}-specific IgG as the sole marker of pulmonary aspergillosis. Patients with AIDS may not form a good antibody response to infection or vaccination in the context of hepatitis B and C\textsuperscript{273,274}. The same problem might occur in relation to antibody response to \textit{Aspergillus}. The sole use of \textit{Aspergillus}-specific IgG as a serological marker for probable acute or subacute pulmonary aspergillosis in patients with AIDS might therefore underestimate the frequency of the disease.

\textbf{Possible selection bias relating to stored sera volume}

There is also a possibility of selection bias as only around three quarters of patients recruited to the MIND-IHOP study had stored sera available. The others did not have sufficient volume of sera left over after previous tests were complete. Although this process is not very likely to introduce bias, it is conceivable that it would remove sicker patients from the analysis, as they might be more difficult to withdraw blood from due to shock and so have lower volumes of stored sera.

The result described here is therefore at best an estimate of the possible prevalence of acute or subacute invasive pulmonary aspergillosis in this group. It is the best that can be achieved from opportunistic testing of sera from a study that was not specifically designed to measure the prevalence of this disease.
While absolute accuracy is not guaranteed, such data does still have significant value. There has never been an attempt to measure the prevalence of subacute invasive aspergillosis in African AIDS patients, or indeed in any living HIV-infected cohort, using serological methods. The fact that this condition is noted in 2-3% of all AIDS autopsies,207,238,239 underlines the potential importance of this disease. 90% of these cases were undiagnosed antemortem. Prospective studies to accurately measure the prevalence of this condition would be expensive, due to the need for CT scans and biopsies. The data from opportunistic testing described here provides the evidence needed to justify such prospective studies.

Interpretation of raised Aspergillus-specific IgG in patients with proven tuberculosis

The population tested in paper five is much more clearly defined, in that all patients included here had proven pulmonary tuberculosis, on the basis of culture, Cepheid GeneXpert PCR testing or smear testing. The difficulty here is the interpretation of the raised levels of Aspergillus-specific IgG.

There is no published evidence that the Aspergillus-specific IgG cross reacts with antibodies to M. tuberculosis. It therefore seems likely that those with raised Aspergillus-specific IgG do have Aspergillus growth in their lungs or airways. This might represent early CPA, but this diagnosis could not reasonably be confirmed until after tuberculosis treatment is complete, as CPA diagnosis requires the presence of symptoms. Any cough or haemoptysis present during active tuberculosis could well be due to the tuberculosis rather than CPA. If the patient became asymptomatic after tuberculosis treatment then the symptomatic criteria for CPA would not be met.

It is equally possible that Aspergillus colonization of the diseased airways at the site of tuberculosis infection is more common than in healthy persons. This might be due to the immunosuppression produced by active tuberculosis infection282. Immune function might return to normal once the tuberculosis infection is cured and Aspergillus colonisation might then resolve as a result.
Selected nature of tuberculosis population in this study

It should also be noted that the population tested here are patients diagnosed with pulmonary tuberculosis in the course of an acute emergency hospital admission. This population is not representative of the overall population with pulmonary tuberculosis. The results presented here cannot therefore be taken as a measure of the overall prevalence of CPA co-existing with active pulmonary tuberculosis. It should be noted, however that the diagnostic threshold used in this survey was defined in relation to healthy Ugandan controls and that 90% of patients with treated tuberculosis tested in paper 2 had normal levels using this cut-off. The high frequency of raised Aspergillus-specific IgG is therefore an unexpected and significant finding.

While this opportunistic study cannot define the exact nature of the Aspergillus disease in these patients it does provide valuable insight into the interaction between *M. tuberculosis* and *Aspergillus* infection. In particular it strongly suggests that the commonly held assumption that CPA develops after tuberculosis is cured may be wrong. This raises the possibility that future programs to screen and treat CPA in patients with pulmonary tuberculosis might need to focus on patients with current tuberculosis rather than past tuberculosis. The results described here are therefore of significant value to those planning future prospective studies to define natural history of CPA in relation to pulmonary tuberculosis.
PAPER 1 - Comparison of six *Aspergillus*-specific IgG assays for the diagnosis of chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA)

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Abstract

Measurement of *Aspergillus*-specific IgG, or precipitating antibodies is a key component of diagnosis of CPA. It also has a role in ABPA, where it is one of three ‘additional features’ of ABPA recognized in recent international society for human and animal mycology (ISHAM) diagnostic guidelines. Many commercial *Aspergillus*-specific IgG assays exist, but there is limited evidence regarding sensitivity and specificity of these assays for the diagnosis of CPA or ABPA. The optimal diagnostic cut-offs for CPA and ABPA are poorly defined.

We performed *Aspergillus*-specific IgG testing on stored sera from the following patients; 1 – CPA (n=241) 2 – ABPA (n=80) 3 – healthy controls (n=100), 4 – severe asthmatic controls (n=100). The following assays were used; ThermoFisher Scientific ImmunoCAP (multi-national), Siemens Immulite (Germany), Serion (Germany), Genesis (UK) and Dynamiker (China) and counterimmunoelectrophoresis (precipitins) using Microgen *Aspergillus* antigens.

Receiver operating curve area under the curve (ROC AUC) results for CPA diagnosis were as follows; ImmunoCAP 0.995 (95% CI 0.991 – 0.999), Immulite 0.991 (0.982-1), Serion 0.973 (0.960 – 0.987), Dynamiker 0.918 (0.89 – 0.946) and Genesis 0.902 (0.871 – 0.933).

ROC AUC results comparing ABPA patients to healthy controls were; ImmunoCAP 0.959 (0.935 – 0.987), Immulite 0.932 (95% CI 0.887 – 0.977), Serion 0.907 (0.866 – 0.949), Dynamiker 0.903 (0.859 – 0.946) and Genesis 0.73 (0.651 – 0.808). Compared to severe asthmatic patients ROC AUC results were; Immulite 0.837 (0.774 – 0.9), Serion 0.826 (0.763 – 0.888), Dynamiker 0.819 (0.754 – 0.885) and Genesis 0.797 (0.728 – 0.866).

Optimal diagnostic cut-offs for CPA were; ImmunoCAP 20 mg/L (96% sensitivity, 98% specificity), Immulite 10 mg/L (96% sensitivity, 98% specificity), Serion 35 U/ml (90% sensitivity, 98% specificity), Dynamiker 65 AU/ml (77% sensitivity, 97% specificity) and Genesis 20 U/ml (75% sensitivity, 99% specificity). Precipitins were 59% sensitive and 100% specific. Sensitivity of these cut offs for diagnosis of ABPA were as follows;
Immulite (81%), ImmuncoCAP (77%), Dynamiker (66%), Serion (62%) and Genesis (46%). Specificity for the diagnosis of ABPA was lower when compared to severe asthmatics and alternative diagnostic thresholds may be appropriate for use in this group.

ROC AUC results for ImmunoCAP and Immulite are both statistically significantly superior to all other assays tested for the diagnosis of both CPA and ABPA in comparison to healthy controls. The Genesis assay was statistically significantly inferior to all other assays for the diagnosis of ABPA in comparison to healthy controls. The currently accepted ImmunoCAP cut-off of 40 mg/L is sub-optimal for CPA diagnosis. Adopting the new proposed thresholds for CPA diagnosis maximizes sensitivity while maintaining specificity of 97% or higher for each assay.

Precipitins testing performed poorly for the diagnosis of CPA and ABPA and should be replaced by IgG ELISA in these contexts, however it performed well for identifying CPA occurring as a complication of ABPA. Most ELISA assays performed poorly in the latter context, with the exception of Immulite which had an ROC AUC of 0.863 and produced sensitivity of 71% and specificity of 91% using an optimal cut off of 100 mg/L for this purpose.
Introduction

CPA is a serious disease that leads to severe disability and death\(^7\), but which can be treated effectively with existing drugs and surgery\(^{15,18,58,198,283}\). The estimated global prevalence of CPA is around 3 million cases\(^{11-13}\). Diagnosis of CPA requires the presence of chronic symptoms, plus appropriate radiological findings and microbiological evidence of disease\(^{5,7,8,50}\). The latter can be provided from biopsy or by culture of either broncho-alveolar lavage (BAL) fluid or sputum. Acquiring samples for these tests either requires an invasive procedure to acquire BAL or biopsy or the production of a sputum sample, which can be troublesome for patients with intermittent cough. Culture also has poor sensitivity with current methods\(^{260}\). As a result many CPA patients never have a positive culture or biopsy\(^8\). By comparison raised levels of Aspergillus-specific IgG are present in the majority of published cases and provide the sole laboratory evidence of *Aspergillus* infection in many cases\(^{5,8}\).

ABPA can complicate asthma and is estimated to complicate around 13% of asthma cases\(^{284}\) and affect around 5 million persons worldwide\(^{13}\). The international society for human and animal mycology (ISHAM) has recently reviewed the diagnostic criteria for ABPA\(^4\). The presence of both raised total IgE and raised *Aspergillus*-specific IgE (or positive skin prick testing) is mandatory for diagnosis. Positive precipitins or *Aspergillus*-specific IgG is one of three additional features, along with raised eosinophil count and radiological features, of which 2 out of 3 are also required to confirm the diagnosis.

Many specialist laboratories have developed their own ‘home-brew’ assays directly from fungal culture, to detect *Aspergillus*-specific IgG\(^{18,73,130,131}\). However replication of such a ‘home-brew’ technique in other laboratories is challenging\(^{141}\) as the mixture of antigens produced varies in relation to factors such as strain, medium pH and length of culture\(^{98,128,134-136,138-140}\). The original Ouchterlony precipitation-in-gel (precipitins) technique\(^{105,123}\) for detection of *Aspergillus*-specific antibodies is time consuming, produces subjective results of a semi-quantitative nature and probably has poor sensitivity\(^{38,74}\). Enzyme-linked immunosorbent assay (ELISA) is now commonly used in its place\(^{130}\).
Multiple commercial tests for *Aspergillus*-specific IgG exist, but published data comparing the performance of these tests is very limited. Research has been hampered by the lack of large cohorts of patients with clearly defined CPA. Some comparisons use small mixed populations of different types of aspergillosis, including invasive disease in addition to CPA or ABPA. However, good intra-laboratory repeatability has been demonstrated for the ImmunoCAP assay (ThermoFisher Scientific, multinational)\(^{180}\).

To our knowledge only two studies have compared the performance of commercial *Aspergillus*-specific IgG assays for the diagnosis of CPA. One study noted that the Bio-Rad (France) and Serion (Germany) assays had respective sensitivity of 94% and 92% for the diagnosis of CPA in 51 cases, with specificity of 87% and 76% respectively\(^{38}\). The other study, published by our team, compared the Bio-Rad assay to ImmunoCAP and precipitins testing using Microgen (UK) antigens. It showed respective sensitivity of 85%, 86% and 56% for the diagnosis of CPA in 116 cases\(^{74}\).

Diagnostic cut-offs for ABPA in the patients with underlying cystic fibrosis have been investigated for the ImmunoCAP assay. One study of 87 patients suggests that a cut-off of 90 mg/L has a sensitivity of 91% and specificity of 88% for the diagnosis of ABPA\(^{78}\), whereas another study of 146 patients suggests that a cut-off of 75 mg/L\(^{66}\) has a sensitivity of 96% and a specificity of 90%. The ImmunoCAP assay has also been assessed in 10 ABPA patients without underlying cystic fibrosis, where a cut-off of 35 mg/L had a sensitivity of 90% and specificity of 86% for the diagnosis of ABPA\(^{73}\).

The diagnostic cut-offs for other assays for ABPA have not been assessed in the published literature. Using the manufacturer’s recommended cut-offs the Serion and Bio-Rad assays had sensitivities of 84% and 92% respectively, in a study of 13 patients without cystic fibrosis\(^{38}\). ImmunoCAP, Bio-Rad and precipitins had sensitivities of 41%, 47% and 15% respectively in a mixed group of 46 patients with either ABPA or Severe Asthma with Fungal Sensitization (SAFS), but no cystic fibrosis\(^{74}\). The Immulite assay (Siemens, Germany) has been shown to have good correlation with the ImmunoCAP assay\(^{180}\), but its sensitivity and specificity for the diagnosis of CPA or ABPA has not been measured directly.
These comparisons are probably too small to detect differences in test sensitivity and specificity and are potentially biased due to the presence of long-term antifungal therapy, which lowers *Aspergillus*-specific IgG levels, in many patients. We are not aware of any publications describing the sensitivity and specificity of Bordier (Switzerland), Dynamiker (China), IBL (Germany) or Genesis (UK) for the diagnosis of CPA or ABPA. The optimal diagnostic thresholds for CPA and ABPA (in patients without cystic fibrosis) have never been assessed for any of the available assays, with the exception of the small ImmunoCAP ABPA study described above.

We have performed a retrospective comparison of six methods in cohorts of CPA patients and ABPA patients. Samples were taken when patients were not taking long-term antifungal medication. Receiver operating characteristic (ROC) curve analysis is used to compare test performance. It is also to define optimal diagnostic cut offs for CPA and ABPA.

### Methods

#### Patients

Eighty patients with ABPA and 241 patients with CPA were identified at the UK National Aspergillosis Centre (NAC). Each had a stored sample of serum taken when either off antifungal treatment or within three months of starting treatment. The median levels of *Aspergillus*-specific IgG in study patients on antifungal therapy and off antifungal therapy are described and compared. Control samples were collected from 100 healthy Ugandan blood donors 100 patients with severe asthma under the care of the North West Lung Centre, UK. Samples were tested for *Aspergillus*-specific IgG by all methods, other than asthmatic controls where ImmunoCAP was not performed as no funding was available to perform this assay in this group.

Diagnosis of CPA or ABPA was taken from patient records. CPA diagnosis at the NAC is based on the composite gold standard comprising symptoms, radiological changes, raised inflammatory markers and microbiological evidence of *Aspergillus* infection as described previously by our group and subsequently accepted in many
studies and in IDSA and ESCMID guidelines. Diagnosis of ABPA at the NAC is based on a composite gold standard comprising raised total IgE, positive Aspergillus-specific IgE or skin prick testing, abnormal radiological findings, raised Aspergillus-specific IgG and raised eosinophil count. All patients with ABPA at our centre are routinely screened for the development of CPA, principally with regular chest imaging. Any patient with progressive lung cavities, paracavitary fibrosis or aspergilloma on imaging was classified as CPA and removed from the ABPA group.

Laboratory techniques

Tests were performed between January and July 2014. Aspergillus-specific IgG levels were measured on all samples using the Siemens Immulite 2000. Manual plate ELISAs were performed on all samples using kits supplied by Serion, Genesis and Dynamiker. All results for plate ELISAs were read on a PolarStar Omega spectrophotometer (BMG Labtech, UK). Optical density readings were converted to arbitrary units using the formulae or software provided by test manufacturers. Results were rejected if the manufacturers’ stated quality control criteria were not met for an individual test plate. If this occurred the tests were re-run on a fresh plate. Where a result was greater than a threshold specified by the manufacturer a 1 in 10 dilution was performed and the assay was repeated.

Precipitation in gel (precipitins) testing was also performed on all samples using the counterimmunoelectrophoresis (CIE) technique and Aspergillus antigens supplied by Microgen (UK). Briefly, 10ml agarose was melted and poured onto a hydrophobic gel bond film (GE Healthcare, USA). Three mm diameter test wells were cut once the gel had set. Twenty µL sera were placed in one row of wells with 20 µL antigens (Microgen, UK) at 2 mg/ml placed in the adjacent row. The gel was placed above a CIE tank filled with veronal buffer and blotting paper wicks were used to connect either end of the gel to the buffer tanks before applying 34V for 90mins. After running the gel was placed in sodium chloride washing solution overnight. After drying it was placed in a Coomassie Blue stain for 15 mins, followed by two serial de-stain solutions of 10 mins each. After further drying the gels were read on a light box with the assistance of a magnifying glass. The presence of any precipitins bands was reported as a positive result. Neat
serum was tested for all samples. Where samples were positive serial dilution to a maximum 1 in 32 dilution was produced to provide dilutional titres. The same technician performed all the above tests. Aspergillus-specific IgG levels were performed on the ThermoFisher Scientific ImmunoCAP system for all CPA and ABPA cases as part of routine clinical care at the time of original sampling. Where a sample produced a result of >200 mg/L a 1 in 10 dilution was performed and the sample was retested. Other assays were performed on these same samples after storage. Healthy control samples were also tested. Where a sample produced the same result (positive or negative) on a single test by all test methods this result was accepted. Where a sample produced divergent results on different assays it was repeated twice. If the two new tests resulted in a different outcome (positive or negative) to the first test then the mean of these two new tests replaced the first test. 72 (14%) samples were repeated for the Dynamiker assay with 13 (2%) results changed. 38 (7%) samples were repeated for the Serion assay with 10 (2%) results changed. 52 (10%) samples were repeated for the Genesis assay with 5 (1%) results changed. 42 (8%) samples were repeated for the Siemens assay with 2 (0.4%) results changed. 112 (21%) samples were repeated for the CIE assay with 15 (3%) results changed. ImmunoCAP samples were not repeated as results from tests performed as part of clinical care at the time of original sampling were being used and no funding was available for repeat testing. We report final results after any repeat testing for all assays. 

**Intra-assay variability**

To calculate intra-assay variability (IAV) we selected two samples for each assay other than ImmunoCAP, with high and low results respectively. No funding was available to perform IAV on ImmunoCAP. Each assay was repeated 20 times per sample. Outliers were identified by study team consensus and removed from the final analysis. For the low level repeats four Serion samples and one Dynamiker sample with readings lower than the substrate blank were removed as outliers, as was one Genesis sample with
apparent cross-well contamination. No samples were removed as outliers for the high level IAV. The Serion IAV was repeated three times with similar results each time. Arbitrary units from different assays cannot be directly compared. Result range, mean, standard deviation and co-efficient of variation (CV) are reported for each assay. CV was calculated as (standard deviation / mean) X 100.

Statistical analysis

Intra assay variation with co-efficient of variation is reported for Immulite, Serion, Genesis and Dynamiker assays. Descriptive statistics are reported for each assay in each patient group, including the frequency of positive, negative and intermediate results by manufacturers current guidelines. The median Aspergillus-specific IgG levels in patients on antifungal therapy and patients not on antifungal therapy are compared by Mann-Whitney U analysis. ImmunoCAP and Immulite both produce results in mg/L. Correlation between these two assays is measured by Spearman's rank analysis.

ROC curve analysis is performed for each ELISA assay and the Area Under the Curve (AUC) for ROC analysis is shown with 95% confidence intervals (95% CI). Wald's statistic is used to compare the significance of differences in ROC AUC between assays. A significant result defined as p<0.005.

Optimal diagnostic cut-offs for each assay are calculated using Youden's J statistic (sensitivity + specificity - 1). Sensitivity and specificity are described for these cut-offs. These comparisons are performed for CPA cases vs. healthy controls, ABPA cases vs. healthy controls, ABPA cases vs. severe asthmatic controls and ABPA cases vs. CPA cases. Statistical analyses were performed using SPSS version 20 (IBM, USA) under license to the University of Manchester, UK.

Results

Stored samples from 241 patients with proven CPA, 80 patients with ABPA and 100 patients with asthma were acquired from the UK National Aspergillosis Centre and North West Lung Centre. One hundred control samples were acquired from healthy
blood donors in Gulu, Uganda. Patient demographics and underlying conditions for the patient groups are compared in table 1.

Table 1 – Patient and control characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CPA n=241</th>
<th>ABPA n=80</th>
<th>Asthmatic controls n = 100</th>
<th>Healthy controls n=100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>101 (42%)</td>
<td>42 (52%)</td>
<td>77 (77%)</td>
<td>55 (55%)</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>65</td>
<td>67</td>
<td>52 years</td>
<td>19</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>23 – 92</td>
<td>25 - 95</td>
<td>19 - 81</td>
<td>17 – 39</td>
</tr>
<tr>
<td>Chronic cavitary pulmonary aspergillosis</td>
<td>238 (99%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Aspergillus</em> nodule disease</td>
<td>3 (1%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HIV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>Prior tuberculosis</td>
<td>37 (15%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>Non-tuberculous mycobacterial infection</td>
<td>28 (12%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>COPD</td>
<td>85 (35%)</td>
<td>3 (4%)</td>
<td>3 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>60 (25%)</td>
<td>43 (54%)</td>
<td>29 (29%)</td>
<td>0</td>
</tr>
<tr>
<td>ABPA</td>
<td>35 (15%)</td>
<td>80 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>9 (4%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malignancy (active or in remission)</td>
<td>33 (14%)</td>
<td>7 (9%)</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>33 (14%)</td>
<td>3 (4%)</td>
<td>2 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>7 (3%)</td>
<td>8 (10%)</td>
<td>4 (4%)</td>
<td>0</td>
</tr>
<tr>
<td>Asthma</td>
<td>41 (17%)</td>
<td>78 (97%)</td>
<td>100 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>0</td>
<td>2 (3%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

HIV = human immunodeficiency virus seropositive; COPD = chronic obstructive pulmonary disease; ABPA = allergic bronchopulmonary aspergillosis

All kits produced results within the manufacturers' stated quality control criteria for the tests included in the analysis, with the exception of Dynamiker. In this case the high concentration control serum was slightly below the stated range in all runs. Forty-two (17%) of CPA patients included in the study had received up to three months antifungal therapy at the time of sampling. The remaining 199 (83%) patients were not on any antifungal therapy. Median *Aspergillus*-specific IgG levels in CPA patients on and off antifungal therapy are shown in table 2. Nine of the 80 (11%) ABPA patients had
received up to 3 months antifungal therapy at the time of sampling. The remaining 71 (89%) were not on any antifungal therapy. Median *Aspergillus*-specific IgG levels in ABPA patients on and off antifungal therapy are shown in table 3. Median levels are compared by Mann-Whitney U test.

**Table 2 – *Aspergillus*-specific IgG levels in CPA patients with and without antifungal therapy**

<table>
<thead>
<tr>
<th>Test</th>
<th>Median <em>Aspergillus</em>-specific IgG level in those with &lt; 3 months antifungals n=42</th>
<th>Median <em>Aspergillus</em>-specific IgG level in those not on antifungals n=199</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmunoCAP</td>
<td>130 mg/L</td>
<td>125 mg/L</td>
<td>0.375</td>
</tr>
<tr>
<td>Immulite</td>
<td>533 mg/L</td>
<td>250 mg/L</td>
<td>0.051</td>
</tr>
<tr>
<td>Serion</td>
<td>143 U/ml</td>
<td>125 U/ml</td>
<td>0.372</td>
</tr>
<tr>
<td>Genesis</td>
<td>90 U/ml</td>
<td>47 U/ml</td>
<td>0.006</td>
</tr>
<tr>
<td>Dynamiker</td>
<td>141 AU/ml</td>
<td>119 AU/ml</td>
<td>0.230</td>
</tr>
</tbody>
</table>

**Table 3 – *Aspergillus*-specific IgG levels in ABPA patients with and without antifungal therapy**

<table>
<thead>
<tr>
<th>Test</th>
<th>Median <em>Aspergillus</em>-specific IgG level in those with &lt; 3 months antifungals n=9</th>
<th>Median <em>Aspergillus</em>-specific IgG level in those not on treatment n=71</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmunoCAP</td>
<td>68 mg/L</td>
<td>45 mg/L</td>
<td>0.337</td>
</tr>
<tr>
<td>Immulite</td>
<td>49 mg/L</td>
<td>36 mg/L</td>
<td>0.819</td>
</tr>
<tr>
<td>Serion</td>
<td>92 U/ml</td>
<td>57 U/ml</td>
<td>0.479</td>
</tr>
<tr>
<td>Genesis</td>
<td>18 U/ml</td>
<td>13 U/ml</td>
<td>0.849</td>
</tr>
<tr>
<td>Dynamiker</td>
<td>331 AU/ml</td>
<td>103 U/ml</td>
<td>0.173</td>
</tr>
</tbody>
</table>
Intra-assay variation for low-level samples is shown in table 4. Intra-assay variation results for high-level samples are shown in table 5. All low level precipitins repeats were negative and all high level repeats were positive, but with dilutional titre results as follows; neat = 1 sample, 1 in 2 = 2 samples, 1 in 4 = 11 samples, 1 in 8 = 4 samples, 1 in 16 = 1 sample.

Table 4 – Intra-assay variation - low

<table>
<thead>
<tr>
<th>Test</th>
<th>Range</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Co-efficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamiker (AU/ml)</td>
<td>45.5 – 66.2 (20.7)</td>
<td>55.8</td>
<td>5.6</td>
<td>10.1%</td>
</tr>
<tr>
<td>Genesis (U/ml)</td>
<td>4.6 – 6.3 (1.6)</td>
<td>5.2</td>
<td>0.4</td>
<td>8.2%</td>
</tr>
<tr>
<td>Serion (U/ml)</td>
<td>6 - 42.5 (36.5)</td>
<td>24</td>
<td>10.5</td>
<td>43.7%</td>
</tr>
<tr>
<td>Immulite (mg/L)</td>
<td>58.4 – 67.8 (9.4)</td>
<td>62.6</td>
<td>2.2</td>
<td>3.6%</td>
</tr>
</tbody>
</table>

AU = arbitrary units, U = units. Both represent arbitrary numbers and no direct comparison can be made between assays producing results in this manner.

Table 5 – Intra-assay variation - high

<table>
<thead>
<tr>
<th>Test</th>
<th>Range</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Co-efficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamiker (AU/ml)</td>
<td>240.7 – 372.7 (132)</td>
<td>287.5</td>
<td>31.9</td>
<td>11.1%</td>
</tr>
<tr>
<td>Genesis (U/ml)</td>
<td>55.4 – 96.5 (41.1)</td>
<td>83.4</td>
<td>10</td>
<td>12.1%</td>
</tr>
<tr>
<td>Serion (U/ml)</td>
<td>59.9-122.1 (62.2)</td>
<td>75</td>
<td>17.4</td>
<td>23.2%</td>
</tr>
<tr>
<td>Immulite (mg/L)</td>
<td>95.3 – 107 (11.7)</td>
<td>99.6</td>
<td>3.4</td>
<td>3.4%</td>
</tr>
</tbody>
</table>

Box and whisker plots with logarithmic scale compare results for cases and control groups for each assay in figures 1-5. Results in cases and control groups are summarized in table 6. Where manufacturers provide instructions on interpretation of results, outcomes are summarized in table 7. Dynamiker, Genesis and Serion advise
reporting of results as positive, intermediate or negative. ImmunoCAP is interpreted with a single diagnostic cut-off (40 mg/L) in line with current UK practice and Immulite does not currently have a recommended diagnostic cut-off.

Precipitins tests produced the following results in CPA cases; negative = 102 cases (42%), neat = 23 cases (10%), 1 in 2 = 34 cases (14%), 1 in 4 = 29 cases (12%), 1 in 8 = 26 cases (11%), 1 in 16 = 23 cases (10%), 1 in 32 = 4 cases (2%). For ABPA cases 4% of cases had positive precipitins results with neat sera only, all others were negative.

The correlation between ImmunoCAP and Immulite in patients with CPA, ABPA and in healthy and asthmatic controls was good (Spearman’s rank analysis 0.876, p 0.000).

Table 6 – Results in CPA cases and healthy controls

<table>
<thead>
<tr>
<th>Test</th>
<th>Controls range (n=100)</th>
<th>Asthma range (n=100)</th>
<th>ABPA cases range (n=80)</th>
<th>CPA cases range (n=241)</th>
<th>Controls mean</th>
<th>Asthma mean</th>
<th>ABPA mean</th>
<th>CPA mean</th>
<th>Controls median</th>
<th>Asthma median</th>
<th>ABPA median</th>
<th>CPA median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamiker (AU/ml)</td>
<td>16-88</td>
<td>26-643</td>
<td>27-5239</td>
<td>23-6118</td>
<td>37</td>
<td>93</td>
<td>334</td>
<td>341</td>
<td>34</td>
<td>46</td>
<td>119</td>
<td>124</td>
</tr>
<tr>
<td>Genesis (U/ml)</td>
<td>0-20</td>
<td>1-25</td>
<td>0-362</td>
<td>1-930</td>
<td>7</td>
<td>6</td>
<td>33</td>
<td>111</td>
<td>6</td>
<td>4</td>
<td>14</td>
<td>60</td>
</tr>
<tr>
<td>Immulite (mg/L)</td>
<td>0-35</td>
<td>0-87</td>
<td>0-149</td>
<td>3-7660</td>
<td>5</td>
<td>11</td>
<td>46</td>
<td>678</td>
<td>4</td>
<td>7</td>
<td>39</td>
<td>392</td>
</tr>
<tr>
<td>ImmunoCAP (mg/L)</td>
<td>2-36</td>
<td>-</td>
<td>3-408</td>
<td>9-1707</td>
<td>6</td>
<td>-</td>
<td>66</td>
<td>216</td>
<td>5</td>
<td>-</td>
<td>46</td>
<td>126</td>
</tr>
<tr>
<td>Serion (U/ml)</td>
<td>0-40</td>
<td>1-416</td>
<td>1-898</td>
<td>4-3436</td>
<td>10</td>
<td>34</td>
<td>112</td>
<td>232</td>
<td>6</td>
<td>16</td>
<td>57</td>
<td>131</td>
</tr>
</tbody>
</table>

ImmuCAP testing could not be performed on sera from asthmatic patients, as no funding was available. Results expressed in U/ml or AU/ml are arbitrary and cannot be directly compared across assays.

