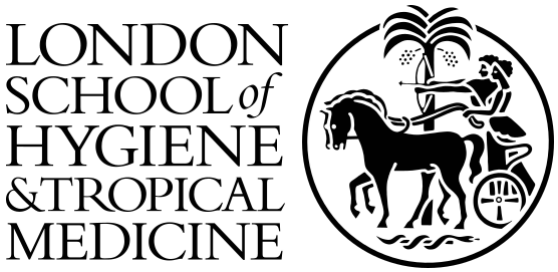
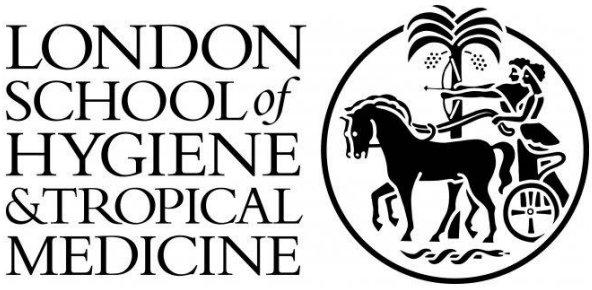


Studies on the Development of Scarring Trachoma in Tanzania



Athumani Mchana Ramadhani

2018

International Centre for Eye Health

Clinical Research Department

Faculty of Infectious and Tropical Diseases

London School of Hygiene and Tropical Medicine

Thesis submitted in accordance with the requirements for the Degree of

Doctor of Philosophy

University of London

Declaration

I, Athumani Mchana Ramadhani, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: 

Date: 28 October 2018

Abstract

Trachoma is an ancient blinding disease which remains a public health and socio-economic threat to many communities, mainly in sub-Saharan Africa. Repeated conjunctival infection with *Chlamydia trachomatis* is thought to cause prolonged inflammation which leads to scarring and blindness, however, the disease is often found to progress in the absence of detected infection. Furthermore, despite apparently similar infection exposure, only some individuals progress to scarring. The relationship between infection and scarring progression and the immunopathological mechanisms underlying it are not clearly understood. A four-year longitudinal study was conducted in northern Tanzania in order to identify associations between *C. trachomatis* infection, conjunctival inflammation and immuno-fibrogenic gene expression with disease and scarring progression.

Children aged 6-10 years at baseline were eligible for inclusion and 666 children were enrolled in the study. Participants were visited every three months for four years. Clinical signs and conjunctival swabs for *C. trachomatis* detection and immuno-fibrogenic gene expression by qPCR were collected at each time-point. Conjunctival photographs from baseline and final timepoints were graded and compared side-by-side to determine scarring incidence and progression. All community members were offered mass drug administration (MDA) with azithromycin for trachoma control annually for three years during the study.

Host immuno-fibrogenic gene expression was profiled at the first five time-points. At baseline, host immune responses were analysed in relation to *C. trachomatis* infection, *trachomatous inflammation – follicular* (TF), papillary inflammation (TP) and scarring. Th1 and NK cell associated pathways were strongly associated with infection, suggesting their importance in the clearance of infection. Growth and matrix factors (MMPs) were strongly associated with the inflammation that persisted after infection was cleared (TF/TP), suggesting they might contribute to scarring development. Th17 pathway-associated cytokines were associated with infection and inflammation, therefore their contribution to protection versus pathology is unclear.

The effect of azithromycin on inflammatory gene expression was investigated due to the reported immunomodulatory role of this antibiotic. Azithromycin treatment was found to have an anti-inflammatory effect on conjunctival gene expression, detectable three months post-treatment, suggesting that it may protect against pathological inflammation and therefore scarring development. The effect was gone by 6 months post treatment.

Of the 448 children with outcome data, 103 (23.0%) had trachomatous scarring progression over the four-year study period. In 48 (10.7%) children this was incident scarring, whereas 55 (12.3%) had progression of pre-existing scarring. Scarring progression was strongly associated with papillary inflammation. Weaker associations between TF and *C. trachomatis* infection with scarring in univariate models were absent in a multivariable model adjusting for TP. These data suggest that the effect of infection and TF is mediated through TP, and that other factors contributing to the development of TP are important in driving scarring progression. Female sex was also associated with scarring progression.

These findings suggest that the use of TP by trachoma control programs might be a more discriminating clinical marker to predict future scarring disease and requirements for trichiasis surgery than TF. In addition to clearing *C. trachomatis* infection, MDA might have an additional independent mechanism for reducing conjunctival inflammation, which may reduce subsequent risk of scarring progression.

Table of Contents

Format of the thesis

This thesis is submitted in the form of published work. All papers include a list of authors involved in the study. The introduction chapter is largely comprised of a general overview of trachoma, which is discussed widely and addresses important areas relevant to the findings presented in the thesis. Chapters 2 and 3 introduce in detail the main objectives of the thesis and the entire work flow methodology from the beginning. Chapters 4 to 8 cover the main findings. These chapters comprise of five papers, two papers systematically review the literature and three present the findings of the current study. Three papers are already published, one has been submitted and the last is in pre-submission format. The ninth and final chapter summarizes the overall findings in the context of what was known before and what this research has contributed. Potential directions for future work are considered.

List of Tables

8. Progression of scarring trachoma in Tanzanian children: a four-year cohort study

List of Figures

1. Literature review of Trachoma

3 Research Methodology

4. The relationship between Active Trachoma and ocular *Chlamydia trachomatis* infection before and after mass antibiotic treatment

Abbreviations

Abbreviation	Full meaning
AFRO	WHO sub-Saharan Africa Region
CD4+	Cluster differentiation antigen 4
CD8+	Cluster differentiation antigen 8
CMI	Cell mediated immunity
CO	Corneal opacification
C. trachomatis	*Chlamydia trachomatis*
ddPCR	droplet digital PCR
DNA	Deoxyribonuclease acid
ECM	Extra cellular matrix
EB	Elementary Bodies
EMT	epithelial-mesenchymal transition
GET2020	Alliance for the Global Elimination of Trachoma by 2020
GTMP	Global Trachoma Mapping Project
HLA	human leukocyte antigen
KCMC	Kilimanjaro Christian Medical Centre
KCRI	Kilimanjaro Clinical Research Institute
KCRI-BL	KCMC-KCRI biotechnology laboratory
LSHTM	London School of Hygiene and Tropical Medicine
MDA	Mass Drug Administration
MHC	Major Histocompatibility Complex
MMP	matrix metalloprotease
MOMP	Major outer membrane protein
NIMR	National Institute for Medical Research
NK cells	Natural killer cells
OMCB	C. *trachomatis* outer membrane complex protein B
Omp1	Outer membrane protein 1 gene
PCR	Polymerase Chain Reaction
RB	Reticulate Body
RNA	Ribonucleic acid
RPP30	*Homo sapiens* RNase P/MRP 30-kDa subunit gene
SNP	single nucleotide polymorphism
TF	Trachomatous Inflammation - Follicular
TH1	Type 1 T-helper cell
TH2	Type 2 T-helper cell
TI	Trachomatous Inflammation - Intense
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TP	papillary hypertrophy/ papillary inflammation
TS	Trachomatous scarring
TT	Trachomatous trichiasis
WHO	World Health Organization

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I would like to thank my supervisors, Professor Matthew Burton, Professor Martin Holland and Dr. Tamsyn Derrick, for their guidance of my study.

Special thanks to Professor Matthew J. Burton for his excellent guidance, encouragement and advice. He was always available for support throughout my entire study, from developing the research proposal up to writing the thesis. He generously gave his time and effort to provide a good working environment at KCMC and LSHTM. Matthew gave me the opportunity to join the trachoma research group, and working alongside him and learning about trachoma inspired me to pursue this degree. I am very grateful to him for giving me this opportunity.

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This project was supported by a wonderful field team. They kindly collected samples from the study participants at their homes and schools every three months for four years. This was not an easy job and they managed to overcome all obstacles including heavy rainfall, intense sunshine, numerous flies and poorly surfaced roads. They managed to complete the work successfully. I am very thankful to them.

The majority of the laboratory work was done in Moshi. My fellow colleagues at KCRI-BL were very supportive and cooperative, and I extend my thanks for their help throughout my PhD

training. I also extend great thanks to the study participants from Tingatinga, Mitimirefu and Roseline villages. They kindly and voluntarily offered to join our study and to give their time and samples.