ROC curves comparing CPA and ABPA to healthy controls are shown in figures 6 and 7. ROC curves comparing ABPA to asthmatic controls are shown in figure 8 and ROC curves comparing Aspergillus-specific IgG levels in CPA and ABPA are shown in figure 9. Results of ROC AUC analysis are presented in table 8. The ROC analyses identified optimal cut-offs for each situation. We report the specificity and sensitivity of each of these for the diagnosis of CPA in tables 9-12. The suggested optimal cut off for each assay is highlighted in bold.
Table 7 – Frequency of positive results by manufacturers’ guidelines

<table>
<thead>
<tr>
<th>Test</th>
<th>Frequency of positive results in controls (intermediate results)</th>
<th>Frequency of positive results in ABPA (intermediate results)</th>
<th>Frequency of positive results in CPA (intermediate results)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamiker</td>
<td>6% (11%)</td>
<td>69% (7%)</td>
<td>78% (5%)</td>
</tr>
<tr>
<td>Genesis</td>
<td>22% (13%)</td>
<td>59% (7%)</td>
<td>82% (5%)</td>
</tr>
<tr>
<td>Immulite*</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>ImmunoCAP</td>
<td>0</td>
<td>54%</td>
<td>88%</td>
</tr>
<tr>
<td>Serion</td>
<td>0 (0)</td>
<td>43% (14%)</td>
<td>74% (10%)</td>
</tr>
<tr>
<td>Precipitins</td>
<td>0</td>
<td>4%</td>
<td>59%</td>
</tr>
</tbody>
</table>

*Immulite do not currently provide diagnostic cut-offs so the number of positive results by manufacturers guidelines cannot be reported.

Table 8– Receiver operating characteristic curve area under curve (ROC AUC) results

<table>
<thead>
<tr>
<th>Test</th>
<th>CPA vs. healthy controls 95% CI</th>
<th>ABPA vs. healthy controls 95% CI</th>
<th>ABPA vs. asthmatic controls 95% CI</th>
<th>CPA vs. ABPA 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmunoCAP</td>
<td>0.996 0.992 - 1 0.961 0.935 - 0.987</td>
<td>0.907 0.866 - 0.949 0.725 0.651 - 0.799</td>
<td>0.725 0.690 - 0.831 0.519 0.443 - 0.596</td>
<td>0.778 0.723 - 0.834</td>
</tr>
<tr>
<td>Immulite</td>
<td>0.991 0.982 - 1 0.932 0.887 - 0.977</td>
<td>0.907 0.866 - 0.949 0.725 0.651 - 0.799</td>
<td>0.725 0.690 - 0.831 0.519 0.443 - 0.596</td>
<td>0.863 0.825 - 0.901</td>
</tr>
<tr>
<td>Serion</td>
<td>0.973 0.960 - 0.987 0.907 0.866 - 0.949</td>
<td>0.907 0.866 - 0.949 0.725 0.651 - 0.799</td>
<td>0.725 0.690 - 0.831 0.519 0.443 - 0.596</td>
<td>0.698 0.629 - 0.768</td>
</tr>
<tr>
<td>Dynamiker</td>
<td>0.918 0.890 - 0.946 0.903 0.859 - 0.946</td>
<td>0.907 0.866 - 0.949 0.725 0.651 - 0.799</td>
<td>0.725 0.690 - 0.831 0.519 0.443 - 0.596</td>
<td>0.759 0.701 - 0.818</td>
</tr>
<tr>
<td>Genesis</td>
<td>0.902 0.871 - 0.933 0.73 0.651 - 0.808</td>
<td>0.797 0.728 - 0.866 0.759 0.701 - 0.818</td>
<td>0.797 0.728 - 0.866 0.759 0.701 - 0.818</td>
<td>0.759 0.701 - 0.818</td>
</tr>
</tbody>
</table>

Nine of 241 sera from CPA cases were negative (<20 mg/L) on testing with the ImmunoCAP assay. Using the new diagnostic cut-offs suggested above 6 of these 9 (67%) were positive on Immulite testing (mean level in positives 93 mg/mL), 3 of 9 (33%) samples were positive on Serion testing (mean level in positive 217 U/ml), 1 sample (11%) was positive by Genesis at 130 U/ml, 1 sample (11%) were positive by Dynamiker at 614 AU/ml and 3 (33%) were positive by CIE (two neat, one at 1 in 2). Two samples were negative on all assays.
Wald’s statistic confirmed the overall difference in ROC AUC performance for the diagnosis of CPA across the five assays is statistically significant (p<0.0001). ImmunoCAP ROC AUC is equivalent to Immulite (p=0.32). ImmunoCAP and Immulite both have significantly superior ROC AUC to the other 3 assays (p=0.0006 for Immulite vs. Serion). Serion has a superior ROC AUC to Dynamiker and Genesis (p<0.0001 for Serion vs. Dynamiker). Dynamiker and Genesis have equivalent ROC AUC (p=0.38).

### Table 9 – Potential diagnostic cut offs for CPA

<table>
<thead>
<tr>
<th>Assay</th>
<th>Diagnostic cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Youden's J statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmunoCAP</td>
<td>10mg/L</td>
<td>100%</td>
<td>86%</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>20 mg/L</td>
<td>96%</td>
<td>98%</td>
<td><strong>0.94</strong></td>
</tr>
<tr>
<td></td>
<td>30 mg/L</td>
<td>91%</td>
<td>99%</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>40 mg/L</td>
<td>87%</td>
<td>100%</td>
<td>0.87</td>
</tr>
<tr>
<td>Immulite</td>
<td>5 mg/L</td>
<td>99%</td>
<td>78%</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>10 mg/L</td>
<td>96%</td>
<td>98%</td>
<td><strong>0.94</strong></td>
</tr>
<tr>
<td></td>
<td>20 mg/L</td>
<td>93%</td>
<td>99%</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>30 mg/L</td>
<td>91%</td>
<td>99%</td>
<td>0.9</td>
</tr>
<tr>
<td>Serion</td>
<td>30 U/ml</td>
<td>91%</td>
<td>95%</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>35 U/ml</td>
<td>90%</td>
<td>98%</td>
<td><strong>0.88</strong></td>
</tr>
<tr>
<td></td>
<td>40 U/ml</td>
<td>88%</td>
<td>99%</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>45 U/ml</td>
<td>85%</td>
<td>100%</td>
<td>0.85</td>
</tr>
<tr>
<td>Dynamiker</td>
<td>60 AU/ml</td>
<td>78%</td>
<td>94%</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>65 U/ml</td>
<td>77%</td>
<td>97%</td>
<td><strong>0.74</strong></td>
</tr>
<tr>
<td></td>
<td>70 AU/ml</td>
<td>75%</td>
<td>98%</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>75 AU/ml</td>
<td>73%</td>
<td>99%</td>
<td>0.72</td>
</tr>
<tr>
<td>Genesis</td>
<td>10 U/ml</td>
<td>86%</td>
<td>65%</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>15 U/ml</td>
<td>79%</td>
<td>95%</td>
<td><strong>0.74</strong></td>
</tr>
<tr>
<td></td>
<td>20 U/ml</td>
<td>75%</td>
<td>99%</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>25 U/ml</td>
<td>71%</td>
<td>100%</td>
<td>0.71</td>
</tr>
<tr>
<td>Precipitins</td>
<td>-</td>
<td>59%</td>
<td>100%</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Wald statistic confirmed an overall difference in the ROC AUC performance of the assays in the comparison of ABPA patients to healthy controls, with ImmunoCAP, Immulite, Serion and Dynamiker all demonstrating statistically significantly superior ROC AUC results to Genesis (p<0.001). There was no statistically significant difference in the performance of the other four assays in this context. ImmunoCAP ROC AUC was equivalent to Immulite (p=0.27), Siemens Immulite ROC AUC was equivalent to Serion (p=0.27) and Serion ROC AUC was equivalent to Dynamiker (p=0.21).
Table 10–Potential diagnostic cut offs for ABPA vs. healthy controls

<table>
<thead>
<tr>
<th>Assay</th>
<th>Diagnostic cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Y ouden's J statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmunoCAP</td>
<td>10 mg/L</td>
<td>94%</td>
<td>86%</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>20 mg/L</td>
<td>77%</td>
<td>98%</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>30 mg/L</td>
<td>64%</td>
<td>99%</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>40 mg/L</td>
<td>54%</td>
<td>100%</td>
<td>0.54</td>
</tr>
<tr>
<td>Immulite</td>
<td>5 mg/L</td>
<td>94%</td>
<td>77%</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>10 mg/L</td>
<td>81%</td>
<td>98%</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>20 mg/L</td>
<td>60%</td>
<td>98%</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>30 mg/L</td>
<td>56%</td>
<td>99%</td>
<td>0.55</td>
</tr>
<tr>
<td>Serion</td>
<td>30 U/ml</td>
<td>65%</td>
<td>95%</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>35 U/ml</td>
<td>62%</td>
<td>98%</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>40 U/ml</td>
<td>57%</td>
<td>100%</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>45 U/ml</td>
<td>55%</td>
<td>100%</td>
<td>0.55</td>
</tr>
<tr>
<td>Dynamiker</td>
<td>55 AU/ml</td>
<td>70%</td>
<td>88%</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>60 AU/ml</td>
<td>69%</td>
<td>95%</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>65 AU/ml</td>
<td>66%</td>
<td>97%</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>70 AU/ml</td>
<td>64%</td>
<td>98%</td>
<td>0.62</td>
</tr>
<tr>
<td>Genesis</td>
<td>10 U/ml</td>
<td>66%</td>
<td>65%</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>15 U/ml</td>
<td>46%</td>
<td>95%</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>20 U/ml</td>
<td>37%</td>
<td>100%</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>25 U/ml</td>
<td>31%</td>
<td>100%</td>
<td>0.31</td>
</tr>
<tr>
<td>Precipitins</td>
<td>-</td>
<td>4%</td>
<td>100%</td>
<td>0.04</td>
</tr>
</tbody>
</table>

In the comparison of assays in ABPA cases vs. asthmatics Wald’s statistic demonstrated no overall difference in the performance of the assays (p=0.1). For the comparison of CPA vs. ABPA there was a difference in the overall performance of the assays (<0.0001). Immulite ROC AUC was superior to all other assays in this context (p=0.0004 for Immulite vs. ImmunoCAP). Immulite, ImmunoCAP and Genesis ROC AUCs were all superior to Serion (p<0.0001 for Genesis vs. Serion). All assays were superior to Dynamiker (p<0.0001 for Serion vs. Dynamiker), which has no diagnostic value in this setting as the lower 95% confidence interval for ROC AUC crosses 0.5.
Table 11 – Potential diagnostic cut offs for ABPA vs. severe asthmatic controls

<table>
<thead>
<tr>
<th>Assay</th>
<th>Diagnostic cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Youden's J statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immulite</td>
<td>10 mg/L</td>
<td>81%</td>
<td>67%</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>20 mg/L</td>
<td>60%</td>
<td>88%</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>30 mg/L</td>
<td><strong>56%</strong></td>
<td><strong>94%</strong></td>
<td><strong>0.5</strong></td>
</tr>
<tr>
<td></td>
<td>40 mg/L</td>
<td>50%</td>
<td>97%</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>50 mg/L</td>
<td>41%</td>
<td>97%</td>
<td>0.38</td>
</tr>
<tr>
<td>Serion</td>
<td>30 U/ml</td>
<td>65%</td>
<td>70%</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td><strong>50 U/ml</strong></td>
<td><strong>55%</strong></td>
<td><strong>86%</strong></td>
<td><strong>0.41</strong></td>
</tr>
<tr>
<td></td>
<td>70 U/ml</td>
<td>41%</td>
<td>89%</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>90 U/ml</td>
<td>34%</td>
<td>93%</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>100 U/ml</td>
<td>27%</td>
<td>95%</td>
<td>0.23</td>
</tr>
<tr>
<td>Dynamiker</td>
<td>60 AU/ml</td>
<td>68%</td>
<td>63%</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>80 AU/ml</td>
<td>60%</td>
<td>74%</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td><strong>100 AU/ml</strong></td>
<td><strong>56%</strong></td>
<td><strong>79%</strong></td>
<td><strong>0.35</strong></td>
</tr>
<tr>
<td></td>
<td>120 AU/ml</td>
<td>50%</td>
<td>80%</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>500 AU/ml</td>
<td>20%</td>
<td>96%</td>
<td>0.16</td>
</tr>
<tr>
<td>Genesis</td>
<td><strong>10 U/ml</strong></td>
<td><strong>66%</strong></td>
<td><strong>83%</strong></td>
<td><strong>0.49</strong></td>
</tr>
<tr>
<td></td>
<td>15 U/ml</td>
<td>46%</td>
<td>93%</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>20 U/ml</td>
<td>38%</td>
<td>95%</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>25 U/ml</td>
<td>31%</td>
<td>99%</td>
<td>0.3</td>
</tr>
<tr>
<td>Precipitins</td>
<td>-</td>
<td>4%</td>
<td>97%</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 12 – Potential diagnostic cut offs for CPA vs. ABPA

<table>
<thead>
<tr>
<th>Assay</th>
<th>Diagnostic cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Youden's J statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmunoCAP</td>
<td>20 mg/L</td>
<td>96%</td>
<td>23%</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>50 mg/L</td>
<td>84%</td>
<td>55%</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>100 mg/L</td>
<td>56%</td>
<td>78%</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>150 mg/L</td>
<td>37%</td>
<td>95%</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>200 mg/L</td>
<td>25%</td>
<td>98%</td>
<td>0.23</td>
</tr>
<tr>
<td>Immulite</td>
<td>10 mg/L</td>
<td>95%</td>
<td>19%</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>50 mg/L</td>
<td>84%</td>
<td>59%</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>100 mg/L</td>
<td>71%</td>
<td>91%</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>125 mg/L</td>
<td>65%</td>
<td>95%</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>150 mg/L</td>
<td>61%</td>
<td>100%</td>
<td>0.61</td>
</tr>
<tr>
<td>Serion</td>
<td>35 U/ml</td>
<td>90%</td>
<td>38%</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>50 U/ml</td>
<td>84%</td>
<td>45%</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>100 U/ml</td>
<td>60%</td>
<td>73%</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>200 U/ml</td>
<td>31%</td>
<td>84%</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>400 U/ml</td>
<td>14%</td>
<td>95%</td>
<td>0.09</td>
</tr>
<tr>
<td>Genesis</td>
<td>15 U/ml</td>
<td>80%</td>
<td>54%</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>50 U/ml</td>
<td>52%</td>
<td>88%</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>75 U/ml</td>
<td>43%</td>
<td>93%</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>100 U/ml</td>
<td>29%</td>
<td>93%</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>125 U/ml</td>
<td>24%</td>
<td>96%</td>
<td>0.2</td>
</tr>
<tr>
<td>Precipitins</td>
<td>-</td>
<td>59%</td>
<td>96%</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Discussion

This is the first study to compare the performance of Aspergillus-specific IgG assays in large populations of well-characterised patients with clinically confirmed CPA and ABPA, who were not on long term antifungal treatment at the time of sampling. The performance of both the ThermoFisher Scientific ImmunoCAP and Siemens Immulite assays was superior to the other four assays, but equivalent to each other.

The Serion assay has statistically superior performance to the other two manual assays produced by Genesis and Dynamiker, but suffers from poor reproducibility. The performance of the manual Dynamiker galactomannan-specific IgG ELISA for the diagnosis of CPA was similar to manual ELISAs using culture extract antigens. We suggest that ImmunoCAP or Immulite assays be adopted as the test of choice for CPA diagnosis wherever possible.
The worst performing assay for CPA diagnosis was precipitins. We confirmed the poor sensitivity found in earlier studies\textsuperscript{74}. In all cases with positive precipitins at least one ELISA was also positive. There are other manufacturers of reagents for precipitin testing, and further studies are needed to confirm that the low sensitivity of precipitin antibody detection was intrinsic to the system and not a peculiarity of the Microgen reagents.

Nonetheless, we argue that \textit{Aspergillus}-specific IgG ELISA should now replace that precipitin testing for the diagnosis of CPA as the precipitins assay has no advantages over ELISA in this context. Furthermore many treatment trials in CPA have previously specified a positive precipitins test as a mandatory diagnostic criteria\textsuperscript{18,108}. This is no longer appropriate. The term ‘precipitins’ has been used in the literature to refer to both precipitation-in-gel and \textit{Aspergillus}-specific IgG ELISA\textsuperscript{8}. This inaccuracy is confusing and should be avoided given the differing performance characteristics of the two techniques. Medical practitioners will need to be educated about this shift as ‘\textit{Aspergillus} precipitins’ testing remains part of the routine vocabulary of respiratory physicians in the English-speaking world.

A small number of CPA sera were negative on the ImmunoCAP assay. In these cases other assays produced strongly positive results in most cases, with Immulite demonstrating the best sensitivity in this group. Each assay will have its own mix of antigens. These patients may be reacting only to certain antigens, present in some assays, but not others. Two patients had no response to any of the assays, suggesting an underlying immune deficit preventing an effective antibody response to \textit{Aspergillus} infection.

When being used for ABPA diagnosis, the Genesis assay had inferior performance to all other ELISAs when diagnosing ABPA in relation to healthy controls. There was no significant difference between the four assays assessed for the diagnosis of ABPA against a severely asthmatic population. For diagnosis of CPA complicating ABPA the Immulite assays was statistically significantly superior to all other assays. The Dynamiker assay performed worst, with no diagnostic value for identification of CPA.
complicating ABPA. Precipitins performed well in this context, with good specificity and superior sensitivity to all ELISAs other than Immulite.

Manufacturers often recommend reporting results as positive, negative or intermediate, with repeat testing in intermediate cases. This may be appropriate in the dynamic context of acute invasive aspergillosis, but in chronic diseases such as CPA and ABPA any change on repeat testing is likely to simply represent the inter-assay variability of the test. We therefore advocate the use of a single diagnostic cut-off for these conditions.

We have confirmed the earlier finding by van Toorenenbergen\textsuperscript{180} that there is good correlation between the ImmunoCAP and Immulite assays. In our study median levels in ABPA and healthy controls were similar for both assays, however the median Immulite level was three times higher than the ImmunoCAP level in CPA cases. This should be taken into consideration when comparing results from different laboratories using different assays, especially with higher levels. Laboratories should identify the assay used when reporting test results.

Patients on long term antifungal therapy were excluded from the study on the grounds that such therapy is likely to reduce \textit{Aspergillus}-specific IgG and so introduce bias to diagnostic cut-off calculations\textsuperscript{58}. However, we included patients who had received up to three months of antifungal therapy in addition to those who were not on antifungal therapy. This was necessary as a common source of stored samples was from sera taken for drug level measurement soon after starting therapy. Most patients included were not on antifungal therapy.

The only significant difference in results between patients on and off antifungal therapy was found in CPA patients using the Genesis assay, where levels were higher in patients on therapy. All other assays showed no significant difference in the median \textit{Aspergillus}-specific IgG levels between CPA patients on no antifungal therapy and those on antifungal therapy for up to three months. No assay produced a significant difference in the ABPA patients. There is therefore no evidence that up to three months antifungal
therapy produces the reduction in *Aspergillus*-specific IgG levels seen with long-term therapy\(^{58}\).

One of the limitations of our study was that it was limited to testing in a single laboratory with tests kits from a single batch. Published evidence of intra-laboratory variability currently exists only for the ImmunoCAP assay\(^{180}\). In addition, ImmunoCAP testing was also performed on fresh samples from CPA patients, while all other tests were performed on frozen stored samples. Long term storage does not appear to significantly reduce other antibody levels in serum\(^{262,288}\) and there is no reason to believe that *Aspergillus*-specific IgG will behave differently to other antibodies in this respect. However, it would have been desirable to demonstrate that this is specifically true for *Aspergillus*-specific IgG by testing stored samples with the ImmunoCAP assay and confirming that there was no significant difference between results obtained before and after storage. Unfortunately no funding was available for such a comparison.

We were also unable to perform intra-assay variability testing for the ImmunoCAP assay, as no funding was available for this. However, three existing studies from separate laboratories have shown between-run CV results from <5% to 23% for this assay\(^{73,78,180}\) and the inter-laboratory CV for ImmunoCAP is 7.3-18.1%\(^{180}\).

In most CPA cases in our cohort microbiological evidence of CPA was provided by raised *Aspergillus*-specific IgG. The ImmunoCAP and precipitins assays were routinely used for clinical testing in our reference laboratory throughout the study period, but with a diagnostic cutoff of 40 mg/L for ImmunoCAP. The Immulite, Serion, Genesis and Dynamiker assays are not used routinely at any UK diagnostic laboratory. While there may therefore be a degree of selection bias in favour of the ImmunoCAP system, this did not prevent Immulite system demonstrating equivalent ROC AUC performance to ImmunoCAP in our study.

Our study defines optimal cut-offs for *Aspergillus*-specific IgG by comparing levels in CPA cases to Ugandan healthy controls. This is appropriate as raised levels of *Aspergillus*-specific IgG form only one aspect of CPA diagnostic criteria\(^{5,250}\). However levels are also raised in other conditions such as *Aspergillus* bronchitis, *Aspergillus*
rhinosinusitis or allergic bronchopulmonary aspergillosis\textsuperscript{4,39,41}. The \textit{Aspergillus}-specific IgG assay cannot therefore be used in isolation to diagnose CPA.

The median ImmunoCAP level in our healthy controls was 5mg/L. The median ImmunoCAP level in Dutch blood donors is 8.75 mg/L\textsuperscript{180} and in healthy female Belgian laboratory workers it is 13.75 mg/L\textsuperscript{73}. These results are consistent with our proposed diagnostic cut-off of 20mg/L, which we suggest should now be adopted for the diagnosis of CPA. The median Serion level in pregnant French women is 20 AU/ml\textsuperscript{38}. This is consistent with the diagnostic cut-off of 35AU/ml produced by our ROC analysis. We are not aware of any prior descriptions of Omega and Dynamiker levels in healthy controls.

The median Immulite value in Dutch blood donors is 13.2 mg/L\textsuperscript{180}. This is higher than both the median level of 4mg/L we found in healthy Ugandan blood donors and the optimal diagnostic cut-off of 10 mg/L produced by our ROC analysis. This significant difference might be explained by different levels of \textit{Aspergillus} exposure in these different environments or differences in the median age of these healthy control groups.

We chose to use healthy Ugandan blood donors as a control group as a key goal of this study was to select an assay for use in a CPA prevalence survey to be conducted in Uganda. While the proposed Immulite cut off of 10mg/L is appropriate for the diagnosis of CPA in Ugandans, further comparisons including European healthy controls may be needed before this cut off can be used with confidence in Europe. The age matching between our controls and cases was sub-optimal, but the use of blood donors is common practice and was the only practical option available to us to acquire a healthy Ugandan control group.

CPA occurs in patients with underlying diseases such as treated tuberculosis, sarcoidosis and COPD\textsuperscript{14}. We have shown that levels of \textit{Aspergillus}-specific IgG are different in asthmatics than healthy controls and levels might also be higher than healthy controls in patients with these other underlying diseases, perhaps as a result of frequent \textit{Aspergillus} colonisation in these patients.
The purpose of the *Aspergillus*-specific IgG assay in the context of CPA diagnosis is to provide evidence of *Aspergillus* infection. Radiological and clinical criteria then need to be met before CPA can be diagnosed. While the optimal diagnostic cut-offs defined here are appropriate to define ‘abnormally high levels of *Aspergillus*-specific IgG’ and provide evidence of *Aspergillus* infection for use as a single aspect of the CPA composite diagnostic criteria, they might frequently produce positive results in a population with underlying lung disease if *Aspergillus* colonisation is common in that population.

Further studies are needed to compare levels of *Aspergillus*-specific IgG in CPA to those in ‘at-risk’ diseased controls.

Optimal cut-offs for ABPA diagnosis have been calculated for each assay, both by comparing ABPA cases to healthy controls and by comparing them to asthmatic controls. Comparison to asthmatic controls is the more valid of these two options, however there are some difficulties with this approach. The asthmatic controls used in this study are patients being treated at a regional referral centre with a sub-specialist interest in fungal lung disease. Patients referred to a centre of this nature are likely to have fungal sensitization or unusually severe asthma, as demonstrated by the fact that 29% of patients in the asthmatic control group had evidence of bronchiectasis.

As such they may be at increased risk of *Aspergillus* colonisation and are probably not representative of the overall asthmatic population in this respect. It is also possible that a population of severe asthmatics such as this may include patients whose disease has been complicated by ABPA, which was not diagnosed at the time of sampling. Our retrospective study design did not allow the identification and exclusion of any such patients. These factors might explain why this asthmatic cohort has a higher median *Aspergillus*-IgG level than healthy controls. It is therefore possible that the cut off produced by the comparison of ABPA cases with healthy controls is more appropriate for use in the diagnosis of ABPA in the asthmatic population as a whole.

Ideally, further studies should be performed involving the testing of samples from a non-selected asthmatic population, ideally one treated at primary care level. An ideal study design would include prospective screening of patients to remove cases of ABPA from the cohort. Such a design would allow the identification of a definitive cut off for
the diagnosis of ABPA in asthmatics. Such an optimal design was not possible within
time and financial constraints associated with this study. We have therefore reported
diagnostic cut offs using both the sub-optimal control groups available to us at the time
of this study. The ideal diagnostic cut off probably lies somewhere between the levels
calculated from the two groups.

ABPA can be complicated by the development of CPA. Aspergillus-specific IgG
measurement might be used to identify cases of CPA complicating ABPA. This is
important as the management of CPA differs from ABPA, with long-term antifungal
therapy being indicated wherever possible and surgery often required\textsuperscript{18,21,250}. We have
suggested optimal cut-offs in terms of Youden's J statistic. However it may be more
clinically useful to select a cut off with high specificity, above which CPA is likely and CT
scan should be recommended to further investigate the possibility. The Immulite had
reasonable sensitivity and specificity for this purpose with an optimal cut off of 100
mg/L, but other assays performed poorly. Precipitins testing also performed reasonably
well in this context. However, no assay performs well enough to be used alone to
diagnose CPA in those with ABPA. CPA must be considered possible in any patient with
ABPA and raised Aspergillus-specific IgG.

All tests were fairly labour intensive to perform. Automated platforms required manual
sample loading and dilution. Plate ELISAs required over 10 pipetting steps. The
precipitins test was the most labour-intensive requiring around 18 gels to be run for
every one ELISA plate used in the comparison. We also undertook preliminary work
with the ELITech (France) Aspergillus-specific haemagglutination assay\textsuperscript{289}, which we
found to be labour-intensive with end point reading that was highly subjective. As a
result this assay was not taken forward to the main analysis.

The Dynamiker Aspergillus-specific IgG assay uses purified galactomannan as its sole
antigen and performed similarly to equivalent manual plate ELISAs for the diagnosis of
CPA and ABPA. This antigen might be appropriate for use in an Aspergillus-specific IgG
lateral flow device (LFD) that would be ideal for use in resource-poor settings. No such
LFD exists at present.
Our study did not include all available commercial Aspergillus-specific IgG ELISAs. Bio-Rad produce an Aspergillus-specific IgG ELISA that uses recombinant antigens. This assay has been shown to have similar sensitivity and specificity to the Serion and ImmunoCAP assays in two small studies, however these studies did not exclude patients on long-term antifungal therapy and used the sub-optimal diagnostic cut-off of 40 mg/L for ImmunoCAP. Other commercial assays exist, but have no published data describing their sensitivity and specificity for the diagnosis of CPA.

We have described the diagnostic performance of six of the most commonly used Aspergillus-specific IgG assays for the diagnosis of CPA and ABPA, the most common forms of pulmonary aspergillosis. The ImmunoCAP assay is currently widely used with a diagnostic cut-off of 40 mg/L. This is sub-optimal for the diagnosis of CPA and should be replaced with a diagnostic cut-off of 20 mg/L. Optimal diagnostic cut-offs for use in CPA have been defined for all assays, which can improve sensitivity for the diagnosis of CPA while maintaining excellent specificity. Further studies are now required to confirm intra-laboratory and batch-to-batch variation for these assays. This will hopefully then allow the roll out of routine testing of at risk patients, including those in areas of high tuberculosis prevalence, where most CPA patients are predicted to reside, but where access to Aspergillus serology is currently extremely limited.

**Hypothesis**

That there are clinically relevant differences in the sensitivity and specificity of different Aspergillus-specific IgG assays or precipitins testing in the context of diagnosis of chronic pulmonary aspergillosis (CPA) and allergic pulmonary aspergillosis (ABPA).

**Aims**

1. To measure the levels of Aspergillus-specific IgG found in groups of patients with untreated CPA or ABPA and in healthy and asthmatic controls, using assays produced by Siemens Immulite, ThermoFisher Scientific ImmunoCAP, Serion, Genesis and Dynamiker, plus precipitins testing using Microgen antigens.
2 – To define the diagnostic performance for each of these assays for CPA and ABPA by performing receiver operating curve (ROC) area under the curve (AUC) analysis comparing patients with CPA and ABPA to healthy and diseased controls.

3 – To define an optimal diagnostic cut-off for each assay in relation to ROC AUC analysis for both CPA and ABPA in relation to both healthy and diseased controls.

4 – To measure intra-assay variability for both high and low level samples for each Aspergillus-specific IgG assay, within funding restrictions.

5 – To measure the correlation between Siemens Immulite and ThermoFisher Scientific ImmunoCAP results.

Ethics

Control samples were acquired as part the ‘Pulmonary aspergillosis in association with tuberculosis’ study. Ethical approval was granted by Gulu University IRB (ref GU/IRC/04/07/12), the Ugandan National Council for Science and Technology (ref HS1253) and the University of Manchester (ref 11424). Stored serum was taken from samples provided by CPA patients for the purpose of Aspergillus-specific IgG testing as part of routine care of CPA and ABPA at the National Aspergillosis Centre, Manchester, UK. Further stored serum samples were acquired from the ManRAB biobank. Ethical approval was granted by the ManRAB REC committee (ref 10/H1010/7).

Funding

Siemens, Serion, Genesis and Dynamiker all donated sufficient test kits to perform this comparison. Serion and Dynamiker each provided grant support to cover the cost of laboratory consumables.

Control samples were acquired as part the ‘Pulmonary aspergillosis in association with tuberculosis’ study (paper two), which was funded by a grant from the University
Hospital of South Manchester Academy charity as part of the established Manchester-Gulu link program.

Acknowledgements

We would like to thank the staff of Gulu Blood Transfusion service, Uganda, for their assistance in recruiting donations of serum from healthy blood donors for use as control samples.

We would like to thank the staff at Manchester Royal Infirmary immunology laboratory for their assistance in performing ImmunoCAP *Aspergillus*-specific IgG testing on control samples.

We would like to thank the staff at Christie Hospital pathology laboratory, Manchester for permitting access to their Siemens Immulite 2000 system to perform this study.

We would like to thank Siemens, Serion, Genesis and Dynamiker for kindly donating test kits to perform this study and for their practical assistance in installing the test kits and relevant software prior to undertaking the study.

We would like to thank the ManRAB biobank at University Hospital of South Manchester for providing stored sera from National Aspergillosis Centre CPA patients for use in this study. ManRAB is supported by the NIHR.
Figure 1 – Dynamiker results in various patient groups
Figure 2 – Genesis results in various patient groups
Figure 3 – ThermoFisher Scientific ImmunoCAP results in various patient groups

Samples from asthmatic patients were not tested with the ImmunoCAP assay, as no funding was available for this.
Figure 4 – Serion results in various patient groups
Figure 5 – Siemens Immulite results in various patient groups
Figure 6 – ROC curve for CPA cases vs. healthy controls
Figure 7 – ROC curves for ABPA cases vs. healthy controls
Figure 8 – ROC curves for ABPA cases vs. asthmatic controls
Figure 9 – CPA cases vs. ABPA cases
PAPER 2 - Aspergillus-specific IgG levels in patients previously treated for pulmonary tuberculosis in Gulu, Uganda

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Abstract

In 1970, 34% of 544 British patients with residual cavities after treated pulmonary tuberculosis were found to have precipitating antibodies to *Aspergillus*. Aspergilloma was detected in 63% of those with antibodies and was often complicated by haemoptysis. Based on this data the global 5-year period prevalence of chronic pulmonary aspergillosis (CPA) is estimated at 0.8 to 1.3 million cases. There are no published surveys from current areas of high tuberculosis prevalence to confirm this prediction and the impact of HIV co-infection on the prevalence of CPA is not known.

We aimed to measure the prevalence of *Aspergillus*-specific IgG in Ugandan patients with treated pulmonary tuberculosis.

We conducted a cross-sectional survey in Gulu, Uganda. Recruitment was open to all persons aged 16 or over who had completed treatment for pulmonary tuberculosis in the last 7 years. Eligible patients were identified with the assistance of clinic staff at Gulu Hospital and the District Health Team. Radio announcements were used to encourage patients to participate. All patients underwent clinical assessment and chest X-ray and had *Aspergillus*-specific IgG measured by Siemens Immulite.

Recruitment was undertaken between October 2012 and February 2013. 400 patients were recruited. 200 (50%) were HIV positive. Median age was 42 years (range 16-83). 39% of patients were female. Median CD4 count in those with HIV was 415 cells/µL (range 0-1400).

Raised *Aspergillus*-specific IgG was found in 10% of patients. Chronic cough was reported by 33% of patients and haemoptysis by 3% of patients. 4% of all patients had suspected fungal ball on chest x-ray, with cavitation present in 16% and pleural thickening in 15%.

This study cannot measure the prevalence of CPA, as it does not include CT scan and serial chest X-ray. However, we suggest that a patient who has ALL of the following should be considered to have possible CPA; 1 – chronic symptoms (over 1 month of cough or haemoptysis), 2 – Raised levels of *Aspergillus*-specific IgG and 3 – Chest X-ray
findings consistent with CPA (cavities or aspergilloma). Overall 12 (3%) met these criteria. A further 2 (0.5%) had simple aspergilloma, without chronic symptoms. HIV status had no statistically significant impact on the frequency of likely CPA.
Introduction

An estimated 9 million people developed tuberculosis in 2013\textsuperscript{215}. It was associated with 1.5 million deaths, of which only 210,000 were estimated to be due to multidrug resistant strains. Many of the other 1.29 million deaths will have been due to late presentation to medical care, lack of diagnosis, poor access to treatment or inadequate adherence, given that they mostly occurred in resource-poor countries with weak health infrastructure. However, misdiagnosis may also have contributed to the problem.

Chronic pulmonary aspergillosis (CPA) is a condition that complicates tuberculosis\textsuperscript{14}. CPA usually presents with progressive pulmonary cavitation associated with weight loss, persistent cough and haemoptysis\textsuperscript{5,7,8}. It has a 5-year mortality of 50 – 80\%\textsuperscript{6,7,264} and has recently been estimated to affect around 3 million people globally\textsuperscript{11-13}, including 1.3 million cases secondary to tuberculosis\textsuperscript{11}. This estimate takes no account of the potential impact of HIV co-infection, which is present in half of the cases of suspected pulmonary tuberculosis notified in Uganda\textsuperscript{215}.

Undiagnosed CPA could be making a substantial contribution to the observed mortality rates currently attributed to tuberculosis. Both conditions present with cavities, pleural thickening and fibrosis on chest X-ray\textsuperscript{266,280}. Aspergillomas are distinctive, but while they are present in all cases of simple aspergilloma, they are present in only 25-36\% of cases of CPA in developed countries\textsuperscript{8,58}. Raised levels of Aspergillus-specific IgG are key to diagnosis of CPA\textsuperscript{5,7,8}, but this test is generally unavailable in Africa\textsuperscript{220}. In Uganda 34\% of all notified cases of pulmonary tuberculosis are clinically diagnosed with no microbiological proof of tuberculosis infection\textsuperscript{215}. Some of these cases may well be CPA that has been misdiagnosed as tuberculosis.

Large CPA case series have been reported in the UK, France, India, China, Korea and Japan, the majority of which are secondary to tuberculosis\textsuperscript{7,8,14,15,18,108,198}. Over 180 cases of CPA have been reported throughout Africa, including South Africa, Nigeria, Ivory Coast, Senegal, Central African Republic, Djibouti, Ethiopia, Tanzania and Uganda\textsuperscript{16,201-212}. Over 90\% of these cases were secondary to pulmonary tuberculosis.
CPA is treatable. Oral treatment with itraconazole, voriconazole or posaconazole prevents clinical and radiological progression\textsuperscript{18,58,108,198,251}. Surgery is curative in selected patients with localized disease\textsuperscript{15,21} and has been safely delivered in resource-poor settings\textsuperscript{16,54,212}.

The prevalence of CPA was measured in 544 patients with residual lung cavities after tuberculosis treatment in the UK in 1968-70\textsuperscript{76,197}. Precipitating antibodies to \textit{Aspergillus fumigatus} were present in 34\%, of whom 63\% had an aspergilloma visible on chest X-ray within 48 months of completion of tuberculosis treatment. Subsequent series have found positive \textit{Aspergillus}-specific antibodies in 20-27\% of patients previously treated for pulmonary tuberculosis in Japan, India and Brazil\textsuperscript{80,146,192,221}.

CPA prevalence in areas where tuberculosis is now common might differ from the UK in 1968-70. Rates of \textit{Aspergillus} rhinitis and keratitis are higher in countries with warm climates and many subsistence farmers\textsuperscript{10}. This might also be true for CPA. Biomass smoke-induced emphysema is common in Africa\textsuperscript{222} and might increase CPA risk\textsuperscript{14}. Crucially HIV co-infection might either result in more CPA cases due to immunosuppression\textsuperscript{52,223,224} or fewer due to reduced the rate of residual cavitation seen in those co-infected with HIV\textsuperscript{225-227}.

We conducted a cross-sectional survey to measure the prevalence of raised \textit{Aspergillus}-specific IgG in persons with treated pulmonary tuberculosis in Gulu, Uganda. We recorded presence of chronic symptoms, performed chest X-ray and measured \textit{Aspergillus}-specific IgG using the Siemens Immulite 2000 system. This assay has specificity of 98\% and sensitivity of 96\% for the diagnosis of CPA (paper 1).

This study cannot definitively measure the prevalence of CPA as it does not include CT scan or serial chest X-ray. However, we have classified patients who meet all of the following conditions as ‘possible CPA’; 1 – chronic symptoms (over 1 month of cough or haemoptysis), 2 – Raised levels of \textit{Aspergillus}-specific IgG and 3 – Chest X-ray findings consistent with CPA (cavities or fungal ball).
We also diagnosed simple aspergilloma in patients with fungal ball on chest X-ray and raised *Aspergillus*-specific IgG, but with no chronic cough or haemoptysis. We targeted recruitment of 50% of patients with HIV co-infection to measure the impact of HIV status on the frequency of raised *Aspergillus*-specific IgG and possible CPA.

Methods

**Study design and participants**

Patients aged 16 or over, who had completed a full course of treatment for pulmonary tuberculosis in 2005 or later, were recruited in Gulu, Uganda from October 2012 to February 2013. Evidence of tuberculosis treatment was taken from tuberculosis treatment cards, completion of treatment certificates, or from the District Health Tuberculosis Team's central records. Patients with documentary evidence of fully treated smear-negative pulmonary tuberculosis were also accepted, but only if they reported complete resolution of all symptoms at the end of tuberculosis treatment.

We aimed to recruit 400 patients, of whom 200 would be HIV negative and 200 HIV positive. We calculated this would have sufficient power to measure the prevalence of CPA in this population with an accuracy of +/- 2.3%.

**Procedures**

Convenience sampling was used. Eligible patients were identified from District Health team records and invited to join the study. Radio announcements were used to encourage patients to enroll. All patients were provided with written study information and written consent was given prior to recruitment. Illiterate patients were provided with verbal information in English or in Acholi via a translator and gave verbal consent.

Patients underwent structured clinical assessment. HIV status was taken from patients’ medical notes or TB treatment records. Where no such record was available patients underwent HIV testing prior to recruitment. Patients with no documented HIV status
who declined testing were not eligible. Serum was tested for *Aspergillus*-specific IgG using the Immulite 2000 system (Siemens, Germany) in July 2014.

Chest X-ray was performed at St. Mary’s Hospital in Lacor and X-rays were photographed with a Nikon DSLR camera. Two radiologists reported results. Where they produced divergent reports the senior consultant respiratory radiologist at the UK National Aspergillosis Centre provided a decisive third report. All were blinded to clinical and serological findings.

**Diagnostic criteria**

Possible CPA was diagnosed when all three of the following criteria were met:-

1 – Symptoms - patients must have been suffering from at least one of the following symptoms for no less than 1 month.
   - Haemoptysis
   - Cough

2 – Radiological changes – at least one of the following features must be present on chest X-ray
   - Fungal Ball
   - Cavitation

3 – Raised *Aspergillus*-specific IgG

In addition, simple aspergilloma was diagnosed in patients with suspected fungal ball on chest X-ray and raised *Aspergillus*-specific IgG, but no chronic cough or haemoptysis. 100 control sera had previously been collected from healthy Ugandan blood donors (paper 1). These were used in receiver operating characteristic curve studies to define the diagnostic threshold of 10 mg/L used in this study.
**Statistical methods**

Statistical analysis was performed using SPSS v20 (IBM, USA). Rates of raised *Aspergillus*-specific IgG and possible CPA in groups of patients with and without potential risk factors were compared using chi-squared test, except for comparisons with less than 5 patients in one group, where Fisher’s exact test was used. Comparison of means for continuous variables in different patient groups with normal distribution was performed using 2-sided t test. Where distribution was skewed Mann Whitney U test was used.

**Results**

400 patients were consented to enter the survey. One patient did not undergo chest X-ray and one patient’s blood sample was lost, leaving 398 patients who completed the assessment process. Patient characteristics are shown in table 1. HIV status was documented on tuberculosis care records in most cases, with only a handful undergoing HIV testing on the day of recruitment. No patient declined HIV testing.

The overall frequency of various symptoms, X-ray abnormalities and *Aspergillus*-specific IgG levels are shown in table 2. Raised *Aspergillus*-specific IgG was found in 9.8% of 398 patients with prior pulmonary tuberculosis and 2% of 100 healthy adult controls (p 0.01)
Table 1 – Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients n=398</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>155 (38.9%)</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>42 years (16-83)</td>
</tr>
<tr>
<td>Positive sputum smear at TB diagnosis</td>
<td>303 (76.1%)</td>
</tr>
<tr>
<td>HIV infection</td>
<td>199 (50%)</td>
</tr>
<tr>
<td>Median 2012 CD4 count in HIV positive persons (range)</td>
<td>424 (14 – 1400) cells/µL</td>
</tr>
<tr>
<td>2012 CD4 count &lt; 200 cells/µL</td>
<td>23 (12%*)</td>
</tr>
<tr>
<td>2012 CD4 count 200 – 499 cells/µL</td>
<td>94 (49.2%*)</td>
</tr>
<tr>
<td>2012 CD4 count ≥ 500 cells/µL</td>
<td>74 (38.7%*)</td>
</tr>
<tr>
<td>Traditional ‘grass-thatch’ home</td>
<td>371 (93.2%)</td>
</tr>
<tr>
<td>Patient reports dampness in home</td>
<td>119 (29.9%)</td>
</tr>
<tr>
<td>Patient is a subsistence farmer</td>
<td>373 (93.7%)</td>
</tr>
<tr>
<td>Patient frequently cooks on open charcoal stove</td>
<td>194 (48.7%)</td>
</tr>
<tr>
<td>Patient smokes tobacco</td>
<td>39 (9.8%)</td>
</tr>
<tr>
<td>Median Aspergillus IgG</td>
<td>4.2 mg/L</td>
</tr>
</tbody>
</table>

The frequency of symptoms and X-ray changes in patients with raised and normal levels of Aspergillus-specific IgG is compared in table 3. The frequency of suspected CPA and simple aspergilloma is shown in table 4, together with the number of patients with suspected fungal ball on chest X-ray, but normal levels of Aspergillus-specific IgG (unspecified fungal ball) and the number of patients with raised Aspergillus-specific IgG in whom the symptomatic and radiological criteria for CPA or simple aspergilloma are not met (raised Aspergillus-specific IgG, but no pulmonary aspergillosis).

Tables 5 – 9 show the frequency of symptoms, test results and diagnoses in relation to gender, prior TB smear status, HIV status, CD4 count and time since tuberculosis diagnosis respectively.
### Table 2 – Symptoms and test findings

<table>
<thead>
<tr>
<th>Result</th>
<th>No patients n=398</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>130</td>
<td>32.7</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>14</td>
<td>3.5</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>191</td>
<td>48</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>193</td>
<td>48.5</td>
</tr>
<tr>
<td>Fevers*</td>
<td>99</td>
<td>24.9</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>131</td>
<td>32.9</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>214</td>
<td>53.8</td>
</tr>
<tr>
<td>Cavities on CXR</td>
<td>65</td>
<td>16.3</td>
</tr>
<tr>
<td>Paracavitary fibrosis on CXR</td>
<td>34</td>
<td>8.5</td>
</tr>
<tr>
<td>Pleural thickening on CXR</td>
<td>58</td>
<td>14.6</td>
</tr>
<tr>
<td>Fungal ball on CXR</td>
<td>15</td>
<td>3.8</td>
</tr>
<tr>
<td>Aspergillus IgG positive</td>
<td>39</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*present for 1 month or longer

### Table 3 – Symptoms and X-ray changes in patients with and without raised *Aspergillus*-specific IgG

<table>
<thead>
<tr>
<th>Results</th>
<th>Raised <em>Aspergillus</em>-specific IgG n=39</th>
<th>Normal <em>Aspergillus</em>-specific IgG n=359</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>5 (12.8%)</td>
<td>60 (16.7%)</td>
<td>0.532</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>6 (15.4%)</td>
<td>8 (2.2%)</td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Fatigue*</td>
<td>20 (51.3%)</td>
<td>171 (47.6%)</td>
<td>0.665</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>21 (53.8%)</td>
<td>172 (47.9%)</td>
<td>0.481</td>
</tr>
<tr>
<td>Fevers*</td>
<td>12 (30.8%)</td>
<td>87 (24.2%)</td>
<td>0.370</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>11 (28.2%)</td>
<td>120 (33.4%)</td>
<td>0.510</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>28 (71.8%)</td>
<td>186 (51.8%)</td>
<td><strong>0.017</strong></td>
</tr>
<tr>
<td>Cavities on CXR</td>
<td>18 (46.2%)</td>
<td>47 (13.1%)</td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Paracavitary fibrosis on CXR</td>
<td>6 (15.4%)</td>
<td>28 (7.8%)</td>
<td>0.108</td>
</tr>
<tr>
<td>Pleural thickening on CXR</td>
<td>15 (38.5%)</td>
<td>43 (14.8%)</td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Fungal ball on CXR</td>
<td>4 (12.9%)</td>
<td>11 (3%)</td>
<td><strong>0.049</strong>**</td>
</tr>
</tbody>
</table>

*present for 1 month or more. **Fisher's exact test used.
### Table 4 – Frequency of various conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of cases</th>
<th>Frequency (%)</th>
<th>Frequency confidence interval (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Likely CPA</td>
<td>12</td>
<td>3</td>
<td>1.7 - 5</td>
</tr>
<tr>
<td>Likely simple aspergilloma</td>
<td>2</td>
<td>0.5</td>
<td>0.1 – 1.6</td>
</tr>
<tr>
<td>Unspecified fungal ball</td>
<td>11</td>
<td>2.8</td>
<td>1.5 – 4.7</td>
</tr>
<tr>
<td>Raised <em>Aspergillus</em> IgG, but no pulmonary aspergillosis</td>
<td>25</td>
<td>6.3</td>
<td>4.2 - 9</td>
</tr>
</tbody>
</table>

### Table 5 - Symptoms and test results by gender

<table>
<thead>
<tr>
<th>Result</th>
<th>Female n=155</th>
<th>Male n=243</th>
<th>p-value by chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>47 (30.3%)</td>
<td>83 (34.2%)</td>
<td>0.426</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>5 (3.2%)</td>
<td>9 (3.7%)</td>
<td>0.801</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>80 (51.6%)</td>
<td>111 (45.7%)</td>
<td>0.248</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>69 (44.5%)</td>
<td>124 (51%)</td>
<td>0.205</td>
</tr>
<tr>
<td>Fevers*</td>
<td>42 (27.1%)</td>
<td>57 (23.5%)</td>
<td>0.413</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>44 (28.4%)</td>
<td>87 (35.8%)</td>
<td>0.125</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>85 (54.8%)</td>
<td>129 (53%)</td>
<td>0.732</td>
</tr>
<tr>
<td>Cavities on CXR</td>
<td>17 (11%)</td>
<td>48 (19.8%)</td>
<td><strong>0.021</strong></td>
</tr>
<tr>
<td>Paracavitary fibrosis on CXR</td>
<td>4 (2.6%)</td>
<td>30 (12.3%)</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Pleural thickening on CXR</td>
<td>11 (7.1%)</td>
<td>47 (19.3%)</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Fungal ball on CXR</td>
<td>3 (1.9%)</td>
<td>12 (4.9%)</td>
<td>0.125</td>
</tr>
<tr>
<td>Positive <em>Aspergillus</em> IgG</td>
<td>11 (7.1%)</td>
<td>28 (11.5%)</td>
<td>0.148</td>
</tr>
<tr>
<td>Median <em>Aspergillus</em> IgG level</td>
<td>3.84 mg/L</td>
<td>4.58 mg/L</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>Likely CPA</td>
<td>3 (1.2%)</td>
<td>9 (3.7%)</td>
<td>0.381***</td>
</tr>
<tr>
<td>Likely simple aspergilloma</td>
<td>0</td>
<td>2 (0.8%)</td>
<td>0.523***</td>
</tr>
<tr>
<td>Unspecified fungal ball</td>
<td>2 (1.3%)</td>
<td>9 (3.7%)</td>
<td>0.214***</td>
</tr>
<tr>
<td>Raised <em>Aspergillus</em> IgG, but no pulmonary aspergillosis</td>
<td>8 (5.2%)</td>
<td>17 (7%)</td>
<td>0.462***</td>
</tr>
</tbody>
</table>

*present for 1 month or longer. ** medians compared by Mann Whitney U test, ***Fisher’s exact test
### Table 6 – Symptoms and test results by prior TB smear status

<table>
<thead>
<tr>
<th>Symptoms/Results</th>
<th>Prior smear positive pulmonary tuberculosis n= 303</th>
<th>Prior smear negative pulmonary tuberculosis n= 95</th>
<th>p-value by chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>107 (35.3%)</td>
<td>23 (24.2%)</td>
<td><strong>0.044</strong></td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>9 (3%)</td>
<td>5 (5.3%)</td>
<td>0.029</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>146 (48.2%)</td>
<td>45 (47.4%)</td>
<td>0.889</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>151 (49.8%)</td>
<td>42 (44.2%)</td>
<td>0.339</td>
</tr>
<tr>
<td>Fevers*</td>
<td>72 (23.8%)</td>
<td>27 (28.4%)</td>
<td>0.359</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>103 (34%)</td>
<td>28 (29%)</td>
<td>0.413</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>165 (54.4%)</td>
<td>49 (51.6%)</td>
<td>0.624</td>
</tr>
<tr>
<td>Cavities on CXR</td>
<td>55 (18.2%)</td>
<td>10 (10.5%)</td>
<td>0.079</td>
</tr>
<tr>
<td>Paracavitary fibrosis on CXR</td>
<td>32 (10.6%)</td>
<td>2 (2.1%)</td>
<td><strong>0.01</strong>*</td>
</tr>
<tr>
<td>Pleural thickening on CXR</td>
<td>50 (16.5%)</td>
<td>8 (8.4%)</td>
<td>0.051</td>
</tr>
<tr>
<td>Fungal ball on CXR</td>
<td>13 (4.3%)</td>
<td>2 (2.1%)</td>
<td>0.537***</td>
</tr>
<tr>
<td>Positive Aspergillus IgG</td>
<td>32 (10.6%)</td>
<td>7 (7.4%)</td>
<td>0.361</td>
</tr>
<tr>
<td>Median Aspergillus IgG level</td>
<td>4.37 mg/L</td>
<td>3.91 mg/L</td>
<td><strong>0.025</strong></td>
</tr>
<tr>
<td>Likely CPA</td>
<td>9 (1.7%)</td>
<td>3 (3.2%)</td>
<td>1***</td>
</tr>
<tr>
<td>Likely simple aspergilloma</td>
<td>1 (0.3%)</td>
<td>1 (1.1%)</td>
<td>0.421***</td>
</tr>
<tr>
<td>Unspecified fungal ball</td>
<td>10 (3.3%)</td>
<td>1 (1.1%)</td>
<td>0.472***</td>
</tr>
<tr>
<td>Raised Aspergillus IgG, but no pulmonary aspergillosis</td>
<td>22 (7.3%)</td>
<td>3 (3.2%)</td>
<td>0.224***</td>
</tr>
</tbody>
</table>

*present for 1 month or longer. ** medians compared by Mann Whitney U test. ***Fishers exact test used.
Table 7 – Symptoms and test results by HIV status

<table>
<thead>
<tr>
<th>Symptoms and test results by HIV status</th>
<th>Positive HIV status n= 199</th>
<th>Negative HIV status n= 199</th>
<th>p-value by chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>51 (25.6%)</td>
<td>79 (39.7%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>5 (2.5%)</td>
<td>9 (4.5%)</td>
<td>0.276</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>96 (48.2%)</td>
<td>95 (47.7%)</td>
<td>0.92</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>94 (47.2%)</td>
<td>99 (49.7%)</td>
<td>0.616</td>
</tr>
<tr>
<td>Fevers*</td>
<td>50 (25.1%)</td>
<td>49 (24.6%)</td>
<td>0.908</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>54 (27.1%)</td>
<td>77 (38.7%)</td>
<td>0.014</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>99 (49.7%)</td>
<td>115 (57.8%)</td>
<td>0.108</td>
</tr>
<tr>
<td>Cavities on CXR</td>
<td>24 (12.1%)</td>
<td>41 (20.6%)</td>
<td><strong>0.021</strong></td>
</tr>
<tr>
<td>Paracavitary fibrosis on CXR</td>
<td>14 (7.1%)</td>
<td>20 (20.1%)</td>
<td>0.282</td>
</tr>
<tr>
<td>Pleural thickening on CXR</td>
<td>23 (11.6%)</td>
<td>35 (17.6%)</td>
<td>0.088</td>
</tr>
<tr>
<td>Fungal ball on CXR</td>
<td>5 (2.5%)</td>
<td>10 (5%)</td>
<td>0.188</td>
</tr>
<tr>
<td>Positive <em>Aspergillus</em> IgG</td>
<td>12 (6%)</td>
<td>27 (13.6%)</td>
<td><strong>0.011</strong></td>
</tr>
<tr>
<td>Median <em>Aspergillus</em> IgG level</td>
<td>3.84 mg/L</td>
<td>4.65 mg/L</td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Likely CPA</td>
<td>4 (2%)</td>
<td>8 (4%)</td>
<td>0.38***</td>
</tr>
<tr>
<td>Likely simple aspergilloma</td>
<td>1 (0.5%)</td>
<td>1 (0.5%)</td>
<td>1***</td>
</tr>
<tr>
<td>Unspecified fungal ball</td>
<td>3 (1.5%)</td>
<td>8 (4%)</td>
<td>0.22***</td>
</tr>
<tr>
<td>Raised <em>Aspergillus</em> IgG, but no pulmonary aspergilosis</td>
<td>7 (3.5%)</td>
<td>18 (9%)</td>
<td><strong>0.023</strong></td>
</tr>
</tbody>
</table>

*present for 1 month or longer. **medians compared by Mann Whitney U test. ***Fishers exact test used.
Table 8 – Symptoms and tests for HIV positive patients by CD4 count groups

<table>
<thead>
<tr>
<th>Results</th>
<th>CD4 count &lt; 200 cells/µL n= 23</th>
<th>CD4 count 200 – 499 cells/µL n=94</th>
<th>CD4 count ≥ 500 cells/µL n=74</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cough</strong></td>
<td>6 (26%)</td>
<td>26 (27.7%)</td>
<td>17 (23%)</td>
<td>0.787</td>
</tr>
<tr>
<td><strong>Haemoptysis</strong></td>
<td>0 (0%)</td>
<td>3 (3.2%)</td>
<td>2 (2.7%)</td>
<td>0.690</td>
</tr>
<tr>
<td><strong>Fatigue</strong></td>
<td>9 (39.1%)</td>
<td>48 (51.1%)</td>
<td>35 (47.3%)</td>
<td>0.580</td>
</tr>
<tr>
<td><strong>Breathlessness</strong></td>
<td>10 (43.5%)</td>
<td>44 (46.8%)</td>
<td>37 (50%)</td>
<td>0.839</td>
</tr>
<tr>
<td><strong>Fever</strong></td>
<td>4 (17.4%)</td>
<td>20 (21.3%)</td>
<td>24 (32.4%)</td>
<td>0.168</td>
</tr>
<tr>
<td><strong>Night sweats</strong></td>
<td>5 (21.7%)</td>
<td>26 (27.7%)</td>
<td>20 (27%)</td>
<td>0.845</td>
</tr>
<tr>
<td><strong>Chest pain</strong></td>
<td>10 (43.5%)</td>
<td>49 (52.1%)</td>
<td>36 (48.6%)</td>
<td>0.737</td>
</tr>
<tr>
<td>Cavities on CXR</td>
<td>2 (8.7%)</td>
<td>14 (14.9%)</td>
<td>8 (10.8%)</td>
<td>0.611</td>
</tr>
<tr>
<td>Paracavitary fibrosis on CXR</td>
<td>1 (4.3%)</td>
<td>7 (7.4%)</td>
<td>6 (8.1%)</td>
<td>0.832</td>
</tr>
<tr>
<td>Pleural thickening on CXR</td>
<td>3 (13%)</td>
<td>13 (13.8%)</td>
<td>7 (9.5%)</td>
<td>0.680</td>
</tr>
<tr>
<td>Fungal ball on CXR</td>
<td>0</td>
<td>4 (4.3%)</td>
<td>1 (1.4%)</td>
<td>0.355</td>
</tr>
<tr>
<td>Positive Aspergillus IgG</td>
<td>0</td>
<td>5 (5.3%)</td>
<td>6 (8.1%)</td>
<td>0.334</td>
</tr>
<tr>
<td>Median Aspergillus IgG level</td>
<td>3.77 mg/L</td>
<td>3.98 mg/L</td>
<td>3.65 mg/L</td>
<td>0.809**</td>
</tr>
<tr>
<td>Likely CPA</td>
<td>0</td>
<td>2 (2.1%)</td>
<td>2 (2.7%)</td>
<td>0.731</td>
</tr>
<tr>
<td>Likely simple aspergilloma</td>
<td>0</td>
<td>1 (1.1%)</td>
<td>0</td>
<td>0.595</td>
</tr>
<tr>
<td>Unspecified fungal ball</td>
<td>0</td>
<td>2 (2.1%)</td>
<td>1 (1.4%)</td>
<td>0.749</td>
</tr>
<tr>
<td>Raised Aspergillus IgG, but no pulmonary aspergillosis</td>
<td>0</td>
<td>2 (2.1%)</td>
<td>4 (5.4%)</td>
<td>0.315</td>
</tr>
</tbody>
</table>

*present for 1 month or longer. ** medians compared by independent samples median test
### Table 9 – Antibody levels and diagnoses by year of starting tuberculosis treatment

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Median <em>Aspergillus</em> IgG level (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 27</td>
<td>4</td>
<td>4.4</td>
<td>4.2</td>
<td>4.8</td>
<td>4.1</td>
<td>4.1</td>
<td>4</td>
<td>0.795*</td>
</tr>
<tr>
<td>Positive <em>Aspergillus</em> IgG</td>
<td>1</td>
<td>7</td>
<td>5</td>
<td>8</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td>0.188</td>
</tr>
<tr>
<td>Likely CPA</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.632</td>
</tr>
<tr>
<td>Likely simple aspergilloma</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.450</td>
</tr>
<tr>
<td>Unspecified fungal ball</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0.564</td>
</tr>
<tr>
<td>Raised <em>Aspergillus</em> IgG, but no pulmonary aspergilosis</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>0.482</td>
</tr>
</tbody>
</table>

* medians compared by independent samples median test

### Discussion

We have demonstrated that raised *Aspergillus*-specific IgG is present in 9.8% of our cohort of Ugandan adults with previously treated pulmonary tuberculosis, but only 2% of healthy controls. This difference was statistically significant with a p-value of 0.01. This is first study to measure this in a community-based survey. Previous publications reported higher frequencies of raised *Aspergillus*-specific antibodies in patients with treated pulmonary tuberculosis \(^{80,146,192,221}\), but these were conducted in highly selected groups of patients attending tertiary referral hospitals for follow up due to ongoing symptomatic illness. Our community-based study provides a more accurate measurement of the overall frequency of this finding in this patient group.