I am extremely thankful to my family. Sometimes I was away from them for many hours, days and months because of this study. They were very patient and supportive and I am immensely grateful to them.

List of people that contributed to the work presented in this thesis

Name	Tittle and affiliation	Activity
Aiweda Malisa	Ophthalmic Nurse & field worker, KCMC	Field clinical signs grading and sample collection
Alex Pallangyo	Field worker, KCMC	Data entry and translator
Antipas Massae	Driver, KCMC	Day to day driving to field
Chrissy h Roberts	Associate Professor, LSHTM	Study design, laboratory and field work supervision
David Macleod	Statistician, LSHTM	Training on statistical analysis and analytical guidance
David Mabey	Professor, LSHTM	Study design, study funding and supervisory committee
Elias Mafuru	Laboratory technologist, KCRI/KCMC	Laboratory work, including DNA/RNA extraction
Henry Marielle	Field worker, KCMC	Conjunctival photography
Karim Mtengai	Laboratory technologist, KCRI/KCMC	Laboratory work
Kelvin Mbuya	Field worker, KCMC	Conjunctival photography
Martin Holland	Professor, LSHTM	Supervisor. Including study design, study funding, laboratory work supervision and thesis writing guidance.
Matthew Burton	Professor, LSHTM	Supervisor. Including study funding, study design, laboratory and field work supervision and thesis writing guidance
Patrick Massae	Ophthalmic Nurse & field worker, KCMC	Field clinical signs grading and sample collection
Phillipo Mollel	Field worker, KCMC	Data entry and translator
Robin Bailey	Professor, LSHTM	Study design, study funding and supervisory committee
Robert Butcher	Research Fellow, LSHTM	Training on ddPCR laboratory work
Tamsyn Derrick	Research Fellow, LSHTM	Supervisor. Including laboratory work supervision, training on data analysis and thesis writing guidance
Tara Mtuy	Eye health project coordinator, KCMC	Study design and project administration support
William Makupa	Head of eye department, KCMC	Study design and project administration support

This thesis is dedicated to my family and my parents.

1. Literature review of Trachoma

1.1. Overview

Trachoma is a leading infectious cause of blindness; it is caused by the bacterium *Chlamydia trachomatis* (*C. trachomatis*).[1] In trachoma endemic populations, the disease starts during early childhood with repeated *Ct* infections of the conjunctiva. This provokes recurrent episodes of chronic conjunctival inflammation, eventually leading to conjunctival scarring. Scarring of conjunctival tissue distorts the upper eyelids: the eyelids roll in towards the eye (entropion) and the eyelashes rub the eye surface (trichiasis). This causes pain and damage to the cornea, leading to irreversible blindness or severe visual impairments.[2] Eyelid surgery to correct trichiasis can reduce the risk of corneal damage and sight loss, but recurrent trichiasis is common, and there is no treatment to halt the progression of conjunctival scarring.[3-9] The time between onset of initial infections during childhood and the development of blinding complications is usually several decades, making it difficult to study the pathogenesis of this disease. However, current evidence suggests that immune responses mediate this process.

There is no known non-human biological reservoir of the forms of *C. trachomatis* that cause endemic trachoma.[10] Transmission of this bacterium is thought to be through physical contact between infected and uninfected individuals. Secretions from the eyes or nose of infected individuals may be transferred through different routes, such as flies, clothes and hands, to uninfected individuals. People who are living in crowded conditions, with limited availability of water and poor sanitation are thought to be at an increased risk of infection transmission and the resulting disease.