Symptoms were common in the study group, with cough reported by 33% of all patients and breathlessness by 48% of all patients. However, the presence of raised *Aspergillus*-specific IgG was associated with a statistically significant increase in the presence of...
both haemoptysis and chest pain. Fatigue, breathlessness and fevers were also more common in the group with raised *Aspergillus*-specific IgG, but these associations were non-significant.

All patients underwent chest X-ray. Radiological abnormalities were less common in the overall study population than reported symptoms, with cavities and pleural thickening both noted in around 15% of X-rays. There was a statistically significant increase in the frequency of cavities, pleural thickening and suspected fungal ball in patients with raised *Aspergillus*-specific IgG compared to those with normal antibody levels. There was also a non-significant increase in the frequency of paracavitary fibrosis associated with raised *Aspergillus*-specific IgG levels.

We compared levels of *Aspergillus*-specific IgG in different groups of patients with previously treated pulmonary tuberculosis. There was a statistically significant increase in the frequency of raised *Aspergillus*-specific IgG levels in HIV negative patients compared to HIV positive patients. The study was not powered to detect differences between other patient groups. There was, however a non-significant trend towards increased frequency of *Aspergillus*-specific IgG in HIV positive patients with higher CD4 cell counts compared to those with lower CD4 cell counts.

There was also a statistically significant increase in the number of patients with cavities on chest X-ray in the HIV negative group, compared to the HIV positive group, as has been observed previously. The presence of residual cavities after tuberculosis might result in increased vulnerability to *Aspergillus* infection, in which case the increased level of raised *Aspergillus*-specific IgG in the HIV negative group might reflect a genuinely higher rate of CPA in this group.

An alternative explanation for this finding would be that patients with HIV infection, especially those with low CD4 counts, might form a less effective antibody response to active *Aspergillus* infection than HIV negative patients. However, the presence of raised *Aspergillus*-specific IgG in 26% of Ugandan in patients with AIDS and sub-acute respiratory disease with no confirmed diagnosis (paper 4), demonstrates that patients with AIDS can mount an antibody response to *Aspergillus*. It therefore seems most likely
that pulmonary aspergillosis does occur more frequently in HIV negative patients, perhaps on account of the increased frequency of pulmonary cavitation in this group.

It is important to note that this study only measured antibodies to *Aspergillus fumigatus*. This species is responsible for over 90% of aspergillosis in Europe \(^5,6,10^8\). In such circumstances measuring *A. fumigatus* specific IgG gives a reasonably reliable measure of the overall prevalence of raised *Aspergillus*-specific IgG. However most aspergillosis in India and the Middle East is due to *A. flavus*\(^10\) and *A. niger* is common in Brazil\(^147\). *A. fumigatus* assays can have poor sensitivity for infection with other *Aspergillus* species\(^147,148\). The sole published study describing the frequency of fungal co-infection in African tuberculosis patients showed two cases of *A. niger* and two cases of histoplasmosis\(^213\). It is not therefore clear whether *A. fumigatus* is likely to be the dominant species of *Aspergillus* causing disease in humans in Uganda. If other species of *Aspergillus* are common in Uganda then our study might significantly underestimate the total prevalence of *Aspergillus*-specific IgG in the study population.

Histoplasmosis is also known to exist in Uganda\(^268\) and blastomycosis elsewhere in Africa\(^232\). These and other chronic fungal lung infections can also cause chronic cough with progressive lung cavitation and fibrosis\(^290–292\). Cross reactivity between other *Aspergillus*-specific IgG assays and *Penicillium* antibodies has been noted\(^269\) and might theoretically also occur with other fungi. If this does occur then in some cases of raised *Aspergillus*-specific IgG found in our study might be due to infection with another fungus.

It is also possible that some of the 11 (2.8%) of patients with ‘unspecified fungal ball’ on chest X-ray might be suffering from chronic infection with another fungus and that cross-reaction with the *Aspergillus*-specific IgG assay is not occurring in these cases. The alternative explanation of erroneous reporting of chest X-rays must also be considered. We aim to perform CT scan on all patients with reported ‘fungal ball’ on chest X-ray during the resurvey to confirm that a fungal ball is indeed present in each case.
This study was not designed to measure the precise prevalence of CPA. All the currently published case cohorts of CPA include CT scan rather than chest X-ray in their diagnostic criteria. They also include progressive lung cavitation and exclusion of chronic lung conditions as mandatory diagnostic features for CPA. None of these features are included in this study. We will later undertake a resurvey of this cohort with repeat chest X-ray, CT thorax and exclusion of recurrent pulmonary tuberculosis with geneXpert sputum PCR testing to permit measurement of the prevalence of CPA in this cohort.

While this study cannot measure the precise prevalence of CPA, it is the first survey of its kind in an area of high HIV prevalence and some useful conclusions can be drawn regarding the prevalence of CPA. 15 (3.8%) of patients had a suspected fungal ball on chest X-ray and 4 (1%) of all patients had a combination of fungal ball on chest X-ray and raised *Aspergillus*-specific IgG. The latter group is highly likely to be suffering from some form of pulmonary aspergillosis. This finding alone suggests that CPA is likely to occur with measurable frequency in Ugandan adults with treated pulmonary tuberculosis.

We have identified ‘likely CPA’ in 12 (3%) patients and simple aspergilloma in 2 (0.5%) patients. We cannot be certain CPA is present in these patients in the absence of progressive radiology or CT scan. However, the Siemens *Aspergillus*-specific IgG assay used has a sensitivity of 96% and specificity of 98% for the diagnosis of CPA (paper 1). The combination of chronic cough or haemoptysis, plus cavities or fungal ball on chest X-ray and raised *Aspergillus*-specific IgG in these patients is therefore likely to be due to CPA. Indeed the 98% specificity of the Siemens *Aspergillus*-specific IgG assay suggests that many of the 25 (6.3%) patients with raised *Aspergillus*-specific IgG in whom the symptomatic and radiological criteria for CPA have not met in this study will be confirmed as having CPA in the resurvey.

Our study suffered from further limitations. The convenience sampling method used is vulnerable to selection bias. Only survivors are recruited. The 5-year mortality of CPA is up to 80%[7]. By allowing recruitment of patients treated for tuberculosis up to 7 years ago we might therefore have missed patients who developed CPA soon after completing...
tuberculosis treatment and subsequently succumbed to the condition. This could be especially important in the HIV positive group, where pulmonary aspergillosis presents in subacute invasive form. Such patients would normally die within months without treatment.

Despite these limitations we have demonstrated that there is a statistically significant increase in the frequency of raised Aspergillus-specific IgG in adults with treated pulmonary tuberculosis, in comparison to healthy controls in Uganda. We cannot definitively state the frequency of CPA in this study, but we can state with reasonable confidence that it must lie between 1% (the number of patients with fungal ball on chest X-ray plus raised Aspergillus-specific IgG) and 10% (the total number of patients with raised Aspergillus-specific IgG).

We have estimated that 3.5% of all patients with treated pulmonary tuberculosis in this cohort are suffering from CPA or simple aspergilloma. We are now conducting a resurvey with repeat chest X-ray and CT thorax to confirm the prevalence of CPA. If this study confirms our estimate of the prevalence of CPA this would constitute evidence of a life threatening and previously neglected complication of tuberculosis occurring with clinically relevant frequency.

**Hypothesis**

That treated pulmonary tuberculosis is complicated by chronic pulmonary aspergillosis in adults and that an increased frequency of raised Aspergillus-specific IgG levels can therefore be detected in patients with treated pulmonary tuberculosis in comparison to healthy controls.

**Aims**

1 – To measure levels of Aspergillus-specific IgG in a group of Ugandan adults with previously treated pulmonary tuberculosis.
2 – To determine the frequency of raised levels of *Aspergillus*-specific IgG in this group, using the diagnostic cut off defined in paper 1.

3 – To record the frequency of various chronic symptoms associated with CPA in this population using a structured questionnaire.

4 – To perform chest X-ray on all patients in this group and record the frequency of abnormalities associated with CPA.

5 – To determine if raised levels of *Aspergillus*-specific IgG are associated with increased frequency of symptoms or X-ray abnormalities.

6 – To estimate the prevalence of CPA in this population, as accurately as possible within the limitations of this study design.

7 – To compare the frequency of raised *Aspergillus*-specific IgG, symptoms, X-ray changes and likely CPA secondary to treated pulmonary tuberculosis in patients with and without HIV infection.

Ethics

Ethical permission for this study was granted by the University of Manchester, UK (ref 11424), Gulu University IRB, Uganda (GU/IRC/04/07/12) and the Ugandan National Council for Science and Technology (ref HS1253).

Funding

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Acknowledgements

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• Gulu Regional Referral Hospital Infectious Diseases Clinic for providing us with space to review patients and assisting in identifying eligible patients from those attending clinic.
• The Joint Clinical Research Centre (JCRC) Gulu laboratory for the storage and cataloging of samples and performing CD4 counts.
• Brother Carlos and the radiology staff at St. Mary’s Hospital, Lacor for their assistance in performing chest X-ray on study patients.
• Andrew Mockridge of Manchester University for his practical assistance in planning the study in Gulu.
• The North West Lung Centre at University Hospital of South Manchester for providing storage of serum samples.
• Department of Pathology at Christie Hospital, Manchester for allowing us to access their Siemens Immulite 2000 to test study samples.
PAPER 3 - Prevalence of chronic pulmonary aspergillosis (CPA) secondary to tuberculosis: a cross-sectional survey in an area of high tuberculosis prevalence.

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Chronic cavitary pulmonary aspergillosis (CPA) is estimated to complicate 1.2 million cases of pulmonary tuberculosis worldwide. This includes both chronic cavitary pulmonary aspergillosis (CCPA) and simple aspergilloma. CPA has a 5-year mortality of 50 – 80%, but is treatable. We measured the prevalence of CPA in an area of high tuberculosis prevalence.

In 2012 to 2013 we surveyed 400 adult patients with treated pulmonary tuberculosis in Gulu, Uganda. Half also had HIV infection. Between October 2014 and January 2015 we conducted a re-survey. Patients underwent clinical assessment and chest X-rays. Those with raised levels of Aspergillus-specific IgG or suspicion of aspergilloma had a CT chest scan. Those with productive cough submitted sputum for GeneXpert PCR.

CCPA was diagnosed in any patient who did not have recurrent active pulmonary tuberculosis, but had all the following: 1 – Cough or haemoptysis for one month or more, 2 – Raised Aspergillus-specific IgG, 3 – Progressive cavitation on serial chest X-ray or cavities with paracavitary infiltrates or aspergilloma on CT scan. Simple aspergilloma was diagnosed in persons with aspergilloma on CT scan and raised Aspergillus-specific IgG, but no chronic cough or haemoptysis.

282 patients were re-surveyed. There was no significant difference in patient characteristics between the surveys. 99 (35%) patients resurveyed had cough and 31 (11%) had haemoptysis. 31 (11%) had progressive cavitation on serial chest X-ray. 29 (10%) patients had raised Aspergillus-specific IgG. 43 (15%) patients had paracavitary fibrosis on CT scan and 14 (5%) had aspergilloma. 25 patients underwent GeneXpert sputum testing and 3 had confirmed active tuberculosis, none of whom had Aspergillus co-infection.

CCPA was present in 16 (6%) patients resurveyed. A further 2 (1%) patients had simple aspergilloma. CPA was diagnosed in 62% of those with raised Aspergillus-specific IgG. 10 of the 12 patients diagnosed with likely CCPA based on chest X-ray findings in the
first survey were re-surveyed. All 10 had CT changes consistent with CCPA. HIV status had no significant impact on CPA prevalence.

CPA complicates pulmonary tuberculosis with clinically relevant frequency. This survey supports the estimated global 5-year point prevalence of CPA secondary tuberculosis of 1.3 million cases. CPA should be considered for in any patient with a background of pulmonary tuberculosis who presents with cough, haemoptysis or progressive cavitation. Access to diagnosis and treatment for CPA is almost non-existent in most areas with high tuberculosis prevalence. Improving this should be an urgent priority in global health.
Introduction

An estimated 9 million people developed tuberculosis in 2013,215. It was associated with 1.5 million deaths, of which only 210,000 were estimated to be due to multidrug resistant strains. Many of the other 1.29 million deaths will have been due to late presentation to medical care, lack of diagnosis, poor access to treatment or inadequate adherence, given that they mostly occurred in resource-poor countries with weak health infrastructure. However, misdiagnosis may also have contributed to the problem.

Chronic pulmonary aspergillosis (CPA) is a condition that complicates tuberculosis14. CPA includes both chronic cavitary pulmonary aspergillosis (CCPA) and simple aspergilloma. CCPA usually presents with progressive pulmonary cavitation associated with weight loss, persistent cough and haemoptysis.5,7,8 It has a 5-year mortality of 50 – 80%6,7,264 and has recently been estimated to affect around 3 million people globally11–13, including 1.3 million cases secondary to tuberculosis11. This estimate takes no account of the potential impact of HIV co-infection, which is present in half of the cases of suspected pulmonary tuberculosis notified in Uganda215.

Undiagnosed CCPA could therefore be making a substantial contribution to the observed mortality rates attributed to tuberculosis. Both conditions present with cavities, pleural thickening and fibrosis on chest X-ray266,280. Aspergillomas are distinctive, but while they are present in all cases of simple aspergilloma, they are present in only 25-36% of cases of CCPA in developed countries8,58. Raised levels of Aspergillus-specific IgG are key to diagnosis of CPA5,7,8, but this test is generally unavailable in Africa220. In Uganda 34% of all notified cases of pulmonary tuberculosis are clinically diagnosed with no microbiological proof of tuberculosis infection215. Some of these cases may well be CCPA misdiagnosed as tuberculosis.

Large CPA case series have been reported in the UK, France, India, China, Korea and Japan and the majority of cases are secondary to tuberculosis7,8,14,15,18,108,198. Over 180 cases of CPA have been reported throughout Africa, including South Africa, Nigeria, Ivory Coast, Senegal, Central African Republic, Djibouti, Ethiopia, Tanzania and Uganda16,201–212. Over 90% of African cases were secondary to pulmonary tuberculosis.
The prevalence of CPA was measured in 544 patients with residual lung cavities after tuberculosis treatment in the UK in 1968-70\textsuperscript{76,197}. Precipitating antibodies to \textit{Aspergillus fumigatus} were present in 34\%, of whom 63\% had an aspergilloma visible on chest X-ray within 48 months of completion of tuberculosis treatment. Subsequent series have found positive \textit{Aspergillus}-specific antibodies in 20-27\% of patients previously treated for pulmonary tuberculosis in Japan, India and Brazil\textsuperscript{80,146,192,221}.

CPA prevalence in areas where tuberculosis is now common might differ from the UK in 1968-70. Rates of \textit{Aspergillus} rhinitis and keratitis are higher in countries with warm climates and many subsistence farmers\textsuperscript{10}. This might also be true for CPA. Biomass smoke-induced emphysema is common in Africa\textsuperscript{222} and might increase CPA risk\textsuperscript{14}. Crucially, HIV co-infection might either result in more CPA cases due to immunosuppression\textsuperscript{52,223,224} or fewer due to reduced the rate of residual cavitation seen in those co-infected with HIV\textsuperscript{225-227}.

CPA is treatable. Oral treatment with itraconazole, voriconazole or posaconazole prevents clinical and radiological progression\textsuperscript{18,58,108,198,251}. Surgery is curative in selected patients with localized disease\textsuperscript{15,21} and has been safely delivered in resource-poor settings\textsuperscript{16,54,212}.

We conducted a cross-sectional survey to measure the prevalence of CPA in persons with treated pulmonary tuberculosis in Gulu, Uganda. We targeted recruitment of 50\% of patients with HIV co-infection to measure the impact of this on CPA rates. We therefore used a case definition\textsuperscript{6,264} that would capture both CCPA and the subacute invasive aspergillosis seen in HIV\textsuperscript{5,6,52,223,236,264}. We diagnosed simple aspergilloma in patients with aspergilloma and raised \textit{Aspergillus}-specific IgG, but no chronic cough or haemoptysis. Taken together these conditions represent the total prevalence of chronic pulmonary aspergillosis.

An initial survey was conducted in 2012 (paper 2). It recorded presence of chronic symptoms, performed chest X-ray and measured \textit{Aspergillus}-specific IgG using the Siemens Immulite 2000 system. This assay has specificity of 98\% and sensitivity of 96\% for the diagnosis of CPA (paper 1). 398 patients were assessed. 39 (9.8\%) were found
to have raised levels of *Aspergillus*-specific IgG and 15 (3.8%) had a suspected fungal ball on chest X-ray. Within the limits of this single survey it was estimated that 12 (3%) of patients were likely to be suffering from CPA as they had a combination of chronic cough or haemoptysis, plus cavitation or fungal ball on chest X-ray and had raised levels of *Aspergillus*-specific IgG. A further 2 (0.5%) asymptomatic patients were suspected to be suffering from simple aspergilloma as they had a combination of fungal ball on chest X-ray and raised levels of *Aspergillus*-specific IgG. However this first survey was limited in its ability to accurately identify cases of CPA due to the lack of CT scans and lack of exclusion of recurrent tuberculosis. It was also impossible to identify progressive cavitation on the basis of a single survey.

A resurvey was therefore conducted in 2014 to allow accurate measurement of the prevalence of CPA. This included repeated clinical assessment, serology and chest X-ray. Patients with raised *Aspergillus*-specific IgG or suspicion of fungal ball on chest X-ray in 2012 also underwent CT chest scan. The impact of potential risk factors, including HIV co-infection, on the frequency of CPA was assessed.

Methods

Study design and participants

398 patients were recruited in 2012 as described in paper 2. Patients enrolled in the first survey were traced by District Health Tuberculosis Team staff and re-assessed between October 2014 and January 2015. All patients underwent repeat clinical examination and chest X-ray, which was reported as before. Repeat Immulite *Aspergillus*-specific IgG were measured on serum. Patient flow and recruitment outcomes are shown in figure 1.

CT scan (GE Duo-slice, USA) was performed at the Kampala Imaging Center on those with raised *Aspergillus*-specific IgG or suspicion of aspergilloma on 2012 chest X-ray. Digital CT scan images were saved and accessed with OsirisX software (Pixmeo SARL, Switzerland). Patient flow for those selected for CT scan is shown in figure 2. Reports were provided by three radiologists, in the same manner as chest X-rays. Verbal
autopsy was performed by district health workers in those that died between surveys\textsuperscript{293}.

Sputum was taken from all patients who were able to provide a sample and underwent GeneXpert IV (Cepheid, USA) \textit{Mycobacterium tuberculosis} nucleic acid amplification testing. Patient flow for patients with productive cough is shown in figure 3.

\textit{Diagnostic criteria}

CCPA was diagnosed when ALL four of the following criteria were met:-

1 – Symptoms - patients must have been suffering from at least one of the following symptoms for no less than 1 month.

- Haemoptysis
- Cough

2 – Radiological changes – at least one of the following features must be present

- Fungal ball on CT scan
- Cavitation with paracavitary fibrosis on CT scan
- New or progressive cavitation on serial chest X-ray

3 – Raised \textit{Aspergillus}-specific IgG

4 – Absence of positive GeneXpert test for \textit{M. tuberculosis}

In addition, simple aspergilloma was diagnosed in patients with fungal ball on CT scan and raised \textit{Aspergillus}-specific IgG, but no chronic cough or haemoptysis. 100 control sera had previously been collected from healthy Ugandan blood donors (paper 1). These were used in receiver operating characteristic curve studies to define the diagnostic threshold of 10 mg/L used in this study.

Chest X-rays and CT scans were both reported by the author on the day of testing and patients were informed of their diagnosis. Where CPA was diagnosed patients were
provided with a written statement of the diagnosis together with an advisory treatment plan, with advice to attend the Gulu Regional Referral Hospital Infectious Diseases clinic for follow up. Patients with resectable disease were referred to the cardiothoracic surgical unit at Mulago Hospital, Kampala for surgical treatment. Where surgery was not appropriate treatment with oral itraconazole was recommended.

Statistical methods

Statistical analysis was performed using SPSS v20 (IBM, USA). Rates of CPA in groups of patients with and without potential risk factors were compared using chi-squared test, except for comparisons with less than 5 patients in one group, where Fisher’s exact test was used. Comparison of means for continuous variables in different groups with normal distribution was performed using 2-sided t test. Where distribution was skewed Mann Whitney U test was used.

Results

Patient characteristics for the original survey and re-survey are compared in table 1. There is no evidence that the resurvey recruitment process introduced bias, as there are no significant differences in characteristics between the groups.

389 patients were recruited in 2012. 282 of these patients were reviewed in the resurvey. Recruitment outcomes for all patients from the 2012 survey are shown in figure 1. 29 of these patients had raised *Aspergillus*-specific IgG in 2012 and were eligible for CT scan. A further 45 patients in the re-survey group had a suspicion of aspergilloma on their 2012 chest X-ray and also underwent CT scan, with 73 persons undergoing CT scan in total. CT scan outcomes are shown in figure 2.

All patients who could provide a sputum sample underwent GeneXpert PCR testing for recurrent tuberculosis. In two cases the GeneXpert machine was not functioning and smear test was performed in its place. Two cases of active pulmonary tuberculosis were identified. A third patient was diagnosed with multi-drug resistant tuberculosis.
between the original study and the resurvey. None of the three patients had evidence of additional CPA. A breakdown of GeneXpert test outcomes is shown in figure 3.

**Table 1 – Patient characteristics**

<table>
<thead>
<tr>
<th>Characteristic in 2012</th>
<th>Original survey n=398</th>
<th>Re-survey n= 282</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>155 (38.9%)</td>
<td>99 (35.1%)</td>
<td>0.308</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
<td>42 (16-83)</td>
<td>42 (16-82)</td>
<td>0.53**</td>
</tr>
<tr>
<td>Positive sputum smear at TB diagnosis</td>
<td>303 (76.1%)</td>
<td>222 (78.7%)</td>
<td>0.427</td>
</tr>
<tr>
<td>HIV infection</td>
<td>199 (50%)</td>
<td>134 (47.5%)</td>
<td>0.524</td>
</tr>
<tr>
<td>Median 2012 CD4 count in HIV positive persons (range)</td>
<td>424 (14 – 1400) cells/µL</td>
<td>424 (59 - 1400) cells/µL</td>
<td>0.867***</td>
</tr>
<tr>
<td>2012 CD4 count &lt; 200 cells/µL</td>
<td>23 (12%*)</td>
<td>15 (11.6%##)</td>
<td>0.911</td>
</tr>
<tr>
<td>2012 CD4 count 200 – 499 cells/µL</td>
<td>94 (49.2%*)</td>
<td>65 (50.4%##)</td>
<td>0.837</td>
</tr>
<tr>
<td>2012 CD4 count ≥ 500 cells/µL</td>
<td>74 (38.7%*)</td>
<td>49 (38%##)</td>
<td>0.891</td>
</tr>
<tr>
<td>Traditional ‘grass-thatch’ home</td>
<td>371 (93.2%)</td>
<td>267 (94.7%)</td>
<td>0.434</td>
</tr>
<tr>
<td>Patient reports dampness in home</td>
<td>119 (29.9%)</td>
<td>76 (27%)</td>
<td>0.402</td>
</tr>
<tr>
<td>Patient is a subsistence farmer</td>
<td>373 (93.7%)</td>
<td>267 (94.7%)</td>
<td>0.599</td>
</tr>
<tr>
<td>Patient frequently cooks on open charcoal stove</td>
<td>194 (48.7%)</td>
<td>127 (45%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Patient smokes tobacco</td>
<td>39 (9.8%)</td>
<td>30 (10.6%)</td>
<td>0.721</td>
</tr>
<tr>
<td>2012 <em>Aspergillus</em> IgG positive</td>
<td>39 (9.8%)</td>
<td>29 (10.3%)</td>
<td>0.836</td>
</tr>
<tr>
<td>2012 median <em>Aspergillus</em> IgG</td>
<td>4.2 mg/L</td>
<td>4.1 mg/L</td>
<td>0.840</td>
</tr>
</tbody>
</table>

*Chi-squared test, except **where means compared by 2-sided t-test ***Mann-Whitney U test #fraction of those with CD4 count available in original survey n=191 ##fraction of those with CD4 count available in re-survey n=129.

Symptoms, chest X-ray results and *Aspergillus*-specific IgG results for re-survey patients are shown in table 2. Breakdowns of these results by gender, original tuberculosis smear status, HIV status and CD4 count are shown in supplementary data. CT scan findings for those who underwent that test are shown in table 3. The median level of
Aspergillus-specific IgG in those who were resurveyed was 4.1 mg/L (range 0.1-1060 mg/L).

Table 2 – Resurvey symptoms and test findings n=282

<table>
<thead>
<tr>
<th>Result</th>
<th>No patients</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>99</td>
<td>35</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>31</td>
<td>11</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>88</td>
<td>31</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>83</td>
<td>29</td>
</tr>
<tr>
<td>Fevers*</td>
<td>26</td>
<td>9</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>51</td>
<td>18</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>76</td>
<td>27</td>
</tr>
<tr>
<td>New cavitation on serial CXR</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Enlarged cavitation on serial CXR</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>New or progressive paracavitary fibrosis</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>New or progressive pleural thickening on CXR</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

*present for 1 month or longer

Table 3 – Chest CT scan findings (n=73)

<table>
<thead>
<tr>
<th>Result</th>
<th>No patients</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavities</td>
<td>49</td>
<td>67</td>
</tr>
<tr>
<td>Paracavitary fibrosis</td>
<td>43</td>
<td>59</td>
</tr>
<tr>
<td>Aspergilloma</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Pleural thickening</td>
<td>35</td>
<td>47</td>
</tr>
<tr>
<td>Nodule</td>
<td>37</td>
<td>51</td>
</tr>
</tbody>
</table>

Frequencies of diagnoses are shown in table 4. We identified 16 cases of CCPA, of which 2 had been complicated by the development of chronic fibrosing pulmonary aspergillosis (CFPA). A further five patients had apparent fungal ball on CT scan, but normal levels of Aspergillus-specific IgG and are recorded as ‘unspecified fungal ball’.
The frequency of CCPA in patients with and without potential risk factors is shown in tables 5 and 6.

Table 4 – Frequency of diagnoses

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of cases N=282</th>
<th>Frequency (%)</th>
<th>Frequency 95% confidence interval (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCPA</td>
<td>16</td>
<td>5.7</td>
<td>3.4 – 8.8</td>
</tr>
<tr>
<td>Simple aspergilloma</td>
<td>2</td>
<td>0.7</td>
<td>0.1 – 2.3</td>
</tr>
<tr>
<td>All CPA</td>
<td>18</td>
<td>6.4</td>
<td>4 – 9.4</td>
</tr>
<tr>
<td>Unspecified fungal ball</td>
<td>5</td>
<td>1.8</td>
<td>0.7 – 3.8</td>
</tr>
<tr>
<td>Raised <em>Aspergillus</em> IgG, but no pulmonary aspergilosis</td>
<td>11</td>
<td>3.9</td>
<td>2.1 – 6.6</td>
</tr>
</tbody>
</table>

Twelve patients were identified as likely cases of CCPA after the first survey on the basis of chronic cough or haemoptysis, plus cavitation on chest X-ray and raised *Aspergillus*-specific IgG. Ten of these patients were reviewed in the resurvey. All ten had radiological features of CCPA confirmed on CT scan, although two reported that their symptoms had resolved at the re-survey assessment and so were not classified as CCPA in the final analysis. The sole patient with likely simple aspergilloma in 2012 and was resurveyed developed symptoms between the surveys and was confirmed as a case of CPA in 2014.

Pulmonary aspergillosis was confirmed in 58% of those with raised *Aspergillus*-specific IgG in 2012. Four (36%) of those with fungal ball on chest X-ray in 2012 had a confirmed fungal ball on resurvey in 2014, but 2 could not be classified as CPA as they had normal levels of *Aspergillus*-specific IgG. The outcomes of all patients from 2012 who were re-surveyed in 2014 are shown in table 7.

Nineteen (5%) patients recruited to the first survey died before the resurvey. None of these were identified as likely CPA in the first survey. Three of the 19 had raised *Aspergillus*-specific IgG, but none of these died in a manner consistent with CPA. Four of the 16 deaths in patients with normal levels of *Aspergillus*-specific IgG were preceded...
by subacute respiratory illness, including three preceded by haemoptysis. These patients may have developed pulmonary aspergillosis after the first survey, but this cannot be confirmed.

Table 5 – Frequency of CCPA in categorical patient groups

<table>
<thead>
<tr>
<th>Factor</th>
<th>CCPA rate where factor is present</th>
<th>CCPA rate where factor is absent</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>16 (5.7%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Female gender</td>
<td>5 (5.1%)</td>
<td>11 (6%)</td>
<td>0.739</td>
</tr>
<tr>
<td>HIV infection</td>
<td>5 (3.7%)</td>
<td>11 (7.4%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Positive smear status at TB diagnosis</td>
<td>13 (5.9%)</td>
<td>3 (5%)</td>
<td>0.799</td>
</tr>
<tr>
<td>Subsistence farmer</td>
<td>16 (6%)</td>
<td>0</td>
<td>0.329</td>
</tr>
<tr>
<td>Traditional dwelling</td>
<td>16 (6%)</td>
<td>0</td>
<td>0.329</td>
</tr>
<tr>
<td>Dampness reported in house</td>
<td>3 (3.9%)</td>
<td>13 (6.3%)</td>
<td>0.447</td>
</tr>
<tr>
<td>Frequently cooks on charcoal</td>
<td>7 (5.5%)</td>
<td>9 (5.8%)</td>
<td>0.915</td>
</tr>
<tr>
<td>Tobacco smoker</td>
<td>2 (6.7%)</td>
<td>14 (5.6%)</td>
<td>0.804</td>
</tr>
</tbody>
</table>

Table 6 – Frequency of CCPA in relation to continuous patient variables

<table>
<thead>
<tr>
<th>Factor</th>
<th>Mean level in those with CCPA</th>
<th>Mean level in those without CCPA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in 2012</td>
<td>43.5 years</td>
<td>42.5 years</td>
<td>0.740</td>
</tr>
<tr>
<td>TB treatment start date</td>
<td>28/6/2009</td>
<td>23/7/2009</td>
<td>0.890</td>
</tr>
<tr>
<td>CD4 count in HIV positive patients</td>
<td>554 cells/µL</td>
<td>472 cells/µL</td>
<td>0.495</td>
</tr>
</tbody>
</table>

Means compared by 2-tailed t-test as each variable had a reasonably normal distribution in the whole survey population.
Table 7 – 2014 outcomes in relation to 2012 survey diagnosis

<table>
<thead>
<tr>
<th>2012 diagnosis</th>
<th>CCPA</th>
<th>Simple aspergilloma</th>
<th>Unspecified fungal ball</th>
<th>No aspergillosis</th>
<th>Died before resurvey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal <em>Aspergillus</em>-specific IgG n= 258</td>
<td>0</td>
<td>0</td>
<td>5 (2%)</td>
<td>248 (96%)</td>
<td>16 (6%)</td>
</tr>
<tr>
<td>All raised <em>Aspergillus</em> specific IgG n=31</td>
<td>16 (52%)</td>
<td>2 (6%)</td>
<td>0</td>
<td>11 (35%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Fungal ball on CXR n=11</td>
<td>2 (18%)</td>
<td>0</td>
<td>2 (18%)</td>
<td>5 (45%)</td>
<td>2 (18%)</td>
</tr>
<tr>
<td>Likely CPA n=10</td>
<td>8 (80%)</td>
<td>0</td>
<td>0</td>
<td>2 (20%)</td>
<td>0</td>
</tr>
<tr>
<td>Likely simple aspergilloma n=1</td>
<td>1 (100%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unspecified fungal ball n=9</td>
<td>0</td>
<td>0</td>
<td>2 (22%)</td>
<td>5 (56%)</td>
<td>2 (22%)</td>
</tr>
<tr>
<td>Raised <em>Aspergillus</em> IgG, but no pulmonary aspergillosis n=20</td>
<td>7 (35%)</td>
<td>2 (10%)</td>
<td>0</td>
<td>9 (45%)</td>
<td>2 (10%)</td>
</tr>
</tbody>
</table>

Discussion

We have demonstrated that CPA is present in 6.4% (95% confidence interval 4 – 9.4%) of patients in our cohort of Ugandan adults with treated pulmonary tuberculosis. If our findings are applied to the 5.28 million cases of pulmonary tuberculosis estimated to occur globally\textsuperscript{215}, then they would be consistent with the predicted global 5-year point prevalence of between 0.85 and 1.37 million cases of CPA secondary to pulmonary tuberculosis\textsuperscript{11}. Further cases of CPA will occur secondary to other conditions\textsuperscript{14}. It is now clear that CPA is sufficiently common to be considered a global public health problem.
The marked similarity between the radiological and clinical presentation of CCPA and tuberculosis, plus the near total absence of *Aspergillus*-specific IgG testing in Africa means that most of these cases are probably being diagnosed as 'smear negative tuberculosis' at present. Correctly identifying these misdiagnosed cases could have a significant impact on the apparent global prevalence of smear negative tuberculosis. Providing treatment with surgery or cheap generic itraconazole could extend many lives.

The diagnosis of 'unspecified fungal ball' refers to cases with apparent fungal ball on CT scan, but negative *Aspergillus*-specific IgG. These are most likely to represent CPA caused by other species of *Aspergillus* not detected by the *A. fumigatus* assay. Three of the 11 patients with 'raised *Aspergillus*-specific IgG, but no pulmonary aspergillosis' had symptoms of chronic cough or haemoptysis, but lacked radiological evidence of CCPA. These may be cases of *Aspergillus* bronchitis, but this diagnosis cannot be confirmed without fungal culture. The other eight did not have cough or haemoptysis. These cases may represent *Aspergillus* rhinosinusitis or simply colonization. It is not known whether these *Aspergillus*-associated conditions are likely to progress to CPA with time. Two (2%) of healthy controls (age 17-39 years) also had raised *Aspergillus*-specific IgG (paper 1).

No risk factors for the development of CPA were identified. There was a non-significant reduction in CPA prevalence in the HIV infected group. However cough was significantly less common in the HIV infected group (p=0.006). The rate of cavitation and paracavitary fibrosis in the HIV positive and negative groups was similar, but there was a statistically significant reduction in the frequency of paracavitary fibrosis in those with low CD4 count (p=0.028). CPA is associated with several forms of immune dysfunction. It is conceivable that AIDS-associated immunosuppression would be protective against the immune dysregulation that leads to fibrosis in CPA.

It is notable that progressive cavitation was noted in 9% of male patients resurveyed but was not noted in any female patients resurveyed. In all female cases with CPA radiological features of CPA were identified on CT scan. This difference might be explained by overlying breast tissue obscuring subtle chest X-ray findings. Progressive
Cavitation on chest X-ray provided the sole evidence of CPA in one case, with CT scan findings diagnostic in the other 15 cases. Overall 8 of 16 (50%) CPA cases showed progressive cavitation on serial chest X-ray. These were all in patients with prior smear positive tuberculosis. This shows that CT scan is more sensitive than serial chest X-ray for the diagnosis of CPA and should be used wherever it is available, however use of serial chest X-ray would allow many cases to be diagnosed in areas where CT scan is not available.

We have shown that the screening of patients with clinical assessment, chest X-ray and measurement of Aspergillus-specific IgG levels undertaken in the first survey in 2012 has some ability to predict the diagnosis of CPA. 80% of the patients diagnosed as ‘likely CPA’ in 2012 and then reviewed in 2014 had confirmed CPA on re-survey with CT scan. The remaining 20% had signs consistent CPA on CT scan but could not be classified as CPA cases as they reported resolution of symptoms in between surveys. If the CT scan had been undertaken immediately in 2012 and produced the same results then 100% of patients with ‘likely CPA’ 2012 would have been confirmed as cases of CPA.

This suggest that when a patient with treated pulmonary tuberculosis has a combination of cough or haemoptysis for 1 month or more, with raised levels of Aspergillus-specific IgG and either cavities or fungal ball on chest X-ray they are likely to have CPA and should undergo further assessment with CT scan wherever possible. However it should be noted that 56% of the patients ultimately diagnosed with CPA were not identified as ‘likely CPA’ in the 2012 survey, demonstrating that chest X-ray alone is insufficient to exclude CPA.

Measurement of Aspergillus-specific IgG alone performed reasonably well as a screening test for the diagnosis of CPA in this population, with 58% of those with raised levels of Aspergillus-specific IgG having confirmed CPA after the re-survey. Raised levels of Aspergillus-specific IgG can also occur in Aspergillus bronchitis or colonization. The remaining patients with raised levels of Aspergillus-specific IgG who did not meet the diagnostic criteria for CPA may be suffering from one of these conditions, but this could be confirmed during our study, as facilities for fungal culture were not available at the study site.
Our study was not without flaws. The convenience sampling method used is vulnerable to selection bias and only identifies those living with CPA. The 5-year mortality of CPA is up to 80%. By allowing recruitment of patients treated for tuberculosis up to 7 years ago we might therefore have missed patients who developed CPA soon after completing tuberculosis treatment and subsequently succumbed to the condition. This could be especially important in the HIV positive group, where pulmonary aspergillosis presents in subacute invasive form. Such patients would normally die within months without treatment.

Our case definition necessarily differed from previous CCPA cohorts. We did not include weight loss as a symptom of CCPA as no records of prior weight were available. We did not include inflammatory markers, as these tests were not available in Gulu. We excluded pulmonary tuberculosis by GeneXpert PCR testing in those who provided a sputum sample, but we did not have access to induced sputum or bronchoscopy. We were also unable to include culture for atypical mycobacteria or testing for alternative fungal infections that might mimic CCPA.

We measured antibodies to Aspergillus fumigatus, which is responsible for most of the CPA in Europe and East Asia. However most aspergillosis in India and the Middle East is due to A. flavus and A. niger is common in Brazil. A. fumigatus assays can have poor sensitivity for other species. The sole study of the frequency of fungal co-infection in African tuberculosis patients showed two cases of A. niger and two cases of histoplasmosis. It is therefore not clear if A. fumigatus is the dominant species of Aspergillus in Ugandan patients. Our study may not have identified cases of CPA due to Aspergillus species other than A. fumigatus.

Histoplasmosis is known to exist in Uganda and blastomycosis elsewhere in Africa. These and other chronic fungal lung infections can also cause chronic cough with progressive lung cavitiation and fibrosis. Cross reactivity between other Aspergillus-specific IgG assays and Penicillium antibodies has been noted. If such cross-reactivity occurred between the Siemens Immulite assay used and other fungal infections potentially present in Uganda then these might be falsely classified as CPA.
Finally it should be noted that CPA also complicates other conditions such as sarcoidosis, COPD, prior pneumothorax, non-tuberculous mycobacterial infection and ABPA\textsuperscript{13,14}. CPA secondary to these conditions is not measured in this survey and as a result the prevalence of CPA will be underestimated if based on our findings alone.

CPA often requires treatment with long term anti fungal drugs and surgery in selected cases\textsuperscript{15,198}. Provision of such long term follow up and treatment was not feasible within the limited time frame of this cross sectional study. The optimal treatment of CPA has not been clearly established in the medical literature. Surgery is effective in selected cases with localized disease\textsuperscript{15}. Some specialist units in the developed world advocate long-term azole therapy\textsuperscript{198}, but six months of generic itraconazole at fixed dose with no drug level monitoring has recently demonstrated efficacy in a randomized controlled trial in India\textsuperscript{18}.

The study team made every effort to maximize the opportunities for patients diagnosed with CPA to access such treatment as was available in Uganda at the time of the study. We provided presentations on CPA and its management to the Gulu District Health Team, the staff of the local Gulu Regional Referral Hospital Infectious Diseases Clinic and the staff of the national referral clinic at the Infectious Diseases Institute in Kampala to raise awareness of CPA amongst potential care providers.

We agreed a patient treatment pathway with the management of Gulu Regional Referral Hospital and the cardiothoracic surgical team at Mulago Hospital, Kampala. Where patients had localized disease amenable to surgery they would be referred to the cardiothoracic team for resection. This team already performs 10-20 resections a year for patients with aspergilloma and severe haemoptysis. Where surgery was inappropriate due to extensive or multifocal disease patients were to be recommended treatment with itraconazole with basic monitoring provided at the Gulu Regional Referral Hospital Infectious Diseases Clinic. We established the itraconazole could normally be purchased at local pharmacies in Gulu at a cost of around US$20/patient/month, which would be affordable to patients with a modest income.
While the results of the Indian RCT\textsuperscript{18} and surgical case series from China\textsuperscript{15} and Senegal\textsuperscript{16} demonstrate that the potential does exist to deliver safe and effective CPA treatment in resource poor settings, our study uncovered a number of practical difficulties. Where patients were candidates for surgical resection there was the potential to cure them within the constraints of the existing Ugandan health service. Unfortunately cost of transport to the cardiothoracic surgery centre in Kampala is a barrier for many patients. At the time of thesis submission the clinical director of Gulu Regional Referral Hospital is trying to make arrangements to transport all the surgical resection candidates to the cardiothoracic surgery department using hospital transport. Clearly we have not been able to provide the same level of care to patients diagnosed as CPA in the course of this study as would have been provided at a referral centre in a well resourced country. We have, however provided patients enrolled in our study with a unique opportunity to be diagnosed with a serious illness and have made every effort to maximize the opportunities for our patients to access the treatment options that are available locally. This includes the possibility of surgical cure of an otherwise fatal disease for some of our patients. Entry to our study has therefore provided a potential benefit to those who chose to enter.

When designing the study we expected that most of our recruits would be urban dwellers with a basic income, most of whom could afford the $20/month cost of itraconazole. However, most patients actually recruited to this study were subsistence farmers who could not afford this cost. Itraconazole is not on the World Health Organization’s essential drugs list and is not provided for free by the Ugandan health service. As itraconazole is off-patent it should be possible to manufacture this drug sufficiently cheaply to allow national health programs in resource poor countries to purchase it and provide it at low or no cost to the poorest patients, but this a significant policy shift at global and national levels would be required to achieve this.

Our epidemiological study is the first to measure the prevalence of CPA in an area of currently high tuberculosis prevalence. Although it has some limitations it does demonstrate that CPA is a frequent complication of pulmonary tuberculosis in this setting. Pulmonary aspergillosis has already been shown to be a frequent complication
of pulmonary tuberculosis in the UK\textsuperscript{76,197}. It should be considered in the differential diagnosis of any patient with a prior history of tuberculosis, who later presents with chronic cough, haemoptysis, progressive lung cavitation or fibrosis.

Further research is now also needed to develop and validate \textit{Aspergillus} serology tests suitable for large-scale use in resource poor settings. The Siemens Immulite assay is performed on a large automated system that requires frequent maintenance. It is not ideal for use in resource-poor settings. A simpler, cheaper test such as a lateral flow device (LFD) is required for large scale testing\textsuperscript{295}. Such an LFD has recently been developed to detect \textit{Aspergillus} antigens and has good performance characteristics for the diagnosis of invasive aspergillosis, even in patients with limited immunosuppression\textsuperscript{187–190}. Its sensitivity and specificity for the diagnosis of CPA has not been investigated, but the Platelia galactomannan antigen ELISA has poor sensitivity in serum in this context\textsuperscript{8,59}.

The efficacy of oral antifungal treatment for CPA and the suitability of these drugs for use without regular monitoring must be confirmed in further trials. Other chronic fungal infections may well be present in resource poor countries and the frequency of these needs to be measured. Such information is a necessary pre-requisite for the design of new health care protocols that might allow routine diagnosis and effective treatment of chronic fungal lung diseases complicating pulmonary tuberculosis in resource poor settings.

We are now planning a larger, prospective multi-center study in Kenya that should provide a more accurate measure of the prevalence of CPA and the timing at which it occurs after pulmonary tuberculosis. This will provide a key piece of evidence to inform the design of healthcare guidelines to allow identification of those patients developing CPA after tuberculosis.

\textbf{Hypothesis}

That chronic pulmonary aspergillosis (CPA) complicates pulmonary tuberculosis at a frequency that is clinically significant and sufficiently large to be measured.
Aims

1 – To complete a cross-sectional survey to measure the prevalence of CPA secondary to pulmonary tuberculosis in an area of high tuberculosis prevalence.

2 – To determine whether HIV co-infection is associated with an altered frequency of CPA secondary to pulmonary tuberculosis.

3 – To determine whether other postulated environmental and clinical risk factors for development of CPA are associated with altered frequency of CPA secondary to pulmonary aspergillosis.

Ethics

Ethical permission for this study was granted by the University of Manchester, UK (ref 11424), Gulu University IRB, Uganda (GU/IRC/04/07/12) and the Ugandan National Council for Science and Technology (ref HS1253).

Funding

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We are indebted to the following persons and organisations;

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- Study assistants Geoffrey Abwola and Thomas Okumu for their work throughout the study.
• Gulu Regional Referral Hospital Infectious Diseases Clinic for providing us with space to review patients and assisting in identifying eligible patients from those attending clinic.

• Gulu Regional Referral Hospital Pathology Laboratory for providing sample storage and Cepheid GeneXpert testing on sputum samples.

• Brother Carlos and the radiology staff at St. Mary’s Hospital, Lacor for their assistance in performing chest X-ray on study patients.

• Matthew Kneale and Michael Clarke of Manchester University for their practical assistance in conducting the study in Gulu.

• The North West Lung Centre at University Hospital of South Manchester for providing storage of serum samples.

• Department of Pathology at Christie Hospital, Manchester for allowing us to access their Siemens Immulite 2000 to test study samples.
Figures

Figure 1 – Recruitment outcomes

Patients recruited in original survey (398)

- Died between surveys (18)
- Moved out of the region (9)
- Declined to participate in the re-survey (11)
- Could not be traced (75)

Living patients traced and consented to enter the re-survey (285)

- CXR not performed as planned (2)
- Patients removed due to incomplete assessment
  - Serum sample not taken as planned (1)

Patients in final re-survey analysis (282)
Figure 2 – CT scan recruitment outcomes

Patients with raised *Aspergillus* IgG levels in original survey (39)

- Died between surveys (2)
- Could not be traced (8)

Consented for CT scan (29)

- Died between consent and CT scan date (1)

Underwent CT scan (28)

Resurvey patients with suspicion of aspergilloma on 2012 chest X-ray, but negative *Aspergillus*-specific IgG (45)

Total number of patients undergoing CT scan (73)
Patients reporting productive cough (39)

- No sputum sample submitted (8)
- GeneXpert testing attempted, but failed (6)

Samples with GeneXpert result available (25)
### S1 - Symptoms and test results in resurvey by gender

<table>
<thead>
<tr>
<th>Result</th>
<th>Female n=99</th>
<th>Male n=183</th>
<th>p-value by chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>31 (31.3%)</td>
<td>68 (37.2%)</td>
<td>0.326</td>
</tr>
<tr>
<td>Productive cough*</td>
<td>9  (9.1%)</td>
<td>25 (13.7%)</td>
<td>0.261</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>11 (11.1%)</td>
<td>20 (10.9%)</td>
<td>0.963</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>34 (34.3%)</td>
<td>54 (29.5%)</td>
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</tr>
<tr>
<td>Breathlessness*</td>
<td>33 (33.3%)</td>
<td>50 (27.3%)</td>
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</tr>
<tr>
<td>Fevers*</td>
<td>8  (8.1%)</td>
<td>18 (9.8%)</td>
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</tr>
<tr>
<td>Night sweats*</td>
<td>15 (15.2%)</td>
<td>36 (19.7%)</td>
<td>0.346</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>23 (23.2%)</td>
<td>53 (29%)</td>
<td>0.301</td>
</tr>
<tr>
<td>New cavitation on serial CXR</td>
<td>0</td>
<td>16 (8.7%)</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>Enlarged cavitation on serial CXR</td>
<td>5  (5.1%)</td>
<td>9  (4.9%)</td>
<td>0.961</td>
</tr>
<tr>
<td>New or progressive paracavitary fibrosis</td>
<td>4 (4%)</td>
<td>11 (6%)</td>
<td>0.482</td>
</tr>
<tr>
<td>New or progressive pleural thickening on CXR</td>
<td>1 (1%)</td>
<td>3  (1.6%)</td>
<td>0.67</td>
</tr>
<tr>
<td>2012 median Aspergillus IgG level</td>
<td>3.77 mg/L</td>
<td>4.41 mg/L</td>
<td><strong>0.016</strong>**</td>
</tr>
<tr>
<td>2012 positive Aspergillus IgG</td>
<td>7  (7.1%)</td>
<td>22 (12%)</td>
<td>0.191</td>
</tr>
</tbody>
</table>

*present for 1 month or longer. ** medians compared by Mann Whitney U

### S2 – CT findings by gender

<table>
<thead>
<tr>
<th>Result</th>
<th>Female n=16</th>
<th>Male n=57</th>
<th>p-value by chi-squared test</th>
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</thead>
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<tr>
<td>Cavities</td>
<td>11 (68%)</td>
<td>38 (66.7%)</td>
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</tr>
<tr>
<td>Paracavitary fibrosis</td>
<td>9  (56.3%)</td>
<td>34 (59.6%)</td>
<td>0.807</td>
</tr>
<tr>
<td>Aspergilloma</td>
<td>2  (12.5%)</td>
<td>12 (21.1%)</td>
<td>0.443</td>
</tr>
<tr>
<td>Pleural thickening</td>
<td>5  (31.3%)</td>
<td>30 (52.6%)</td>
<td>0.130</td>
</tr>
<tr>
<td>Nodule</td>
<td>4  (25%)</td>
<td>33 (57.9%)</td>
<td><strong>0.02</strong></td>
</tr>
</tbody>
</table>
### S3 - Symptoms and test results in resurvey by HIV status

<table>
<thead>
<tr>
<th>Result</th>
<th>HIV positive n=134</th>
<th>HIV negative n=148</th>
<th>p-value by chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>36 (26.9%)</td>
<td>63 (42.6%)</td>
<td><strong>0.006</strong></td>
</tr>
<tr>
<td>Productive cough*</td>
<td>13 (9.7%)</td>
<td>21 (14.2%)</td>
<td>0.248</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>17 (12.7%)</td>
<td>14 (9.5%)</td>
<td>0.387</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>39 (29.1%)</td>
<td>49 (33.1%)</td>
<td>0.469</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>38 (28.4%)</td>
<td>45 (30.4%)</td>
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</tr>
<tr>
<td>Fevers*</td>
<td>12 (9%)</td>
<td>14 (9.5%)</td>
<td>0.884</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>22 (16.4%)</td>
<td>29 (19.6%)</td>
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</tr>
<tr>
<td>Chest pain*</td>
<td>31 (23.1%)</td>
<td>45 (30.4%)</td>
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<tr>
<td>New cavitation on serial CXR</td>
<td>7 (5.2%)</td>
<td>9 (6.1%)</td>
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<tr>
<td>Enlarged cavitation on serial CXR</td>
<td>5 (3.7%)</td>
<td>9 (6.1%)</td>
<td>0.364</td>
</tr>
<tr>
<td>New or progressive paracavitary fibrosis</td>
<td>8 (6%)</td>
<td>7 (4.7%)</td>
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</tr>
<tr>
<td>New or progressive pleural thickening on CXR</td>
<td>2 (1.5%)</td>
<td>2 (1.4%)</td>
<td>0.92</td>
</tr>
<tr>
<td>2012 median Aspergillus IgG level</td>
<td>3.77 mg/L</td>
<td>4.63 mg/L</td>
<td><strong>0.001</strong>**</td>
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<tr>
<td>2012 positive Aspergillus IgG</td>
<td>10 (7.5%)</td>
<td>19 (12.8%)</td>
<td>0.138</td>
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</table>

*present for 1 month or longer. ** medians compared by Mann Whitney U

### S4 – CT findings by HIV status

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<tr>
<th>Result</th>
<th>HIV positive n=26</th>
<th>HIV negative n=47</th>
<th>p-value by chi-squared test</th>
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<tr>
<td>Cavities</td>
<td>17 (65.4%)</td>
<td>32 (68.1%)</td>
<td>0.814</td>
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<tr>
<td>Paracavitary fibrosis</td>
<td>13 (50%)</td>
<td>30 (63.8%)</td>
<td>0.250</td>
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<tr>
<td>Aspergilloma</td>
<td>5 (19.2%)</td>
<td>9 (19.1%)</td>
<td>0.993</td>
</tr>
<tr>
<td>Pleural thickening</td>
<td>9 (34.6%)</td>
<td>26 (55.3%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Nodule</td>
<td>11 (42.3%)</td>
<td>26 (55.3%)</td>
<td>0.287</td>
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S5 - Symptoms and test results in resurvey by prior TB smear status

<table>
<thead>
<tr>
<th>Result</th>
<th>Smear positive (n=222)</th>
<th>Smear negative (n=60)</th>
<th>p-value by chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>80 (36%)</td>
<td>19 (31.7%)</td>
<td>0.529</td>
</tr>
<tr>
<td>Productive cough*</td>
<td>31 (14%)</td>
<td>3 (5%)</td>
<td>0.058</td>
</tr>
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<td>Haemoptysis*</td>
<td>25 (11.3%)</td>
<td>6 (10%)</td>
<td>0.782</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>67 (30.2%)</td>
<td>21 (35%)</td>
<td>0.475</td>
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<tr>
<td>Breathlessness*</td>
<td>63 (28.4%)</td>
<td>20 (33.3%)</td>
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</tr>
<tr>
<td>Fevers*</td>
<td>22 (9.9%)</td>
<td>4 (6.7%)</td>
<td>0.441</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>40 (18%)</td>
<td>11 (18.3%)</td>
<td>0.955</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>60 (27%)</td>
<td>16 (26.7%)</td>
<td>0.955</td>
</tr>
<tr>
<td>New cavitation on serial CXR</td>
<td>16 (7.2%)</td>
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<td><strong>0.032</strong></td>
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<td>Enlarged cavitation on serial CXR</td>
<td>10 (4.5%)</td>
<td>4 (6.7%)</td>
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<td>New or progressive paracavitary fibrosis</td>
<td>12 (5.4%)</td>
<td>3 (5%)</td>
<td>0.901</td>
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<tr>
<td>New or progressive pleural thickening on CXR</td>
<td>3 (1.4%)</td>
<td>1 (1.7%)</td>
<td>0.855</td>
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<td>2012 median Aspergillus IgG level</td>
<td>4.29 mg/L</td>
<td>3.7 mg/L</td>
<td>0.16**</td>
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<td>2012 positive Aspergillus IgG</td>
<td>24 (10.8%)</td>
<td>5 (8.3%)</td>
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*present for 1 month or longer. ** medians compared by Mann Whitney U Test

S6 – CT findings by prior TB smear status

<table>
<thead>
<tr>
<th>Result</th>
<th>smear positive (n=60)</th>
<th>smear negative (n=13)</th>
<th>p-value by chi-squared test</th>
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<tr>
<td>Cavities</td>
<td>40 (66.7%)</td>
<td>9 (69.2%)</td>
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</tr>
<tr>
<td>Paracavitary fibrosis</td>
<td>37 (61.7%)</td>
<td>6 (46.2%)</td>
<td>0.303</td>
</tr>
<tr>
<td>Aspergilloma</td>
<td>11 (18.3%)</td>
<td>3 (23.1%)</td>
<td>0.694</td>
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<tr>
<td>Pleural thickening</td>
<td>30 (50%)</td>
<td>5 (38.5%)</td>
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<td>Nodule</td>
<td>34 (56.7%)</td>
<td>3 (23.1%)</td>
<td><strong>0.02</strong></td>
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### Symptoms and test results in resurvey by CD4 count groups

<table>
<thead>
<tr>
<th>Result</th>
<th>CD4 count &lt; 200 cells/µL n=15</th>
<th>CD4 count 200 - 499 cells/µL n=65</th>
<th>CD4 count ≥ 500 cells/µL n=49</th>
<th>p-value by chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough*</td>
<td>3 (20%)</td>
<td>15 (23.1%)</td>
<td>16 (32.7%)</td>
<td>0.433</td>
</tr>
<tr>
<td>Productive cough*</td>
<td>3 (20%)</td>
<td>4 (6.2%)</td>
<td>5 (10.2%)</td>
<td>0.241</td>
</tr>
<tr>
<td>Haemoptysis*</td>
<td>2 (13.3%)</td>
<td>7 (10.8%)</td>
<td>7 (14.3%)</td>
<td>0.847</td>
</tr>
<tr>
<td>Fatigue*</td>
<td>4 (26.7%)</td>
<td>22 (33.8%)</td>
<td>13 (26.5%)</td>
<td>0.667</td>
</tr>
<tr>
<td>Breathlessness*</td>
<td>4 (26.7%)</td>
<td>17 (26.2%)</td>
<td>16 (32.7%)</td>
<td>0.737</td>
</tr>
<tr>
<td>Fevers*</td>
<td>0</td>
<td>4 (6.2%)</td>
<td>8 (16.3%)</td>
<td>0.075</td>
</tr>
<tr>
<td>Night sweats*</td>
<td>2 (13.3%)</td>
<td>8 (12.3%)</td>
<td>12 (24.5%)</td>
<td>0.213</td>
</tr>
<tr>
<td>Chest pain*</td>
<td>4 (26.7%)</td>
<td>13 (20%)</td>
<td>13 (26.5%)</td>
<td>0.678</td>
</tr>
<tr>
<td>New cavitation on serial CXR</td>
<td>1 (6.7%)</td>
<td>3 (4.6%)</td>
<td>2 (4.1%)</td>
<td>0.917</td>
</tr>
<tr>
<td>Enlarged cavitation on serial CXR</td>
<td>0</td>
<td>3 (4.6%)</td>
<td>2 (4.1%)</td>
<td>0.703</td>
</tr>
<tr>
<td>New or progressive paracavitary fibrosis</td>
<td>1 (6.7%)</td>
<td>4 (6.2%)</td>
<td>2 (2.1%)</td>
<td>0.867</td>
</tr>
<tr>
<td>New or progressive pleural thickening on CXR</td>
<td>0</td>
<td>2 (3.1%)</td>
<td>0</td>
<td>0.368</td>
</tr>
<tr>
<td>2012 median Aspergillus IgG level</td>
<td>3.7 mg/L</td>
<td>3.75 mg/L</td>
<td>4.38 mg/L</td>
<td>0.954**</td>
</tr>
<tr>
<td>2012 positive Aspergillus IgG</td>
<td>0</td>
<td>4 (6.2%)</td>
<td>5 (10.2%)</td>
<td>0.372</td>
</tr>
</tbody>
</table>

*present for 1 month or longer. ** medians compared by independent samples median test
### S8 – CT findings by in HIV positive persons by CD4 count groups

<table>
<thead>
<tr>
<th>Result</th>
<th>CD4 count &lt; 200 cells/µL n=2</th>
<th>CD4 count 200 – 499 cells/µL n=11</th>
<th>CD4 count ≥ 500 cells/µL n=11</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavities</td>
<td>1 (50%)</td>
<td>6 (54%)</td>
<td>9 (81.8%)</td>
<td>0.348</td>
</tr>
<tr>
<td>Paracavitary fibrosis</td>
<td>0</td>
<td>4 (36.4%)</td>
<td>9 (81.8%)</td>
<td><strong>0.028</strong></td>
</tr>
<tr>
<td>Aspergilloma</td>
<td>0</td>
<td>1 (9.1%)</td>
<td>4 (36.4%)</td>
<td>0.217</td>
</tr>
<tr>
<td>Pleural thickening</td>
<td>1 (50%)</td>
<td>3 (27.3%)</td>
<td>5 (45.5%)</td>
<td>0.631</td>
</tr>
<tr>
<td>Nodule</td>
<td>0</td>
<td>5 (45.5%)</td>
<td>6 (54.5%)</td>
<td>0.363</td>
</tr>
</tbody>
</table>
PAPER 4 - An estimate of the prevalence of pulmonary aspergillosis in HIV-positive Ugandan in patients diagnosed as smear-negative pulmonary tuberculosis.