Trachoma affects mostly poor communities in Sub-Saharan Africa and some limited parts of Asia.[11, 12] In 2016, 42 countries were recognised as being trachoma endemic, of which the majority are in the World Health Organization (WHO) sub-Saharan Africa Region (AFRO).[12] The Global Trachoma Mapping Project (GTMP) was conducted to fill the gaps in epidemiological data about potentially endemic regions. Recently updated trachoma burden estimates from the WHO *Weekly Epidemiology Record* showed that about 157.7 million people lived in trachoma endemic areas requiring interventions, as they were at risk of blindness from trachoma,[13] 2.8 million were suffering from sight threatening trichiasis and need surgery[14] and 1.9 million had either already developed blindness or had severe visual impairments, of which about 90% are from WHO AFRO countries.[15] Encouragingly, in the last year Nepal, Ghana and Iran have been validated by WHO as having eliminated trachoma as a public health problem. Elimination as a public health problem is defined as evidence that all endemic districts have reached prevalence thresholds

of <5.0% for TF in children 1–9-years old and <0.2% for TT in people aged ≥15 years or an approximate <1 case per 1000 in individuals of all ages.[16]

Trachoma is a neglected tropical disease. It affects some of the world's poorest communities. The morbidity from trichiasis and sight loss can reduce individual productivity, promoting cycles of poverty. There is a lack of awareness and resources available for treatment and research. During late stages of trachoma women are usually more frequently affected than men.[2, 12] Vision loss causes social and economic problems to individuals, families and the community at large. Several years ago it was estimated that trachoma costs up to US$8 billion per year in lost productivity.[17] In addition, the mortality rate among individuals blind from trachoma was higher than a normally sighted control group.[18]

To meet this health, social and economic challenge, the WHO-led Alliance for the Global Elimination of Trachoma by 2020 (GET2020) was established. This set the target of eliminating blinding trachoma by 2020 through the implementation of the **SAFE** Strategy: **S**urgery to correct trichiasis, **A**ntibiotics distribution to treat *C. trachomatis* infection, **F**acial cleanliness and **E**nvironmental improvements to suppress infection transmission.[19]

1.2. Clinical Features of Trachoma and Grading Systems

Clinically, trachoma has acute and chronic stages. The acute stage, "Active Trachoma", is mostly seen in children who are the major reservoir of infection in trachoma endemic settings. The disease is mainly focused in the conjunctiva of the upper eyelid (Figure 1.1a). It is characterised by the presence of conjunctival follicles and redness due to papillae (Figure 1.1b-c). These clinical signs are thought to develop in response to *C. trachomatis* infection.

The chronic stage of scarring complication tends to develop later in life and includes conjunctival scarring, in-turning of the eyelid (entropion), in-turning of the eyelashes and scratching on the eyeball (trichiasis) and corneal blindness (Figure 1.1d-f).[20, 21] Active trachoma signs and *C. trachomatis* infection are at their peak during pre-school age children between 3 and 5 years, although the disease and infection may begin at any age.[2, 22] Development of scarring can become visible during childhood and early adulthood, depending on the endemicity of the setting.[23]

There are several systems for grading trachoma signs that have been developed and used over the last century, that are discussed below. These have evolved as our understanding of the disease process and natural history have developed. The main system that is used today in the context of trachoma control programs is the Simplified WHO Trachoma Grading System (Table 1.1 and Figure 1.1).[20]

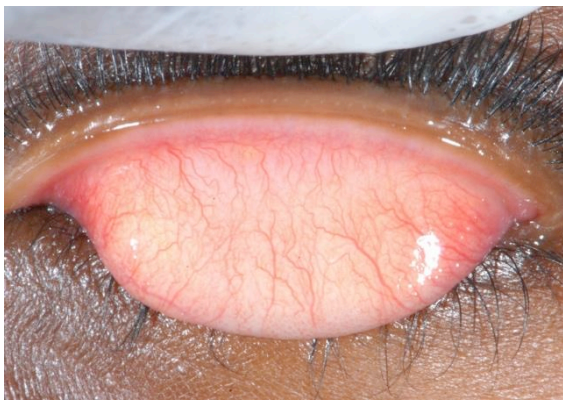
Table 1.1: The WHO Simplified Trachoma Grading System.[20]

Grade	Description
Trachomatous inflammation, follicular (TF)	The presence of five or more follicles (0.5 mm or greater in diameter on the central upper tarsal conjunctiva)
Trachomatous inflammation, intense (TI)	Inflammatory thickening of the upper tarsal conjunctiva with obstruction of more than half the normal tarsal vessels
Trachomatous conjunctival scarring (TS)	The presence of easily visible scarring in the upper tarsal conjunctiva
Trachomatous trichiasis (TT)	The presence of at least one eyelash rubbing the eyeball, or evidence of eyelash removal
Corneal opacity (CO)	The presence of easily visible central corneal opacity sufficiently dense to obscure the pupillary margin