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In resource-poor settings pulmonary tuberculosis is often diagnosed on the basis of 'smear-negative' criteria. Microbiological proof of tuberculosis infection is not required. In Ugandan HIV positive in-patients these clinical protocols have negligible diagnostic value. Subacute invasive pulmonary aspergillosis, also known as chronic necrotizing pulmonary aspergillosis (CNPA) also occurs in HIV infected persons and is 100% fatal without treatment. Autopsy studies show that it is present in 2-3% of all AIDS deaths and went undiagnosed ante-mortem in over 90% of these cases.

Diagnosis of CNPA / subacute invasive pulmonary aspergillosis requires one month of cough or haemoptysis, plus radiological evidence of cavitating lung lesions with paracavitary infiltrates and raised Aspergillus-specific IgG. Aspergilloma is not always present. Such patients would likely be classified as 'smear-negative pulmonary tuberculosis' in the absence of specific testing for aspergillosis. We aimed to estimate the likely prevalence of pulmonary aspergillosis in an at-risk African in-patient population.

Stored sera were available from adult patients admitted to Mulago Hospital, Kampala with a cough of 2 weeks to 6 months duration between March 2010 and March 2011. These patients were thoroughly investigated for tuberculosis. We selected 39 sera from HIV infected persons with abnormal chest X-rays with no evidence of tuberculosis or other clear diagnosis after full investigation. We measured Aspergillus-specific IgG in these samples using the Siemens Immulite assay, which has a specificity of 98% and sensitivity of 96% for the diagnosis of chronic pulmonary aspergillosis. 100 control sera had previously been collected from healthy Ugandan blood donors. These were used in receiver operating characteristic curve studies to define the diagnostic threshold of 10 mg/L used in this study.

The mean patient age was 35 years and 59% of patients were female. Mean CD4 count was 109 cells/µL. 44% of patients had CD4 count <50 cells/µL. Raised Aspergillus-specific IgG was present in 2% of healthy controls, but 26% of patients (95% CI 14 – 41%, p 0.000). 40% of those with a positive test died within 2 months of sampling.
While this study does not conclusively prove the existence of subacute invasive aspergillosis in this population, it is likely that the majority of these patients with raised Aspergillus-specific IgG had either chronic or subacute pulmonary aspergillosis. Pulmonary aspergillosis is probably being misdiagnosed as ‘smear-negative tuberculosis’ in many patients with HIV infection. Further prospective studies with CT scanning, plus effective fungal culture and serology should be performed to investigate this possibility.
Introduction

In 2012 4.4 million patients were diagnosed with pulmonary tuberculosis following a sputum smear test. In 1.9 million of these cases the smear test was negative. In the resource-poor settings where tuberculosis and HIV are common, pulmonary tuberculosis is often diagnosed on the basis of World Health Organization (WHO) approved ‘smear-negative’ criteria. Microbiological proof of tuberculosis infection is not required. Currently 54% of Ugandan HIV positive out-patients commencing tuberculosis therapy are smear-negative. Tuberculosis is only confirmed in 35% of these patients when sputum is cultured. In Ugandan HIV positive in-patients these clinical diagnostic protocols have negligible diagnostic value for tuberculosis.

The 2-year mortality of smear-negative TB is 34%. The hazard ratio for mortality in smear-negative tuberculosis against smear-positive tuberculosis is 1.49 for 2-month mortality in HIV positive cases in DR Congo.

The WHO diagnostic criteria for smear negative tuberculosis require all of the following; HIV infection, cough for two weeks or more, two negative sputum acid alcohol fast bacilli (AAFB) smear tests, no response to broad-spectrum antibiotics and radiological changes potentially consistent with tuberculosis.

Fungal lung infections would also meet these criteria. Chronic pulmonary aspergillosis (CPA) presents with cough of at least 3 months duration, plus haemoptysis, weight loss and fatigue. Subacute pulmonary aspergillosis, also known as chronic necrotizing pulmonary aspergillosis (CNPA), has similar symptoms, but is more rapidly progressive, with a duration of illness of only one month.

Cavities, pleural thickening and fibrosis are found on chest X-ray in both tuberculosis and aspergillosis. Aspergilloma are distinctive, but are present in only 36% of cases of CPA. Raised levels of Aspergillus-specific IgG differentiate CPA from similar conditions, but this test is essentially unavailable in Africa.
CPA has a global distribution, with large case series reported in the UK, France, India, China, Korea and Japan\(^7,8,15,18,108,198\). Over 180 cases of CPA have been reported throughout Africa, including South Africa, Nigeria, Ivory Coast, Senegal, Central African Republic, Djibouti, Ethiopia, Tanzania and Uganda\(^16,201–212\). A recent survey in Uganda demonstrated that CPA complicates 6% of all treated pulmonary tuberculosis cases (paper 3). The global burden of CPA secondary to tuberculosis has recently been estimated at around 1.2 million cases\(^11\).

Invasive aspergillosis can complicate AIDS in the absence of tuberculosis. It is associated with corticosteroid therapy and pulmonary infection, including *Pneumocystis jirovecii* or bacterial pneumonia. Drug-induced neutropenia is present in most cases, however 44% of AIDS related cases occur in patients with normal neutrophil counts, but CD4 counts below 100 cells/\(\mu\)L\(^235\). Advanced AIDS is associated with impaired neutrophil function\(^237\), which might result in increased susceptibility to acute aspergillosis. In patients who are not profoundly neutropenic, a subacute course of one to two months duration is typical\(^52,223,224,235\). Survivors who receive effective HIV treatment can develop CPA\(^223\).

Autopsy studies from Italy, India and Uganda have demonstrated that aspergillosis is present in 3-11% of all AIDS related deaths and that only 10% of these cases were diagnosed antemortem\(^207,238–240\). A recent study identified *Aspergillus fumigatus* growth in BAL samples from 6% of patients admitted to hospital in Uganda with subacute respiratory disease, the majority of whom were HIV positive\(^277\).

The mortality of untreated invasive aspergillosis is 100% within weeks\(^19,248,249\). CPA has 5-year mortality rate of 40-85%\(^7,8,264\). If cases of pulmonary aspergillosis occur commonly and are being misdiagnosed as smear-negative tuberculosis then this could be making a substantial contribution to the excess mortality observed in this group.

Effective treatment is available. Most patients survive invasive aspergillosis if treated with voriconazole or amphotericin\(^247\) and oral itraconazole prevents disease progression in CPA\(^18,198\). Surgical resection of individual lesions by lobectomy can be safely delivered in resource-poor settings\(^16,54,212\) and is potentially curative\(^15,21\).
We aimed to estimate the prevalence of subacute invasive pulmonary aspergillosis in HIV positive patients treated as ‘smear-negative tuberculosis’. We performed opportunistic testing of stored sera at Mulago Hospital, Kampala. These were acquired during an earlier study to measure the frequency of different conditions in patients admitted with chronic cough.

Stored sera were selected from adult in-patients who met the diagnostic criteria for smear-negative tuberculosis and who had no evidence of tuberculosis after extensive investigation including smear testing, GeneXpert PCR testing and culture on sputum and/or broncho-alveolar lavage fluid. Samples were tested for *Aspergillus*-specific IgG using the Siemens Immulite assay, which has recently been shown to have a sensitivity of 96% and specificity of 98% for the diagnosis of CPA.

Methods

The Mulago Inpatient Noninvasive Diagnosis – International HIV Opportunistic Pneumonia (MIND-IHOP) study recruited patients between March 2010 and March 2011. During this period all adults admitted to the casualty department of Mulago Hospital, Kampala on weekdays, with a cough of between 2 weeks and 6 months duration were offered admission to the study. Clinical details were recorded and sputum samples taken for acid and alcohol fast bacteria (AAFB) smear testing, culture and GeneXpert automated nucleic acid amplification assay (Cepheid, USA). Induced sputum was acquired if necessary.

CD4 count (in HIV infected patients) and cryptococcal antigen testing (Imuno Mycologics, USA) were performed on blood. Bronchoscopy was offered to any HIV positive patient with persistent symptoms and negative sputum smear test. Broncho-alveolar lavage (BAL) specimens underwent culture and staining for mycobacteria and fungi including *Pneumocystis jirovecii*. Patients were reviewed at two months after recruitment. The mortality rate at this point was recorded.

Stored sera were available from around three quarters of patients originally recruited to the study. Sera were retrospectively selected from patients meeting the following
criteria; 1 - HIV infection, 2 - abnormal chest X-ray, 3 - No diagnosis made after all investigations complete, including no evidence of pulmonary tuberculosis. All patients were treated with a broad-spectrum antibiotic, normally ceftriaxone, on admission. Those who responded were diagnosed as 'likely bacterial pneumonia' and so were not included in the 'no diagnosis' group. 100 control sera had previously been collected from healthy Ugandan blood donors. These were used in receiver operating characteristic curve studies to define the diagnostic threshold of 10 mg/L used in this study (paper 1).

Levels of Aspergillus-specific IgG were measured in each selected sample by Immulite 2000 (Siemens, Germany) assay. Samples with a level greater than 200mg/L underwent a 1 in 10 dilution and were repeated.

Statistical analysis

Median Aspergillus-specific IgG levels and CD4 counts are compared with Mann-Whitney U test. Mean ages are compared by 2-sided t-test. Categorical results are compared by 2-sided Fisher's exact test.

Results

Sera from 39 patients that met the stated criteria were identified. 23 (59%) patients were female and the mean age was 35 years (range 21-54). Mean CD4 count was 109 cells/µL (range 3 -399 cells/µL). 23 (62%) patients had CD4 count < 100 cells/µL and 17 (44%) patients had CD4 count <50 cells/µL. Thirteen (33%) patients underwent bronchoscopy as part of their investigations. Chest X-ray showed infiltrates in 34 (87%) of cases, military appearance in one (3%) case and "likely tuberculosis", with no further details in 4 (10%) cases.

Results of Aspergillus-specific IgG are shown in table 1. 100 control samples from healthy Ugandan blood donors were also tested (paper 1). The frequency of raised Aspergillus-specific IgG in controls was 2% (paper 1). The frequency of raised
Aspergillus-specific IgG levels in patients meeting the study criteria was 26% (95% confidence interval 14% - 41%).

Table 1 – Aspergillus-specific IgG testing in patients and controls

<table>
<thead>
<tr>
<th>Result</th>
<th>Healthy controls n=100</th>
<th>Study patients n=39</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Aspergillus-specific IgG level</td>
<td>4 mg/L</td>
<td>7 mg/L</td>
<td>0.000</td>
</tr>
<tr>
<td>Aspergillus-specific IgG range</td>
<td>0-35 mg/L</td>
<td>2-26 mg/L</td>
<td>-</td>
</tr>
<tr>
<td>Number of positive tests</td>
<td>2 (2%)</td>
<td>10 (26%)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 2 – Characteristics of patients with and without raised Aspergillus-specific IgG

<table>
<thead>
<tr>
<th>Result</th>
<th>Normal Aspergillus-specific IgG n=29</th>
<th>Raised Aspergillus-specific IgG n=10</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>15 (52%)</td>
<td>8 (80%)</td>
<td>0.105</td>
</tr>
<tr>
<td>Mean age</td>
<td>34 years</td>
<td>37 years</td>
<td>0.052</td>
</tr>
<tr>
<td>Median CD4 count</td>
<td>59 cells/µL</td>
<td>59 cells/µL</td>
<td>-</td>
</tr>
<tr>
<td>CD4 &lt;100 cells/µL</td>
<td>16 (55%)</td>
<td>7 (70%)</td>
<td>0.48</td>
</tr>
<tr>
<td>2 month mortality</td>
<td>8 (27%)</td>
<td>4 (40%)</td>
<td>0.463</td>
</tr>
</tbody>
</table>
Discussion

These results suggest that subacute invasive or chronic pulmonary aspergillosis are important differential diagnoses in Ugandan in-patients with AIDS who are currently diagnosed and managed as ‘smear-negative tuberculosis’. As the mortality rate of pulmonary aspergillosis is very high this is likely contribute the excess mortality observed in this population.

We are unable to differentiate acute, subacute invasive and chronic pulmonary aspergillosis in patients diagnosed with ‘smear-negative tuberculosis’ due to differences in case definition. CPA requires chronic cough, defined as three months or more\textsuperscript{5,250,251}, whereas subacute invasive pulmonary aspergillosis requires one month of cough\textsuperscript{6,264}. Our cohort includes patients with cough for two weeks or more. This would capture both conditions and acute invasive disease.

Progressive cavitation, paracavitary fibrosis or aspergilloma are also required for the diagnosis of pulmonary aspergillosis. Radiological information was only available from chest X-ray, which is unreliable for the diagnosis of pulmonary tuberculosis, especially when patients are co-infected with HIV\textsuperscript{265}. The findings in acute pulmonary aspergillosis are often non-specific\textsuperscript{218,280}. We excluded patients with normal chest X-rays, but accepted those with any chest X-ray abnormality. While any chest X-ray abnormality is potentially consistent with pulmonary aspergillosis a CT scan would have been required to state that radiological features of pulmonary aspergillosis were definitely present.

Raised levels of \textit{Aspergillus}-specific IgG are consistent with pulmonary aspergillosis, but can also occur in colonization\textsuperscript{38}, \textit{Aspergillus} bronchitis\textsuperscript{39} or tracheobronchitis\textsuperscript{43}. Active pulmonary aspergillosis is, however the most likely of these options in our patients as they all had persistent respiratory symptoms and abnormal chest X-ray.

While fungal culture on BAL samples did not identify any cases of aspergillosis in this group, the sensitivity of culture for \textit{Aspergillus} is very low with standard techniques\textsuperscript{260} and so the absence of culture growth does not exclude pulmonary aspergillosis. Testing
BAL for galactomannan might also have provided confirmation of pulmonary aspergillosis, but no stored samples were available.

We measured antibodies to Aspergillus fumigatus, which is responsible for the vast majority of CPA in Europe and East Asia. However, most aspergillosis in India and the Middle East is due to A. flavus and A. niger is common in Brazil. The dominant species of Aspergillus in Africa is not known. A. fumigatus based assays can have poor sensitivity for CPA due to other Aspergillus species, potentially resulting in false negative results. Profound immunosuppression dampens antibody responses in AIDS and the performance of Aspergillus-specific IgG in a group with low CD4 counts is not well-described. These factors may lead to false negative results.

The Aspergillus-specific IgG assay can cross-react with Penicillium-specific antibodies. Little is known about its cross-reactivity with other fungal infections. Histoplasmosis is present in Uganda and blastomycosis elsewhere in Africa. These fungal infections are among those known to complicate HIV/AIDS. False positives might occur if other fungal infections are present in our patients and they cross-reacted with the Siemens Immulite assay.

This study was conducted in an opportunistic manner, using stored sera from a prior study. Not all sera had sufficient volume to allow Aspergillus serology. The sickest patients may have been excluded from our study as obtaining large volumes of blood can be difficult in these cases due to shock. This might lead to an underestimate of the prevalence of a rapidly fatal condition such as pulmonary aspergillosis.

To our knowledge this is the first attempt to estimate the prevalence of pulmonary aspergillosis in an African cohort with AIDS and ‘smear-negative tuberculosis’. While our study design does not include all the standard diagnostic tests for pulmonary aspergillosis in highly immunocompromised patients, the Siemens Immulite assay used has a specificity of 98% for the diagnosis of CPA (paper 1). It is therefore likely that pulmonary aspergillosis, or other chronic fungal lung disease, is present in many of the patients in this cohort.
Given the combination of high mortality and good response to treatment in these conditions, there is now an urgent need to perform thorough prospective studies in this population including CT scan, effective fungal culture with optimal techniques, extensive fungal serology and ideally biopsy to definitively measure the prevalence of fungal lung disease in Africans with AIDS and subacute respiratory infection.

**Hypothesis**

That a proportion of patients who presented to hospital with AIDS and sub-acute respiratory disease in an area of high tuberculosis prevalence, but who had no evidence of tuberculosis after thorough investigation were suffering from undiagnosed primary sub-acute invasive pulmonary aspergillosis.

**Aims**

1 – To measure the levels of *Aspergillus*-specific IgG in stored sera from HIV infected patients admitted to hospital with sub-acute respiratory disease in an area of high tuberculosis prevalence, but who had no evidence of tuberculosis after thorough investigation.

2 – To compare these levels of *Aspergillus*-specific IgG to those found in healthy controls from the same country.

3 – To compare two-month mortality outcomes in HIV infected patients with sub-acute respiratory disease in an area of high tuberculosis prevalence with no evidence of tuberculosis after thorough investigation, who had either raised or normal levels of *Aspergillus*-specific IgG.

**Ethics**

Ethical permission for this study was granted by the University of Manchester, UK (ref 11424), Makerere University, Kampala, Uganda (ref 2006-017) and the Ugandan National Council for Science and Technology (ref – HS259).
Funding

Funding to transport samples to the UK for analysis was provided by the Manchester Academy academic charity. Test kits for use in this study were kindly donated by Siemens.

Acknowledgements

We would like to thank all those involved in the MIND-IHOP study group for their kind decision to share serum samples for use in this collaborative study. Thanks to the North West Lung Centre, University Hospital of South Manchester for storage of samples. Thanks to the pathology laboratory staff at Christie Hospital, Manchester, UK for allowing the study group access to their Siemens Immulite 2000.
PAPER 5 - *Aspergillus* co-infection may be common in Africans with active pulmonary tuberculosis

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CPA is estimated to affect 3 million people globally\textsuperscript{11-13}. A recent survey demonstrated that chronic pulmonary aspergillosis (CPA) is present in 6.5\% of Ugandan adults with previously treated pulmonary tuberculosis and raised \textit{Aspergillus}-specific IgG present in 10\% (papers 2 and 3). These cases occurred in patients who no longer had active tuberculosis infection. However, active co-infection with atypical mycobacteria frequently occurs in CPA and co-infection with active \textit{Mycobacterium tuberculosis} and \textit{Aspergillus} has been described in several case reports. We aimed to estimate the prevalence of this problem in an area of high tuberculosis prevalence.

Stored sera were available from 57 adult patients admitted to Mulago Hospital, Kampala between March 2010 and March 2011. All patients had between 2 weeks and 6 months cough and were diagnosed with pulmonary tuberculosis based on culture or GeneXpert PCR testing of sputum and/or broncho-alveolar fluid. We measured \textit{Aspergillus}-specific IgG in these samples using the Siemens Immulite assay, which has a specificity of 98\% and sensitivity of 96\% for the diagnosis of chronic pulmonary aspergillosis.

46 (81\%) patients were HIV positive. Mean CD4 count in those with HIV was 99 cells/\(\mu L\) (range 2 - 581 cells/\(\mu L\)). 35 (61\%) patients had CD4 count < 100 cells/\(\mu L\) and 24 (42\%) patients had CD4 count <50 cells/\(\mu L\).

\textit{Aspergillus}-specific IgG levels were raised in 2 (2\%) of controls and 27 (47\%) tuberculosis patients. 3 (11\%) of those with raised \textit{Aspergillus}-specific IgG died within 2 months of sampling.

This is a select group of patients requiring emergency hospital admission and may not be representative of all patients with pulmonary tuberculosis. False positive IgG results might occur due to cross-reaction with other fungi and false negative IgG tests might occur in patients with CPA caused \textit{Aspergillus} species other than \textit{A. fumigatus}.

However, given the diagnostic accuracy of the Siemens Immulite assay it is likely that active \textit{Aspergillus} co-infection is present in many of those with positive results. This possibility should be considered in patients who fail to improve or clinically relapse in
spite of appropriate tuberculosis therapy. Prospective studies are needed to record the outcome of patients with pulmonary tuberculosis and raised *Aspergillus*-specific IgG and define the prevalence of pulmonary aspergillosis in this group.
An estimated 9 million people developed tuberculosis in 2013. It was associated with 1.5 million deaths, of which only 210,000 were estimated to be due to multidrug resistant strains. Many of the other 1.29 million deaths will have been due to late presentation, lack of diagnosis, poor access to treatment or inadequate compliance, given that they mostly occurred in resource-poor countries with weak health infrastructure. However, other factors may also be have been present.

Chronic pulmonary aspergillosis (CPA) is an important sequel of pulmonary tuberculosis. It presents with progressive pulmonary cavitation associated with weight loss, persistent cough and haemoptysis. It has a 5-year mortality of 50–80% and has recently been estimated to affect around 3 million people globally, including 1.2 million cases secondary to tuberculosis. A recent survey confirmed that CPA is present in 6% of Ugandan adults with previously treated pulmonary tuberculosis (paper 3).

CPA is treatable. Oral itraconazole, voriconazole or posaconazole all prevent clinical and radiological progression. Surgery is curative in selected patients with localized disease and has been safely undertaken in resource-poor settings.

While it is now clear that CPA frequently follows tuberculosis, the natural history of CPA is not well established. Published CPA cohort studies are all from countries where tuberculosis is now uncommon. Atypical mycobacteria, however, commonly co-infect persons with CPA, in addition to their role as an antecedent condition. Evidence from countries with high tuberculosis prevalence is limited to case reports, but co-
infection with active pulmonary tuberculosis and pulmonary aspergillosis has been
documented in India, Tunisia and Egypt. A recent study identified
Aspergillus fumigatus growth in BAL samples from 6% of patients admitted to Mulago
Hospital, Kampala with suspected tuberculosis. This may well be an underestimate
as standard culture techniques have very poor sensitivity for Aspergillus.

Co-infection with Aspergillus at the time of active pulmonary tuberculosis might also
result in subacute invasive aspergillosis. This condition occurs in patients with mild to
moderate immunosuppression and has been noted in a wide range of conditions
including HIV infection, diabetes, alcohol abuse and COPD. It presents with
progressive pulmonary cavitation associated with weight loss, persistent cough and
haemoptysis and is associated with 50% mortality within a few months.

Infection with Mycobacterium tuberculosis results in impaired immunity and decreased
macrophage function. This might well place a patient at risk of subacute invasive
pulmonary aspergillosis. As the clinical and radiological presentation of this condition is
essentially identical to pulmonary tuberculosis itself it would be very difficult to detect
it without performing specific Aspergillus serological testing.

We aimed to estimate the frequency of Aspergillus co-infection in patients recently
diagnosed with active pulmonary tuberculosis. We performed opportunistic testing of
stored sera at Mulago Hospital, Kampala. These were acquired during an earlier study
to measure the frequency of different conditions in patients admitted with cough. Mycobacterium tuberculosis infection was proven in all cases on the basis of smear
testing, GeneXpert nucleic amplification or culture. Samples were tested for Aspergillus-specific-IgG using the Siemens Immulite assay, which has recently been shown to have a
sensitivity of 96% and specificity of 98% for the diagnosis of CPA (paper 1).

Methods

The Mulago Inpatient Noninvasive Diagnosis – International HIV Opportunistic
Pneumonia (MIND-IHOP) Study recruited patients relevant to this study between March
2010 and March 2011. During this period all adults admitted to the casualty department
of Mulago Hospital, Kampala on weekdays, with a cough of between 2 weeks and 6 months duration were offered admission to the study. All patients submitted sputum, on which smear testing for acid alcohol fast bacilli, nucleic acid amplification (GeneXpert, Cepheid, USA) and culture for *Mycobacterium tuberculosis* were performed. Bronchoscopy was also performed in selected patients.

Stored sera were available from around three quarters of patients originally recruited to the study. Sera were retrospectively selected from patients with proven pulmonary tuberculosis following the above investigations. 100 control sera had previously been collected from healthy Ugandan blood donors. These were used in receiver operating characteristic curve analysis to define the diagnostic threshold of 10 mg/L used in this study (paper 1).

Levels of *Aspergillus*-specific IgG were measured in each selected sample (Siemens Immulite 2000, Germany). Samples with a level greater than 200mg/L underwent a 1 in 10 dilution and were repeated.

Statistical analysis

Median *Aspergillus*-specific IgG levels in patients and controls and CD4 counts in those with and without raised *Aspergillus*-specific IgG levels are compared with Mann-Whitney U test. Mean age in those with and without raised *Aspergillus*-specific IgG levels is compared by 2-sided t-test. Categorical variables are compared with Chi-squared test, except for comparison of number of positive *Aspergillus*-specific IgG tests in tuberculosis cases vs. healthy controls, where Fisher’s exact test is used.

Results

Fifty-seven sera that met the stated criteria were identified. 29 (51%) patients were female. Mean age was 35 years (range 18 – 79 years). 46 (81%) patients were HIV positive. Mean CD4 count in those with HIV was 99 cells/µL (range 2 -581 cells/µL). 35 (61%) patients had CD4 count < 100 cells/µL and 24 (42%) patients had CD4 count <50
cells/µL. All patients had a chest X-ray that was reported as abnormal and potentially consistent with pulmonary tuberculosis.

100 control samples were acquired from healthy blood donors in Gulu, Uganda. The frequency of raised *Aspergillus*-specific IgG in controls was 2% (paper 1). The frequency of raised *Aspergillus*-specific IgG levels in patients with proven pulmonary tuberculosis was 47% (95% confidence interval 35% - 60%).

Table 1 – *Aspergillus*-specific IgG testing in patients and controls

<table>
<thead>
<tr>
<th>Result</th>
<th>Healthy controls n=100</th>
<th>Pulmonary tuberculosis n=57</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean <em>Aspergillus</em>-specific IgG level</td>
<td>5 mg/L</td>
<td>11 mg/L</td>
<td>0.000</td>
</tr>
<tr>
<td><em>Aspergillus</em>-specific IgG range</td>
<td>0-35 mg/L</td>
<td>4 - 36 mg/L</td>
<td>-</td>
</tr>
<tr>
<td>Number of positive tests</td>
<td>2 (2%)</td>
<td>27 (47%)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 2 – Characteristics of patients with and without raised *Aspergillus*-specific IgG

<table>
<thead>
<tr>
<th>Result</th>
<th>Normal <em>Aspergillus</em>-specific IgG n=30</th>
<th>Raised <em>Aspergillus</em>-specific IgG n=27</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>12 (40%)</td>
<td>17 (63%)</td>
<td>0.08</td>
</tr>
<tr>
<td>Mean age</td>
<td>38 years</td>
<td>38 years</td>
<td>-</td>
</tr>
<tr>
<td>HIV positive</td>
<td>24 (80%)</td>
<td>22 (81%)</td>
<td>0.887</td>
</tr>
<tr>
<td>Median CD4 count in those with HIV</td>
<td>49 cells/µL</td>
<td>46 cells/µL</td>
<td>0.560</td>
</tr>
<tr>
<td>CD4 &lt;100 cells/µL in those with HIV</td>
<td>17 (71%)</td>
<td>18 (82%)</td>
<td>0.761</td>
</tr>
<tr>
<td>2 month mortality</td>
<td>5 (17%)</td>
<td>3 (11%)</td>
<td>0.547</td>
</tr>
</tbody>
</table>
Discussion

Overall, 27 (47%) of patients had raised levels of *Aspergillus*-specific IgG during admission for microbiologically confirmed pulmonary tuberculosis. These results add to the growing body of evidence that pulmonary aspergillosis is a common complication of pulmonary tuberculosis. They inference is that CPA may well begin when active tuberculosis infection is still present.

We cannot, however state that all patients with raised *Aspergillus*-specific IgG definitely have CPA. The diagnosis of CPA also requires all of the following in addition to raised *Aspergillus*-specific IgG; 1 – productive cough or haemoptysis of at least 3 months duration, 2 – radiological findings of either progressive cavitation, paracavitary fibrosis or aspergilloma, 3 - exclusion of conditions with a similar presentation.

The MIND-IHOP study allowed recruitment of patients with only 2 weeks of cough. The patients in this group also clearly have another condition confirmed, rather than excluded. If their symptoms resolve entirely with tuberculosis treatment then they could not reasonably be considered cases of CPA. However the possibility of sub-clinical CPA, that might cause symptoms months or years later, cannot be excluded without prolonged follow up.

Given that CPA is only found in 6% of patients who have completed treatment for pulmonary tuberculosis, it is possible that many of those with raised *Aspergillus*-specific IgG at the time of active pulmonary tuberculosis are simply simply colonized with *Aspergillus* and that this colonization frequently resolves after the tuberculosis is treated, without developing CPA.

Alternatively it may be that co-infection with both HIV and *Aspergillus* results in a worse clinical course in pulmonary tuberculosis, perhaps through the development of invasive pulmonary aspergillosis. If this were the case then higher rates of hospitalization would be seen in those with active *Aspergillus* co-infection than would be seen in the tuberculosis population as a whole. This would also explain the unexpectedly high rate of raised *Aspergillus*-specific IgG seen in this in patient population.
Raised levels of *Aspergillus*-specific IgG are consistent with CPA, but can also occur in colonization \(^{38}\), *Aspergillus* bronchitis \(^{39}\) or tracheobronchitis \(^{43}\). The radiological features of CPA are normally confirmed on CT scan, which was not included in this study. While every patient in this study had an abnormal chest X-ray, the only radiological finding that differentiates CPA from tuberculosis is aspergilloma, which is absent in the majority of cases of CPA \(^{8}\) and harder to detect with chest X-ray than CT scan (paper 3). We cannot therefore differentiate CPA from these other conditions in patients with active tuberculosis and no aspergilloma, as it is unclear whether *M. tuberculosis* or *Aspergillus* is primarily responsible for the abnormal radiological findings. Repeat imaging after tuberculosis treatment is complete would be required to identify definite CPA cases.

We measured antibodies to *Aspergillus fumigatus*, which is responsible for the vast majority of CPA in Europe and East Asia \(^{5-8,108}\). However most aspergillosis in India and the Middle East is due to *A. flavus* \(^{10}\) and *A. niger* is common in Brazil \(^{147}\). The dominant species of *Aspergillus* in Africa is not known. *A. fumigatus* based assays can have poor sensitivity for other species \(^{147,148}\), potentially resulting in false negative results. Antibody responses are also generally poor in AIDS \(^{273,274}\), which affected a large number of patients in our cohort, although we noted in an earlier study that levels of *Aspergillus*-specific IgG are often raised in patients with AIDS and sub-acute respiratory disease (paper 4).

The *Aspergillus*-specific IgG assay can cross-react with *Penicillium* antibodies \(^{269}\). Little is known about its cross-reactivity with other fungal infections. Histoplasmosis is present in Uganda \(^{268}\) and blastomycosis elsewhere in Africa \(^{232}\). All these fungal infections are among those known to complicate HIV/AIDS \(^{292,298}\). False positives might occur if other fungal infections are present in our patients and they cross-reacted with the Siemens Immulite assay.

This study was performed in a population diagnosed with pulmonary tuberculosis during acute admission to hospital. The rate of HIV co-infection in this group is higher than the overall frequency of HIV co-infection seen in Ugandan tuberculosis patients \(^{215}\). Pulmonary aspergillosis might well be common in this study population, which has
severe disease and unusually severe immunosuppression. The study group is therefore not representative of all newly diagnosed pulmonary tuberculosis.

Our study does not therefore definitively measure the prevalence of CPA in patients with active pulmonary tuberculosis. However the Siemens Immulite assay has good sensitivity and specificity for the diagnosis of CPA (paper 1). It is therefore likely that many of the patients identified here are suffering from some form of pulmonary aspergillosis, or other fungal lung disease. This possibility should be actively considered in any patient with pulmonary tuberculosis who is failing to respond to appropriate therapy or who has symptomatic relapse after initial response to tuberculosis therapy.

Prospective studies including CT thorax, fungal serology and fungal culture using sensitive high volume techniques are now needed to confirm the frequency of *Aspergillus* co-infection in pulmonary tuberculosis. Follow up is required to identify if and when these patients develop CPA and the optimal treatment strategy for them. Given the recently confirmed high prevalence of CPA complicating tuberculosis (paper 3) and the high mortality rate of pulmonary aspergillosis these studies should be performed urgently.

Hypothesis

That chronic pulmonary aspergillosis (CPA) begins to develop during active infection with pulmonary tuberculosis.

Aims

1 – To measure the levels of *Aspergillus*-specific IgG in stored sera from HIV infected patients admitted to hospital with proven active pulmonary tuberculosis.

2 – To compare these levels of *Aspergillus*-specific IgG to those found in healthy controls from the same country.
3 – To compare two-month mortality outcomes in HIV infected patients admitted to hospital with active pulmonary tuberculosis with either raised or normal levels of *Aspergillus*-specific IgG.

Ethics

Ethical permission for this study was granted by the University of Manchester, UK (ref 11424), Makerere University, Kampala, Uganda (ref 2006-017) and the Ugandan National Council for Science and Technology (ref – HS259).

Funding

Funding to transport samples to the UK for analysis was provided by the Manchester Academy academic charity. Test kits for use in this study were kindly donated by Siemens.

Acknowledgements

We would like to thank all those involved in the MIND study group for their kind decision to share serum samples for use in this collaborative study. Thanks to the North West Lung Centre, University Hospital of South Manchester for storage of samples. Thanks to the pathology laboratory staff at Christie Hospital, Manchester, UK for allowing the study group access to their Siemens Immulite 2000.
SUMMARY

The study of pulmonary aspergillosis in persons without gross immunosuppression has been neglected. Although the existence of pulmonary aspergillosis in non-immunosuppressed persons has been documented for over 200 years, the clinical syndrome of chronic pulmonary aspergillosis was only properly defined 12 years ago. Since then significant studies have been published that describe cohorts of patients with this condition in several countries in Europe and Asia. The central importance of Aspergillus-specific IgG measurement to the diagnosis of CPA has been established in these studies. The link between CPA and many underlying conditions has been established and the dominance of tuberculosis as the most common underlying cause of CPA on a global scale is now clear.

Recent cohort studies have demonstrated that CPA is associated with a high mortality rate over the course of a few years. Fortunately the response of CPA to treatment with itraconazole has been also established in a randomized controlled trial and the efficacy and safety of surgical treatment (in suitable cases) has been demonstrated in large cohort descriptions. The potential for intervention to prolong the lives of the estimated 3 million persons living with CPA therefore exists.

Unfortunately, it is likely that the majority of persons with CPA are currently going undiagnosed and untreated. The major barriers to progress in this area are lack of confirmation of the predicted prevalence of CPA in areas with currently high tuberculosis prevalence and lack of validation of tests for the diagnosis of CPA.

This work provides a substantial contribution to the field by answering three major questions in relation to chronic pulmonary aspergillosis.

First the optimal diagnostic cut-offs for CPA have been defined for the first time for five of the available commercial Aspergillus-specific IgG ELISAs, including the assay most commonly used in the UK. While diagnostic cut-offs were provided by most
manufacturers these were defined in relation to tiny numbers of patients with CPA, often pooled with patients with invasive or allergic aspergillosis. Recent studies had shown the cut-offs in common use were sub-optimal for the diagnosis of ABPA in cystic fibrosis patients. There was no certainty that they were appropriate for CPA. Lack of clearly validated CPA diagnostic cut-offs for *Aspergillus*-specific IgG was a major barrier to any attempts to improve access to CPA diagnosis. Defining these cut-offs was also a pre-requisite for any measurement of the prevalence of CPA.

By accessing stored sera from the world’s largest CPA cohort it was possible to identify a suitably large number of sera to perform a meaningful analysis. Crucially these sera were taken from patients not on antifungal treatment. Such sera are representative of patients being diagnosed with CPA for the first time. As antifungal treatment lowers *Aspergillus*-specific IgG levels any cut-off defined in relation to CPA patients on treatment may not be applicable to those being tested for initial diagnosis. This was probably a major methodological flaw in the limited number of prior studies in this area. This study identified optimal diagnostic thresholds by performing ROC analysis of results obtained from this unique cohort of untreated CPA patients and healthy controls. In the case of several assays, including ThermoFisher Scientific ImmunoCAP, the assay currently in use in most of the UK, the existing cut-offs were shown to be too high. By lowering the cut-offs to optimal levels it is possible to markedly increase sensitivity, while maintaining high specificity. These results will change practice at the UK National Aspergillosis Centre and are highly likely to inform changes to guidelines. This will allow those units with access to testing to correctly identify around 10% more cases than was previously possible.

The second major contribution of this work to the field is to define the sensitivity and specificity of the five *Aspergillus*-specific IgG ELISAs, plus precipitins, for the diagnosis of CPA. The large cohort descriptions for CPA all suggested that *Aspergillus*-specific IgG has excellent sensitivity for CPA, but these studies all used a single *Aspergillus* antibody
assay as their main serological test for aspergillosis. While a few small studies have recently compared the sensitivity and specificity of different tests, these have not directly compared the ELISAs included in this study in patients with CPA. These studies were also potentially flawed on account of sera being taken from patients who were on antifungal therapy. Defining the sensitivity and specificity of these assays was required before any assay could be selected for use in a CPA prevalence study.

The performance of each ELISA was described in terms of ROC area under the curve. A sufficiently large number of cases were assessed to allow the detection of statistically significant differences in the diagnostic performance of the various assays. No previous study had achieved this. It was confirmed that the assay currently in regular use in the UK (ThermoFisher Scientific ImmunoCAP) does indeed have good sensitivity and specificity for the diagnosis of CPA.

While there was some unavoidable bias in our cohort, due to the fact that Aspergillus-specific IgG serology, including the ThermoFisher Scientific ImmunoCAP assay forms an integral part of the diagnostic process for patients at our unit, this study is still the definitive work in this field. The Siemens Immulite assay was shown to have equivalent sensitivity and specificity in spite of this potential bias in favour of ThermoFisher Scientific ImmunoCAP. Other assays performed less well. It was then possible to use the donated Siemens Immulite assay with confidence in a survey to measure the prevalence of CPA in an area of high tuberculosis prevalence.

The optimal diagnostic cut offs and comparative sensitivity and specificity of the assays for the diagnosis of ABPA was also defined. This was performed in relation to both healthy controls and to asthmatics. The appropriate cut offs for Aspergillus-specific IgG for the diagnosis of ABPA in patients with cystic fibrosis and the appropriate cut-offs for both total and Aspergillus-specific IgE for the diagnosis of ABPA in general have both been assessed in recent studies. This is, however the first study to define optimal cut-offs for Aspergillus-specific IgG in relation to the diagnosis of ABPA. It is also the first comparison of the sensitivity and specificity of the five Aspergillus-specific IgG serology assays for this purpose. The analysis defining the appropriate cut-offs for Aspergillus-specific IgG to diagnose CPA complicating ABPA is unique.
The third and perhaps most important contribution to the field is the first measure of the prevalence of CPA in an area of high tuberculosis prevalence. The cross-sectional study was a major undertaking that required two surveys two years apart and the transportation of patients for 700 km for CT scan. The author spent a total of 14 months in Uganda undertaking the study.

The study demonstrated the presence of CPA in 6% of all patients with previously treated pulmonary tuberculosis. A cross-sectional study with convenience sampling was the only realistic option in light of the financial and time constraints in place. A degree of selection bias may exist with this method. However, the study does provide the first clear evidence that CPA is a sufficiently common problem in an area of high tuberculosis prevalence to be considered a public health issue and provides the first validation of the predicted global prevalence of 3 million cases.

Evidence from opportunistic testing of stored samples provided by collaborators at Mulago Hospital, Kampala is also presented. It suggests that *Aspergillus* infection is probably present in many patients with current active pulmonary tuberculosis and that subacute invasive pulmonary aspergillosis is probably the correct diagnosis in a significant proportion of HIV positive patients currently labeled as 'smear-negative tuberculosis'.

These results raise major questions about the appropriateness of the diagnostic and management protocols currently in place for pulmonary tuberculosis in resource poor settings. They suggest that further studies to accurately define the prevalence of fungal lung diseases in patients presenting with suspected tuberculosis are now urgently required. The frequency of raised *Aspergillus*-specific IgG found in each Ugandan patient group is shown in table 1 below.
Table 1 – Frequency of raised *Aspergillus* disease in Ugandan patient groups

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Frequency of raised <em>Aspergillus</em>-specific IgG</th>
<th>Prevalence of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ugandan healthy controls n = 100</td>
<td>2%</td>
<td>Not measured</td>
</tr>
<tr>
<td>Ugandans with previously treated pulmonary tuberculosis n = 282</td>
<td>10%</td>
<td>6.5%</td>
</tr>
<tr>
<td>Ugandans admitted to hospital with HIV and sub-acute lung disease, but no evidence of tuberculosis n = 39</td>
<td>26%</td>
<td>Not measured</td>
</tr>
<tr>
<td>Ugandans admitted to hospital with proven active pulmonary tuberculosis n = 57</td>
<td>47%</td>
<td>Not measured</td>
</tr>
</tbody>
</table>

The results from this thesis have been presented to policy leaders at large global health institutions. They suggest that CPA is an important neglected disease in global health terms, due to the number of persons likely to be affected and the high morbidity and mortality associated with the illness. The author is part of a team planning of a new, larger, prospective multi-centre study to confirm the prevalence of CPA in Kenya. Further studies will be needed to confirm the prevalence of CPA in other countries around the world. If these confirm the prevalence of CPA described here it will be necessary to amend Global policies relating to the investigation and treatment of tuberculosis to include diagnosis and treatment of CPA. This process could ultimately result in large-scale roll out of testing and treatment of CPA and potentially extend millions of lives.
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### Appendix 1 - *Aspergillus* IgG ELISA comparison sheet

<table>
<thead>
<tr>
<th></th>
<th>DYNAMIKER</th>
<th>GENESIS</th>
<th>SERION</th>
</tr>
</thead>
</table>
| **DILUTION**         | Add 1 µL sample to 1ml diluent | Add 5 µL sample to 1ml diluent | 2 STEP  
1st add 10 µL sample to 1ml diluent  
THEN  
add 50 µL from above to 200 µL diluent |
| **STANDARD SERA**    | FIVE               | FIVE PLUS POSITIVE CONTROL | TWO PLUS NEGATIVE CONTROL |
| **VOLUME OF SERA / STANDARDS TO ADD** | 100 µL | 100 µL | 100 µL |
| **INCUBATION LOCATION (for all steps)** | 37°C incubator with seal on plate | Bench at room temperature | 37°C incubator in moist chamber |
| **1ST INCUBATION DURATION** | 60 mins | 30 mins | 60 mins |
| **FIRST WASH STEP**  | 3 washes           | 3 washes             | 4 washes             |
| **VOLUME OF CONJUGATE** | 100 µL | 100 µL | 100 µL |
| **2ND INCUBATION DURATION (with conjugate)** | 30 mins | 30 mins | 30 mins |
| **SECOND WASH STEP** | 3 washes           | 4 washes             | 4 washes             |
| **VOLUME OF SUBSTRATE** | 100 µL | 100 µL | 100 µL |
| **3rd INCUBATION DURATION** | 15 mins | 10 mins | 30 mins |
| **VOLUME OF STOPPING SOLUTION** | 50 µL | 100 µL | 100 µL |
| **QUALITY CONTROL CRITERIA** | SB od <0.1  
Sa od 0.1-0.5  
Se od 1.6-2.0 | PC 32-60 | STANDARD od 0.42 – 1.43 |
| **INDICATION FOR DILUTION AND RETESTING** | Result > Se | Result > 100 U/ml | Software reports as HIGH or >1000 units |
| **HIGH IAV RANGE**   | 132 UNITS OR 11%  | 41 UNITS or 12%     | 62 UNITS or 23%     |
Appendix 2 – Patient Information Sheet for Paper 2 - Prevalence of chronic pulmonary aspergillosis secondary to tuberculosis: a cross-sectional survey in an area of high tuberculosis prevalence.

ASPERGILLOSIS STUDY

PATIENT INFORMATION SHEET

We would like to invite you to join our study. You have been selected because you are being treated for TB, or because you have been treated for TB in the past. Research in England has shown that patients who have been treated for TB sometimes develop a second illness called Chronic Pulmonary Aspergillosis or CPA.

This illness is caused by a fungus growing in the lungs. This fungus is very similar to mould on bread. It can be breathed into the lungs as an invisible dust. If you are healthy this is normally harmless, but if your lungs have been damaged by TB or other illnesses then it can grow in your lungs and make you ill. This illness can make you very tired or very short of breath. It can kill you by causing bleeding inside your lungs. There is treatment available for this illness. Some people can have an operation to cure them. Others can be made less ill by taking a drug called Itraconazole. This drug can be bought in pharmacies in Gulu.

In England this illness was found in around 1 person in 20 after they had TB. No one has ever tested to see how many African patients get the illness after TB treatment. The number might be bigger or smaller than it is in England. Also, no one has ever tested for this illness in patients who have had both TB and HIV. It might be that people with HIV are more likely to get this illness because their immune systems are weaker.

We hope this study will tell us how many patients get this illness in Gulu. We will tell other doctors the results of our study. If we find that this disease is a big problem we hope that this study will convince doctors and government ministers to find all the people with the illness and give them treatment. This could help many people all over the world! We also hope to develop a better blood test for this illness, which will be cheap and can be used at any African clinic – even ones that don’t have electricity.

It is your choice whether you want to join this study or not. If you choose to enter the study we will listen to your chest for signs of the illness and ask you some questions about your health and where you live and work. This will help us work out if some people are more likely to get this illness than others.

We will take some blood. This will be used to test for the illness. We will take some blood back to Manchester University in England. We will use it to help make the new test for Africa. We will also test it for other types of fungal disease. This will help us decide what diseases to look for in our next study. Lastly we will use the blood to find genetic markers of the illness. This will not give us an immediately useful result, but in many years we hope it will let us design a much better test for people who are at risk of this sickness. If you are coughing we will take a sample of your cough sputum and test it for fungus infection. After this you will go to Lacor hospital by car where you will have a chest X ray to see if there is any sign of disease in your lungs. You will then come back to Gulu by car. We expect the whole process to take a few hours. You will be provided with some cold drink for refreshment.

We will get results from the tests in a few months. We will pass this result to your doctor if you are coming back to clinic. If you do not come to clinic we will give the result to the District Health
Officer. He will phone your village health worker if your test is positive and ask you should come to the Infectious Diseases Clinic at Gulu Hospital. They will advise you if you need treatment. If you do you can buy it from the pharmacy in town. You are welcome to ask any questions you like before you decide to join the study. If you change your mind and decide not to be in the study later on we can remove your details from the study and this will not have any impact on your health care now or in the future.

If you wish to complain about the conduct of the study please contact me in the first instance and if you are still unhappy you can contact my supervisor Professor Denning (david.denning@manchester.ac.uk) or the Research Governance Office at the University of Manchester (research-governance@manchester.ac.uk).

Finally we would like to contact you again to take part in further studies. This is optional and you can still take part in this study if you do not want to take part in the other studies.

This project was reviewed by the University of Manchester Research Ethics Committee 1.
Appendix 3 – Patient Consent form for Paper 2- Prevalence of chronic pulmonary aspergillosis secondary to tuberculosis: a cross-sectional survey in an area of high tuberculosis prevalence.

ASPERGILLOSIS STUDY
CONSENT FORM

If you agree to enter the Aspergillosis study please sign the consent form below. In doing so you agree to the following:

I have read the patient information sheet, have had the opportunity to consider it’s contents and ask questions and had these answered satisfactorily.

I will undergo a medical examination. The doctor will record the results of this and my answers to his questions.

I will give blood to be used in the study. I understand these samples will be taken to Manchester University in England and that they will be used for research including developing a new test and identifying genetic risk factors for Aspergillosis.

I agree to my blood being tested for Aspergillosis and other fungal lung diseases. If I am HIV positive I understand my CD4 count may be re-checked as part of this study.

I agree that the results of the Aspergillosis study will be sent to my doctor at Gulu Hospital or to the District Health and that they may contact me with the result.

I agree to travel to Lacor Hospital and have a chest X-ray when I arrive there.

I understand I may be contacted by study staff in future if I am suitable to enter further studies.

I understand that I may at any time withdraw my approval for tissue and information to be stored without giving any reason and without it affecting my treatment. If I do this my tissue samples will be destroyed and my information will not be used for future research.

……………………………………..X
Name of participant Date Signature

I have explained the request for research purposes and answered such questions as the patient has asked. I am satisfied that the donor signing this form understands the content and the purpose and nature of this consent process

……………………………………..X
Name of person Date Signature

taking consent

Study number -
Appendix 4 – Medical Research Council Dyspnoea Scale (MRC Scale)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Degree of breathlessness related to activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Not troubled by breathlessness except on strenuous exercise</td>
</tr>
<tr>
<td>2</td>
<td>Short of breath when hurry on the level or walking up a slight hill</td>
</tr>
<tr>
<td>3</td>
<td>Walks slower than most people on the level, stops after a mile or so, or stops after 15 minutes walking at own pace</td>
</tr>
<tr>
<td>4</td>
<td>Stops for breath after walking about 100 yards or after a few minutes on level ground</td>
</tr>
<tr>
<td>5</td>
<td>Too breathless to leave the house, or breathless when undressing</td>
</tr>
</tbody>
</table>
Thank you for joining our study in 2012/2013. Your blood test results from the first survey showed that you might be suffering from pulmonary aspergillosis or CPA. Blood tests alone are not enough to diagnose this disease as people can have positive tests even when they don’t have the disease. We performed chest X-ray as well as blood tests to provide a picture of the lungs to help us see if aspergillosis was really present in the lungs.

While chest X-ray is the best test available for this purpose in Gulu it is better to do a test called a CT scan of the chest. This gives a much better picture of the inside of the chest and so is much better for identifying aspergillosis in the chest. By having this test we will be able to give you a clear answer as to whether you are suffering from CPA or not. The test also tells us exactly where in the lungs the CPA is. This is important as some patients can be cured of CPA by an operation, but it depends on where in the lung the disease is. A chest X-ray does not give a good enough picture to decide if an operation is possible.

We therefore plan to transport you to Kampala to undergo a CT scan of the chest at the Kampala Imaging Centre. We will arrange transport and provide accommodation for you. You will be provided with an allowance to spend on food while you are away.

A CT scan has a bigger dose of radiation than a chest X-ray. There is a very small risk this might cause cancer, but this risk is much smaller than the risk that you will become sick because of aspergillosis if it is not confirmed and treated. We therefore recommend you have this test.

As well as being used to decide if you have aspergillosis or not as part of our study, your scans will also be available for you to show to a surgeon at Mulago hospital to decide if you can be cured by surgery or not. We hope to arrange a trial of surgery and if this goes ahead you will be offered the chance to join it. Any treatment as part of a trial would be free. If there is no trial you would still be able to access surgical treatment at Mulago Hospital (if the scan shows this is possible) as part of Mulago hospitals standard provision of care.

If you wish to complain about the conduct of the study please contact me in the first instance and if you are still unhappy you can contact my supervisor Professor Denning (david.denning@manchester.ac.uk) or the Research Governance Office at the University of Manchester (research-governance@manchere.ac.uk).

Finally we would like to contact you again to take part in further studies. This is optional and you can still take part in this study if you do not want to take part in the other studies.

This project was reviewed by the University of Manchester Research Ethics Committee 1.
Appendix 6 – Consent form for CT scan

ASPERGILLOSIS STUDY

CONSENT FORM FOR CT SCAN

If you agree to undergo CT scan as part of the aspergillosis study please sign the consent form below. In doing so you agree to the following:

1. I have read the patient information sheet, have had the opportunity to consider its contents and ask questions and had these answered satisfactorily.
2. I will travel to Kampala where I will undergo a CT scan of my chest.
3. I understand I have been selected for this test because my blood tests suggest I may have aspergillosis and that this test will decide if I have aspergillosis or not.
4. I am aware that there is a very small risk of developing cancer from the CT scan.
5. I understand that the results of my scan will be assessed to decide whether it is possible to cure my aspergillosis through an operation or not. This consent is limited to the scan and does not mean I have decided to undergo surgery.
6. If surgery is possible I agree to be contacted to discuss the option of having surgery.
7. I consent to the results of my scan being stored as part of the study.
8. I agree that images from my scan may be included in publications or presentations relating to this study.
9. I understand that I may at any time withdraw my approval for information to be stored or presented without giving any reason. If I do this my tissue samples will be destroyed and my information will not be used for future research. I realize that it will not be possible to assess my suitability for surgery if my images are destroyed.

……………………………………
Name of participant

……………………………………
Name of person

X

Name of participant                      Date                      Signature

I have explained the request for research purposes and answered such questions as the patient has asked. I am satisfied that the donor signing this form understands the content and the purpose and nature of this consent process.

……………………………………
Name of person                      Date                      Signature

taking consent
Appendix 7 – MIND-IHOP study protocol

Mulago Inpatient Noninvasive Diagnosis – International HIV Opportunistic Pneumonia (MIND-IHOP) Study

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Respiratory infections are a leading cause of death in Africa, especially among Human Immunodeficiency Virus (HIV)-infected patients, and the lack of understanding of host and pathogen biology constitutes a major barrier to developing new management approaches for improving outcomes. Over the past 4 years, rapid, noninvasive tests and
strategies have been validated for the diagnosis of tuberculosis (TB), yet substantial
improvements in mortality have yet to be realized. Understanding the fundamental
biological principles underlying human-microbial interactions in patients with
respiratory illness offers the possibility for reshaping current approaches to care.
Therefore, we propose minor modifications to our current platform for study of the
diagnosis and epidemiology of HIV-associated pulmonary infections and the human
responses to those infections that will combine earlier and more frequent patient
assessment with the latest technologies for studying the biology of host-pathogen
interaction.

Objectives:

Our specific aims are:

1. To determine the frequency, quantity, and diversity of bacterial, mycobacterial,
fungal, and viral organisms in respiratory specimens using microbiologic,
serologic, and nucleic-acid amplification techniques to determine the
relationship between presence of these organisms and clinical outcomes;
2. To evaluate the performance and impact of novel independent and integrated
approaches to TB diagnosis using both smear microscopy and automated nucleic
acid amplification testing;
3. To evaluate the operational and performance characteristics of novel approaches
to treatment monitoring using intensified measures including clinical
characteristics, microbiologic results, automated nucleic acid amplification, and
cytokine profiling for prediction of clinical and microbiologic outcomes among
patients with TB and other pneumonias;
4. To describe mycobacterial and host gene expression profiles and cytokine
responses in blood and respiratory specimens to gain insights into the
pathophysiology of TB and to more accurately classify TB disease states.
5. To describe the influence of airway pathogens on the gastrointestinal
microbiome by comparing respiratory samples, stool samples, and clinical
outcomes

Some of our associated hypotheses are:

1. The frequency, quantity, and diversity of microbial species in oral and
respiratory specimens will generate new hypotheses about the predictors and
roles of microbial communities and provide insights about clinical outcomes.
2. Novel approaches to microscopy will have equivalent sensitivity to existing
approaches but integrated approaches to TB diagnosis and treatment monitoring
will optimize diagnostic accuracy and maximize clinical impact.
3. Two independent hypotheses:
   a. Disease response markers (e.g. quantitative nucleic acid
      amplification results, host or pathogen gene expression, quantitative
      microbiologic results, host cytokine and inflammatory marker responses)
      measured early during the course of anti-tuberculosis treatment will provide
      insights into the kinetics and biology of treatment response in HIV-infected and
      HIV-uninfected patients;
   b. Molecular or microscopy markers measured during treatment of
      PCP will have a high positive predictive value for treatment failure.
4. Description of mycobacterial and host gene expression profiles and host cytokine profiles will provide insights into pathogenesis and correctly classify TB disease states.