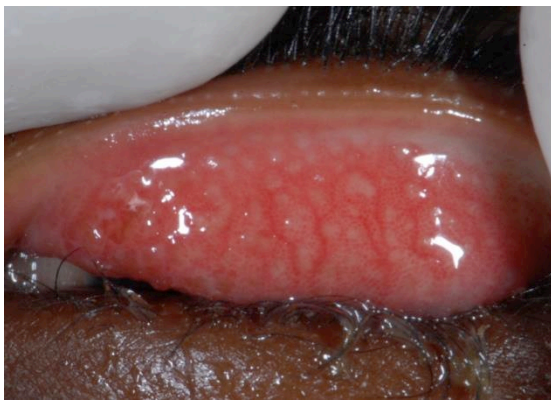
Figure 1.1: Clinical signs of trachoma:[20]

(a) Normal conjunctiva is pink, smooth, thin and transparent. (b) Trachomatous Inflammation - Follicular (TF) Follicles are round swellings of the conjunctiva, appearing white, grey or yellow and associated with ocular irritation and slightly watery discharge. (c) Trachomatous Inflammation - Intense (TI) predominately diffuse inflammatory thickening with redness, more than half of the blood conjunctival vessels are obscured. (d) Trachomatous scarring (TS) the scars are visible as white lines, bands, or sheets (fibrosis) in the conjunctiva, which may distort or obscure tarsal blood vessels. (e) Trachomatous trichiasis (TT) eyelashes rub on the surface of the eye or evidence of epilation. It is associated with pain. (f) Corneal opacification (CO) scarring of the cornea because of TT. Vision may be significantly impaired at this stage.

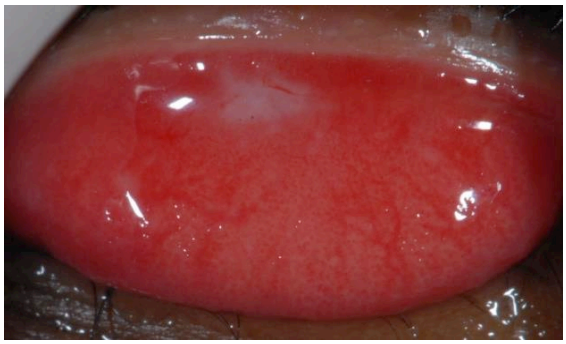
(a) Normal Conjunctiva



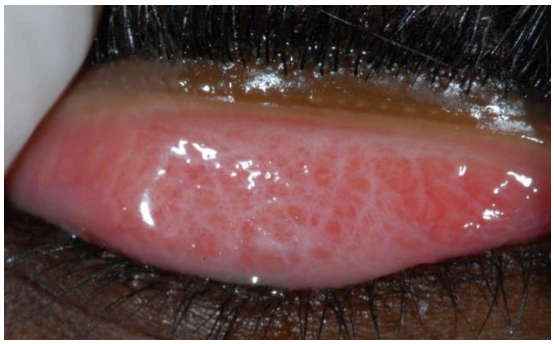
(b) Follicular Conjunctivitis (TF)



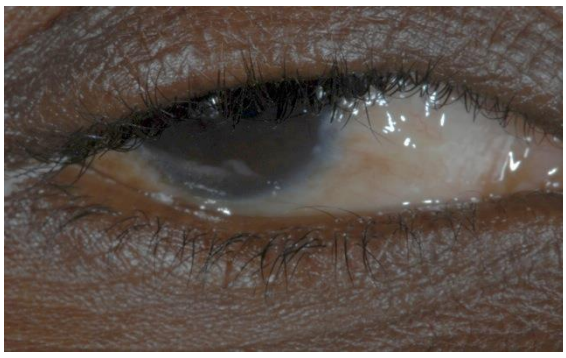
(c) Papillary Inflammation (TI)