Background:

Overview.

Respiratory infections are a leading cause of death in Africa, especially among HIV-seropositive adults and children (Lopez, 2006; Ansari, 2003; Lucas, 1993). Definitive diagnosis of respiratory infections in Africa is difficult because resources are often limited and because non-invasive techniques for diagnosing opportunistic infections lack adequate sensitivity and specificity.

Key Findings to Date.

Since 1998, members of the study team have been refining molecular methods in clinical studies of non-invasive diagnosis of PCP and tuberculosis (Huang, 2000; Fischer, 2001; Zelazny, 2004). We have applied some of these tools to study respiratory infections at Mulago Hospital in Kampala, Uganda. Since March, 2007, we have safely enrolled almost 2000 patients and have facilitated a thorough diagnostic evaluation for the etiology of pneumonia in each of these patients including chest radiography, CD4 T-lymphocyte count measurement in HIV-infected patients, sputum smear microscopy and culture, and bronchoscopy with BAL when requested by the treating physician.

Through this process, we have produced the following key findings:

- HIV seroprevalence is over 80% among patients admitted to Mulago Hospital with pneumonia
- Tuberculosis is the most common cause of pneumonia, accounting for over 50% of cases.
- Pneumocystis pneumonia is a rare cause of respiratory infections.
- Clinical symptoms and chest radiography have poor positive and negative predictive values for TB diagnosis.
- Nucleic acid tests have moderate sensitivity and substantial clinical impact for the diagnosis of smear-negative TB.
- T-cell interferon-gamma release assays perform poorly for diagnosis of TB.
- Same-day microscopy has equivalent sensitivity to conventional two-day microscopy for TB diagnosis.
- LED fluorescence microscopy increases the sensitivity of smear-examination for TB.

Aim 1 – Lung microbial diversity. Increasing evidence for other lung diseases such as cystic fibrosis suggests that alterations in host bacterial communities contribute to the pathogenesis of lower respiratory tract infections. No studies have been conducted to determine the composition of host communities present in the HIV-infected lung. We will evaluate sputum, tongue scrapings, oro-pharyngeal washes, and BAL using bacterial, mycobacterial, fungal, and viral nucleic acid tests (including microarrays), complemented by conventional microbiologic, serologic, and other biochemical assays.
to detect with the presence of these organisms, and correlate results with clinical outcomes. We will describe the types and variation of microbial populations resident in a variety of respiratory specimens.

Aim 2 – Diagnosis of active TB. TB is the leading cause of mortality in HIV-infected patients in sub-Saharan Africa. Failure to promptly diagnose TB has adverse consequences including disease progression, acceleration of HIV-related immunodeficiency in dually infected persons, and increased TB transmission in the community (WHO, 2004; Steen, 1998). Despite these consequences, failure to rapidly diagnose TB is common, in part due to inadequate diagnostic tests. We will evaluate the clinical impact of integrated algorithms employing combinations of traditional microbiologic (sputum smear microscopy and culture) and novel nucleic acid testing (Xpert MTB/Rif) for TB diagnosis on patient- and health-system important outcomes. In addition, we will collect biological specimens (sputum, blood, urine) for evaluation of novel diagnostic biomarkers for development into new TB diagnostic assays.

Aim 3 – Surrogate markers of response to anti-TB and pneumonia chemotherapy. Surrogate markers of treatment response are needed to decrease the cost and duration of clinical trials of new anti-tuberculosis medications. Documenting clinical cure and absence of relapse currently requires following patients for up to two years after treatment completion. Some studies have used two-month culture conversion as a surrogate endpoint, but recent data suggests this approach has limited sensitivity and specificity. Some studies have explored the role that pathogen and host specific markers may play in predicting treatment outcomes. However, new measurement tools (e.g. whole genome gene expression studies, quantitative nucleic acid amplification testing, multiplex cytokine assays) are now available to improve the precision of our measures. We will also explore novel approaches to monitoring with smear microscopy, the standard method for monitoring treatment response in TB patients, by measuring serial levels of inflammatory markers. For other pneumonias, we will explore other surrogate markers such soluble TREM-1 (bacterial pneumonia), and serum S-adenosylmethionine and co-trimoxazole drug levels (PCP) (Gibot, 2004; Skelly 2003).

Aim 4 – TB pathophysiology. There is an urgent need to distinguish between people who are not infected with MTB, infected but without active disease, and infected with active disease. Novel techniques can provide insights into host and pathogen characteristics in different disease states, potentially leading to novel diagnostic interventions. We will assess (1) Mycobacterial gene expression in respiratory specimens and (2) Host gene expression and cytokine profiles in respiratory and blood specimens. We will correlate these results among patients with different MTB disease states.

Aim 5 – Gastrointestinal Microbiome. We would like to compare the lung microbiome to the gastrointestinal microbiome in order to better understand the influence of gastrointestinal microflora on opportunistic pulmonary conditions. We will do this by comprehensively comparing the bacterial populations in respiratory specimens (tongue scraping, oral wash, bronchoalveolar lavage specimens) to those in gastrointestinal specimens (stool specimens).
Design:

This is a prospective cohort study of patients with pneumonia admitted to Mulago Hospital. We will enroll consenting patients with cough, and collect respiratory specimens (including bronchoalveolar lavage fluid) and blood as indicated to obtain as definitively as possible a diagnosis for the pulmonary complaints. A subset of patients will be followed for 2 months as part of a treatment monitoring sub-study. An overview of the protocol follows.

After identifying eligible patients with the assistance of the medical and nursing staff, the study medical officers will screen and enroll patients. This will take place on the casualty ward (emergency department, 3BE) on weekdays. All alert, English- or Luganda-speaking adults with respiratory complaints (cough) will be invited to join the study on the day of hospital admission, through a verbal and/or written invitation in English or Luganda. Interested patients will subsequently be enrolled at the bedside. The study team (which includes physicians, medical officers, nurses, and laboratory technicians) will administer a brief questionnaire to enrolled patients, in English or Luganda, and collect several biological samples.

These will include expectorated or induced (if the patient is unable to expectorate spontaneously) sputum. (Please see description below in Part 3: Procedures). Sputum will be processed, and will undergo staining, interpretation, mycobacterial culture, and other clinical tests as necessary for care of the patient on site at Mulago Hospital and at the National Tuberculosis and Reference Laboratory (NTRL). When clinically indicated, sputum will also undergo testing for M. tuberculosis and rifampicin drug resistance using the GeneXpert™ MTB automated nucleic acid amplification assay as well as smear microscopy at Mulago Hospital. The results of sputum acid-fast bacilli (AFB) smears and GeneXpert testing will be available to the treating clinicians within 24 hours.

During enrollment, samples of blood (totaling up to 42 mL) will be drawn from all subjects and an additional 22mls will be drawn for patients undergoing bronchoscopy. This blood will be used for CD4 count in the majority of patients who are HIV-infected, and research assays in all patients. Clinical testing will be performed at the Makerere University–Johns Hopkins University (MU-JHU) Core Lab, the Makerere University College of Health Sciences Clinical Lab, or the Mulago Hospital Clinical Lab, unless services become unavailable, in which case alternative local labs will be used. Unused blood will be separated into its constituents (erythrocytes, mononuclear cells, plasma, serum, etc) and stored for research studies in patients who specifically provide informed consent.

As soon as testing is complete, sputum AFB results will be collected from the laboratory and delivered to the ward. Additional sputum will be collected on Day 2. A portion of this sample will be delivered to the NTLP for smear microscopy and culture. These AFB results will again be collected and delivered to the ward on the following working day. Patients with evidence of rifampicin drug resistance on the GeneXpert assay will have drug susceptibility testing performed, with the results provided to patients. Patients with drug-resistant TB will be registered for treatment with second line TB drugs at the National TB and Leprosy Programme once a drug-resistant-TB treatment program has
been introduced. The Uganda NTLP has received approval from the WHO Green Light Committee to acquire second line drugs for treatment of drug-resistant TB. The remainder of all sputum, as well as BAL specimens, and all culture isolates will be stored for future studies.

HIV results will be received from the hospital-run HIV-testing service as soon as they are available after admission. Ward physicians will be encouraged to refer any patient who is HIV-infected, with persistent respiratory symptoms and negative-AFB smears, to the pulmonary service for bronchoscopy as soon as two sputum samples have been examined for acid-fast bacilli. Bronchoscopy is routinely performed in such patients in high-income countries and increases the yield for diagnosis of PCP, pulmonary Kaposi’s sarcoma, fungal pneumonias, and possibly TB. This study will pay all costs associated with bronchoscopy and testing of BAL fluid such that all consenting patients can undergo bronchoscopy unless the clinicians deem it unsafe.

On the morning of bronchoscopy, the bronchoscopist and a bronchoscopy nurse will consent the patient for the procedure. All patients will be monitored with continuous pulse oximetry and receive continuous oxygen supplementation, if required. An oropharyngeal wash (OPW) specimen will be collected at this time by having the patient gargle 10 mL of sterile normal saline for 60 seconds and expectorate it into a cup. In addition, 22mL of blood will be collected as well as gentle tongue scrapings, an oral rinse specimen, and sputum. Baseline vital signs will be recorded. In preparation for the procedure, the patient may receive intramuscular midazolam for anxiolysis, at the clinician’s judgment. The nurse will then anesthetize the upper airway with 10 mL of 2% lignocaine, to be administered by nebulizer. Additional aliquots of lignocaine, not to exceed a total dose of 5 mg/kg of body weight, may be delivered topically to diminish coughing. Multiple 25 mL aliquots of sterile normal saline will be lavaged through the bronchoscope channel into a bronchus occluded by the bronchoscope and suction applied to return a target of at least 50 mL of lavage fluid. After the procedure, the patient will be monitored by nursing staff to see that vital signs and clinical status have stabilized before returning the patient to the ward. Bronchoalveolar lavage specimens will be delivered to the study’s microbiology technician, who will stain the specimens for PCP, and send them for mycobacterial and fungal stains and cultures. A portion of the BAL will be saved for research studies. Patients with Pneumocystis pneumonia will have an additional 5 mL of blood taken from them for sulfa steady-state drug levels after the 5th dose of treatment with any sulfa antibiotic.

Patients undergoing bronchoscopy for clinical indications will be asked to participate in a sub-study in which we will ask them to provide a stool sample near to or on the day of bronchoscopy. This sample will be used to compare the microbiome of the gastrointestinal tract to the microbiome of the lung.

A subset of approximately one hundred smear-positive TB patients will be asked to submit serial sputum specimens and provide additional blood during the initial days of treatment to evaluate treatment response. These patients will undergo serial sputum sampling prior to and following initiation of standard 4-drug TB therapy. Smear, culture, and automated nucleic acid amplification testing on sputum will be done at baseline (pre-treatment) and after 2 months (60 days) of therapy; automated nucleic acid amplification testing alone will be done on sputum around days 2, 4, 7, 14, and 30...
of therapy; and up to 30 mL of blood will be collected to assess gene expression and
cytokine responses at baseline and around days 7, 14, 30, and 60 of therapy. A finger
prick will also be performed for point-of-care C-reactive protein (POC CRP) testing at
the time of enrollment and at each follow-up visit. The data for all 100 patients will be
analyzed to identify the 1-2 measurement time points during treatment (minimum
sampling frame) that most accurately represent the slope of decline in quantitative
sputum MTB DNA and CRP concentrations described by the full set of time points
(maximum sampling frame). QPCR and cytokine assays will be performed at additional
time points following treatment initiation based on the minimum sampling frame. We
will also collect a small amount of clinical data from those enrolled on treatments taken
after discharge.

Sputum samples provided by patients at 5 month follow-up for AFB treatment may be
used for GeneXpert testing including staining/culture for acid-fast bacilli and other
pathogens as clinically indicated.

All respiratory specimens will subsequently be processed, de-identified, divided into
triplicate sets, and stored frozen in the MIND study freezers located in Mulago Hospital.
At least one set of specimens will remain at Mulago Hospital/Makerere University and
offered to local investigators for research studies. One or more sets (depending on the
yield of each specimen after processing) of non-personally-identifiable specimens of
sputum, blood, oral specimens, BAL fluid, and culture isolates will be shipped to the U.S.
for testing by laboratory collaborators with different areas of expertise. The
investigators will analyze them according to previously validated protocols and return
the results to the clinical investigators.

Statistical Methods and Sample Size Calculation:
Our sample-size generating hypothesis relates to Aim 2, in which we expect to show the
equivalent sensitivity of portable fluorescence microscopy to conventional LED
fluorescence microscopy (FM). To calculate sample size, the following equation is
necessary:

Equation for proportions: \[ N = C \times \left\{ \left[ \left( P_1 \right)^2 \right] \left( 1 - P_1 \right) + \left( P_2 \right)^2 \times \left( 1 - P_2 \right) \right\} \times \frac{1}{d^2} + \frac{\left[ 2/d \right]}{2} + 2 \]

Sample size \(N\) for a study whose endpoint is a difference in proportions can be
estimated using this equation where \(P_1\) is the expected proportion in group 1 and \(P_2\) is
the expected proportion in group 2, and \(d\) is the difference between \(P_1\) and \(P_2\),
expressed as a positive quantity, and where \(C\) is a constant that depends on the values
chosen for alpha and beta. (Fleiss, 1981)

We used PASS 11.0 (NCSS, Kaysville, USA), a comprehensive and validated software
program for Power and Sample Size calculations, to determine the number of patients
needed to demonstrate that portable LED FM is no less sensitive than conventional LED
FM, as defined by a non-inferiority margin of 10%. To demonstrate non-inferiority with
80% power and a 5% significance level using a one-sided equivalence test of correlated
proportions (Liu JP, Stat Med 2002), 370 tuberculosis patients will be needed. This
calculation is based on the 60% sensitivity of conventional LED FM using culture as the
gold standard in our cohort to date and assumes the actual difference in sensitivity
between conventional and portable LED FM will be 0%. Given the 50% prevalence of
Clinical data will be reviewed, interpreted, cleaned, and analyzed by the clinical investigators. Chest radiographs will be interpreted according to standardized categories by a clinical investigator who will be blinded to the diagnoses of the patients. All data will be entered using study numbers for identification. Data will be entered in duplicate and compared using SAS Corporation Statistical Software. Data will be analyzed using Microsoft Access, Microsoft Excel, and STATA Corporation Statistical Software. The data will be managed by members of the MIND team working in the MU-UCSF Research Collaboration Data Centre. The data will be accessible through a secured, password protected web server stored in the MU-UCSF Data Centre, or on secured servers at UCSF. Bivariate and multivariate analyses of associations between clinical data and the outcome of disease will be performed. Receiver operating curves will be generated using measures of test accuracy at various thresholds of results. Other statistical comparisons between the data points may be performed to test other hypotheses that arise.

**STUDY PROCEDURES**

**Study Instruments, Procedures, and Location:**

- **Questionnaire:** The medical officer will interview the patient at the bedside to gather demographic information and obtain a clinical history (Please see Appendix).

- **Sputum:** Subjects will submit sputum on Day 0 to the laboratory technician. Sputum will be delivered to the microbiology lab for smear examination and culture. If negative, a portion of the sputum samples will be used for GeneXpert testing including staining/culture for acid-fast bacilli and other pathogens as clinically indicated, while the remainder of the samples will be used for research. Also, additional sputum will be sent for culture on Day 2 if the GeneXpert test is negative (Please see Appendix, Flow Diagram).

In addition, for the monitoring sub-study, sputum will be collected daily during inpatient hospitalization and up-to-weekly during the follow-up period. If patients are unable to expectorate sputum spontaneously, sputum induction may be performed.

- **At 5 month follow-up, sputum will be collected and used for GeneXpert testing.**

- **Oropharyngeal Wash/Oral Rinse:** The subject will pour 10 milliliters of normal saline into the mouth, and then “gargle” for sixty seconds. The timing and quality of the procedure will be recorded. These will be collected at the time of bronchoscopy. Patients will also be asked to rinse the mouth without gargling and expectorate.
• Tongue Scraping: A sample of oral microbiologic flora will be obtained by a trained lab technician applying a wooden stick with a smooth edge to the tongue immediate prior oropharyngeal wash collection.

• Urine: A urine sample will be collected in a 30 mL specimen cup on the day of admission.

• Finger prick: The laboratory technician will obtain 1-2 drops of whole blood via finger prick. The skin will be prepped with an alcohol prep pad and dried with cotton. A lancet will be applied to a fingerpad and blood expressed. A capillary will draw up 1-2 drops of blood which will then be mixed with a reagent for POC CRP measurement. POC CRP will be performed at baseline (Day 0) and at each follow-up visit (Days 2, 4, 7, 14, 28, and 56). This will allow evaluation of the accuracy and acceptability of this assay.

• Blood: The laboratory technician will collect one ~1 mL sample EDTA-containing tube to measure CD4+ T-cell count, one ~10 mL tube for serum/plasma studies, and three 8 mL tubes for measurement of T-cell telomerase enzymes. For patients undergoing bronchoscopy, an additional 22 mL of blood will be collected for gene expression and telomerase assays. For patients with TB enrolled in the treatment monitoring sub-study, up to 30 mL of additional blood will be collected for gene-expression and cytokine profiling studies in a subset of patients at baseline and around days 7, 14, 30, & 60. For patients without TB in the gene expression profiling study, 5 mL will be collected around day 60. 5 mL more will be gathered in patients with PCP after the 5th dose of trimethoprim sulfamethoxazole. Clinical blood tests will be performed in the MU-JHU lab, which is certified by the Clinical Laboratory Improvements Amendments (CLIA) Advisory Committee.

• Clinical data: The medical officer will measure the patient’s vital signs, lung physical exam results, and clinician diagnosis from the bedside chart at the time of admission.

• Chest radiographs: Chest radiographs will be taken routinely at enrollment, and for the IM patients, at the 8-month follow-up visit. If a chest x-ray has not been performed just prior to admission, one will be obtained. The medical officer will photograph x-rays with patient-identifying text obscured. If additional x-rays are requested for clinical purposes while the patient is being followed in the study, the study will provide those as well. Chest radiographs will be interpreted using a standardized research form.

Bronchoscopy: For HIV-infected patients who are not shown to have tuberculosis after sputum analysis, bronchoscopy will be performed in the bronchoscopy suite upon request of the treating physician according to the local protocol. Bronchoscopy will be deferred in patients with unstable respiratory status.

• Stool Collection: We will ask bronchoscopy subjects for a stool sample. We will ask that patients place a paper collection device onto the toileting area just prior to having a bowel movement. After depositing the stool on the device, it will be
follow-up during 3 30-minute home visits around Days 14, 30, and 60:

- Collection of 5 mL sputum volume, by expectoration or induction
- Collection of 1-2 drops of blood via finger prick
- Collection of 30 mL blood via venipuncture

Follow-up for up to 50 non-TB patients for collection of 5 mL blood for gene expression profiling. (60 minutes)
Total patient time for patients for the standard inpatient procedures and follow-up is estimated as 2 hours, 55 minutes. Total additional patient time for patients enrolled in the intensive monitoring sub-study is estimated as 5 hours.

All study procedures will be reviewed by the Makerere University School of Medicine Research Ethics Committee, the University of California, San Francisco, Committee on Human Research, and the Mulago Hospital Institutional Review Board. They will also be reviewed annually by the Uganda National Council for Sciences and Technology.

Specimen Testing and Data Review:

Specimen analysis and data review will take place in the laboratories/offices of co-investigators, listed above, according to well-defined protocols that include the testing of positive and negative controls as indicated. Any positive result will be communicated to the primary team. DNA and RNA will be processed and analyzed in laboratories of the investigators in accordance with the above proposed protocols and the banking permissions granted. All specimens will be coded and de-identified, and non-clinical investigators will not have access to the key.

One specimen, blood will be subjected to tests of host gene expression at the laboratory of Mark Geraci, M.D., and collaborating core lab facilities under the scientific direction of Dr. Geraci and Dr. Walter at the University of Colorado. These specimens will be used to study host responses to pulmonary infections. We will not collect DNA or analyze individual-specific genetic characteristics. Instead we will analyze patterns of RNA expression (gene expression). This analysis therefore does not meet the definition of genetic research. Once laboratory testing is complete, the results will be linked to the clinical outcome data by the clinical investigators, who will perform the primary analysis. Only researchers listed as investigators for this protocol will have access to the specimens and clinical data.

Tissue Banking Procedures:

De-identified specimen material remaining after the completion of study assays will be stored in secured freezers at three sites: 1) Mulago Hospital; 2) San Francisco General Hospital, and 3) the National Institutes of Health. At Mulago Hospital, the specimens will be stored in a locked freezer in the Department of Microbiology on the 2nd floor. At San Francisco General Hospital, the specimens will be stored in a locked laboratory located in Building 100, Room 109, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA 94110, USA. At the National Institutes of Health (NIH), the specimens will be stored in the Lung HIV Specimen Bank at the National Heart Lung and Blood Institute. The code and all identifiable clinical information will be stored separately on password-protected computer servers located at the MU-UCSF Research Collaboration, Mulago Hospital, and at the University of California, San Francisco. If outside investigators request use of these specimens for research suited to the scientific aims listed in the consent form (i.e. research to learn about, prevent, or treat other respiratory infections or diseases and diseases related to HIV, as we deem appropriate), we would release the specimens under our control after our scientific merit review of the proposed research and after receipt of a copy of the IRB-approval letter for the new
During the procedure. To guard against the possibility of oxygen desaturation, patients

If the participant decides that he or she does not wish for his or her oral wash specimen

or clinical information to be used for future research, he or she may tell us, and we will
destroy any remaining identifiable sample and information, and ask our collaborators to
do so also.

A material transfer agreement governing this study has been approved by legal and
governing authorities at Makerere University, UCSF, the University of Colorado and the
National Institutes of Health. The Foundation for Innovative New Diagnostics, a non-
governmental organization supporting some of this work and carrying out laboratory
assays on some of these specimens, is also party to a material transfer agreement
governing this study.

RISKS AND BENEFITS

Alternatives to Participation:

Patients who choose not to enroll in the study may receive whatever care they would
have ordinarily received had they not been approached to participate in the study. This
might include bronchoscopy with bronchoalveolar lavage, which we will provide for
HIV-infected inpatients who require a procedure to diagnose a pneumonia of
undetermined etiology. Otherwise, only noninvasive tests (AFB-sputum-smears) are
available for diagnosing pneumonia at Mulago Hospital.

Risks and Discomforts:

Subjects performing oropharyngeal wash may gag, experience nausea, or feel short of
breath as a result of gargling. These symptoms are likely caused by the act of gargling
rather than by the solution gargled. These side effects are rare and usually self-limited.

Tongue scraping with a smooth wooden spatula should be painless in subjects with
normal oral mucosa and should have no lasting consequences. If the mucosa is damaged
such that the procedure could induce pain, the test will be omitted.

Patients having blood drawn via finger prick or venipuncture may experience local
discomfort at the site of the needle puncture, where the skin may become dark or tender.

Patients with anemia may be unable to tolerate having large amounts (>25 mL) of blood
drawn. To guard against this possibility, patients with evidence of conjunctival pallor or
other clinical signs of anemia will be screened with a hemocue hemoglobin
measurement. Anyone with a hemoglobin less than 7 mg/dL will not have blood drawn.

Patients performing sputum induction may gag, experience nausea, or feel short of
breath as a result of the nebulization procedure. These symptoms are likely caused by
the act of saline inhalation rather than by the solution gargled. These side effects are
rare and usually self-limited. Severely ill patients may develop oxygen desaturation
during the procedure. To guard against the possibility of oxygen desaturation, patients
with respiratory rates greater than 30 or requiring oxygen supplementation will be excluded from sputum induction for research purposes. Patients may still be referred for sputum induction for clinical purposes, but only at the request of the primary clinical team caring for the patient. Such referrals will need approval of the attending physician on the 4C Pulmonology ward.

Bronchoscopy is performed regularly at Mulago for patients who can afford it. Study participants undergoing bronchoscopy assume the same risks as any patient undergoing bronchoscopy at Mulago. These risks include coughing, gagging, aspiration, minor bleeding, pneumothorax, respiratory failure, and death. The risks of bronchoscopy will be disclosed in the consent form. Of the risks described above, coughing and gagging are common but self-limited. To prevent them, all patients receive a pre-procedure treatment with nebulized lignocaine, and are treated with additional topical lignocaine to control coughing or discomfort at the clinician’s discretion during the procedure. Aspiration is rare, but to prevent its dangerous consequences (acute pneumonitis or pneumonia) patients take nothing by mouth for 8 hours prior to the procedure. Any patients not in compliance with this requirement will have bronchoscopy postponed until they comply. Bleeding and pneumothorax are extremely uncommon adverse effects of bronchoscopy, especially when biopsies are not planned. Respiratory failure may occur in patients undergoing bronchoscopy for evaluation of pneumonia because of worsening of the underlying disease process with lavage of the lung. To guard against this possibility, all patients referred for bronchoscopy are routinely screened by a Pulmonary physician before the procedure. If bronchoscopy is deemed unsafe, the procedure will not be performed and the patient will be returned to the ward. Death from bronchoscopy is extremely rare. When fatal complications occur, they are usually the result of bleeding, pneumothorax, or respiratory failure. To screen for early signs of such adverse events, all patients will be monitored with continuous pulse oximetry and receive continuous oxygen supplementation, if required.

Finally, testing for TB using sputum induction or bronchoscopy produces aerosols that may be infectious, and pose a risk to individuals who are subsequently exposed to these aerosols. To reduce this risk of nosocomial TB transmission, sputum induction will be performed in a well-ventilated room on the Pulmonology ward. After sputum induction, fan ventilation out of the open window will be performed for at least 15 minutes for another patient enters the room. Similarly, in the bronchoscopy suite, fan ventilation through an open window will be used to remove infectious aerosols. N95 respirators will be supplied to all staff working with patients in these settings.

Stool should be handled with gloved hands at all time. Direct contact between stool and ungloved broken skin may lead to infection. Some people, particularly those sensitive to odor, develop nausea and occasionally vomiting.

Benefits:

All patients will have sputums stained for AFB on the first hospital day. In addition, sputum samples will be tested for TB using rapid nucleic acid testing (PCR), and cultured for mycobacteria. Patients will be notified of the results of these tests as soon as they are available. Both patients and providers have described this as a major benefit of the study. In our previous study, a large proportion of smear-negative patients were
Inclusion Criteria:

We will screen over 10,000 patients, and grounds of Mulago Hospital or associated clinics with a clinical suspicion of pneumonia. Our target population consists of all HIV-infected adults undergoing evaluation on the grounds of Mulago Hospital or associated clinics with a clinical suspicion of pneumonia. We will screen over 10,000 patients, and enroll at least 3300 patients.
Adults undergoing evaluation on the grounds of Mulago Hospital or associated clinics with cough may be screened and invited to enroll.

Exclusion Criteria:

Patients who are under the age of 18, unable to provide consent, or unable to communicate in English or Luganda will be excluded. We will also exclude patients with heart failure.

Inclusion criteria for monitoring sub-study:

Sputum AFB or GeneXpert automated nucleic acid test positive for TB and willing to participate in intensive follow-up program

Exclusion criteria for monitoring sub-study:

Patients residing >30 km from Mulago Hospital and patients otherwise unable to adhere to intensive follow-up plans will be excluded.

Screening Procedures:

The medical officer/nurse will screen all patients as they undergo clinical evaluation. Patients meeting inclusion criteria will be invited to join the study, through a process described in more detail below in the “Recruitment” section. Patients enrolled in the sub-study will be identified after TB diagnosis according to inclusion and exclusion criteria above.

RECRUITMENT

The medical officer will approach patients identified as they undergo evaluation in the clinic or after hospital admission in their beds on the open ward. Patients will be asked if they would like to participate in a study to evaluate the etiology of respiratory infections, without any reference to HIV-status. HIV-status will not be mentioned to protect study subjects from disclosure of HIV status. If an individual expresses interest in the study, his/her name and bed number will be recorded, with enrollment deferred until after screening. If the patient needs more time to decide (e.g. because he/she needs to consult his/her attendant or family member), the coordinator will attempt to return later. After the screening described above, patients enrolled in the sub-study will be randomly enrolled after TB diagnosis.

INFORMED CONSENT PROCEDURES

At the time of enrollment, a study officer will introduce himself or herself, and explain the study by reading the standardized consent form to the subject. One of these officers, who are all bilingual, will read the consent in English or Luganda, according to the subject’s preference. The subject will be provided with a copy of the consent form to read, but literacy will not be required for consent (For patients unable to read, a witness will be required to co-sign the consent form). (Please see Appendix). After the coordinator has read through the document, which is written in a question and answer format, the subject will be asked if he or she has any questions. Then, the subject will be
asked whether he or she wishes to grant, refuse, or defer a decision on participation in the study. If the subject is unable to decide before the coordinator leaves the ward for the day, he or she will not be enrolled. If the subject agrees to participate, he or she will be asked to sign the consent form. A separate consent form will be used for specimen banking.

Patients will be asked to enroll in the stool sub-study if they are going to undergo bronchoscopy. Patients enrolling in the intensive follow-up for treatment monitoring study will be consented according to a separate consent process discussing fully the issues related to longitudinal follow-up. We will draw on our experience in previously approved SOM-REC MIND protocols which involved longitudinal follow-up.