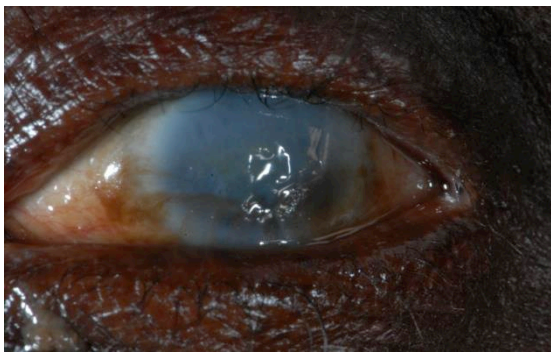
(d) Conjunctival Scarring (TS)



(e) Entropion and Trichiasis (TT)



(f) Corneal Opacification (CO)



Trachomatous inflammation – follicular **(TF);** refers to localised areas of lymphoid hyperplasia. Follicles are white or grey and characterized by a round swelling of 0.5-2mm diameter encircled by tiny blood vessels. They contain lymphocytes (a type of white blood cell) and are clearly visible on the inner surface of the upper eyelid (conjunctiva). Follicles are often accompanied by a mucopurulent discharge, itching and irritation.

Trachomatous inflammation – intense **(TI);** refers to redness caused by papillae and thickening of the conjunctiva in severe cases. Papillae are visible by magnification as small red dots and contain a central vascular core. Visibility of deep blood tarsal vessels varies depending on the severity of inflammation, ranging from slightly hazy to completely obscured. It is also accompanied by a mucopurulent discharge, light sensitivity, itching and irritation.

Trachomatous scarring (TS); As a result of prolonged inflammation, scars appear as spots or white lines on the conjunctiva. In severe cases the scars grow and cover most of the conjunctiva. Due to tissue fibrosis, glandular tissues in the lids including the lacrimal glands (tear-producing glands) can be affected resulting in dryness of the eye.

Entropion/Trichiasis (TT); Accumulating scar tissue causes contraction of the conjunctiva, resulting in in-turning of eyelids (entropion) and eyelashes (trichiasis). The eyelashes then rub and scratch the cornea, leading to corneal epithelial abrasions and more extensive damage. This causes severe pain and the eye may become even drier than before. At this stage lubricating glands are severely distorted. Mechanical damage to the cornea increases risk of secondary infections.

Corneal opacity (CO)**;** As a result of the mechanical damage caused by in-turned eyelashes, the cornea develops scars and becomes cloudy. If in-turned eyelashes/eyelids are left uncorrected individuals may develop visual impairment and eventually irreversible blindness. The epithelial layer/barrier breakdown can allow other pathogens into the cornea, leading to rapid loss of sight from microbial keratitis.

A detailed analysis of the published literature on the rates and risk factors for the natural history of disease progression is presented in Chapter 5.

The earliest formal grading system was developed by A. F. MacCallan in 1905. This includes four categories of disease (Table 1.2).[24, 25] This consisted of upper tarsal immature follicles (presumed infection stage), presence of large follicles and papillary hypertrophy, presence of conjunctival

scarring with follicles and papillary hypertrophy, and scarring without any other clinical signs. In this grading system trichiasis and corneal scarring were not included.

Table 1.2: MacCallan's system for grading of trachoma.[25]

Stages	Description
Stage I or Trachoma I (Tr. I)	Early stage of "pin head" follicles.
Stage II or Tr. II Subdivided into:	
Tr. IIa	Follicles are large and gelatinous
Tr. IIb'	Papillary enlargement as well as follicles
Tr. IIb"	Follicles with the added complication of spring catarrh
Tr. IIc	Trachoma complicated by gonococcal conjunctivitis
Stage III or Tr. III	Where cicatrization has commenced; often non-contagious
Stage IV or Tr. IV	Where cicatrization of the conjunctiva is complete. This stage is non-contagious

The Fourth WHO Scientific Meeting on trachoma, held in 1966, recognised 22 clinical signs of disease. In this grading system detailed information about each clinical sign was included. This was considered to be too complex. Therefore, the WHO subsequently reduced the 1966 detailed grading system to five clinical signs called the 1981 "FPC detailed grading system".[26] Each sign category has a four point scale, starting with normal and increasing in severity. This grading system is presented in Table 1.3 below and the grading area is as illustrated in Figure 1.2.

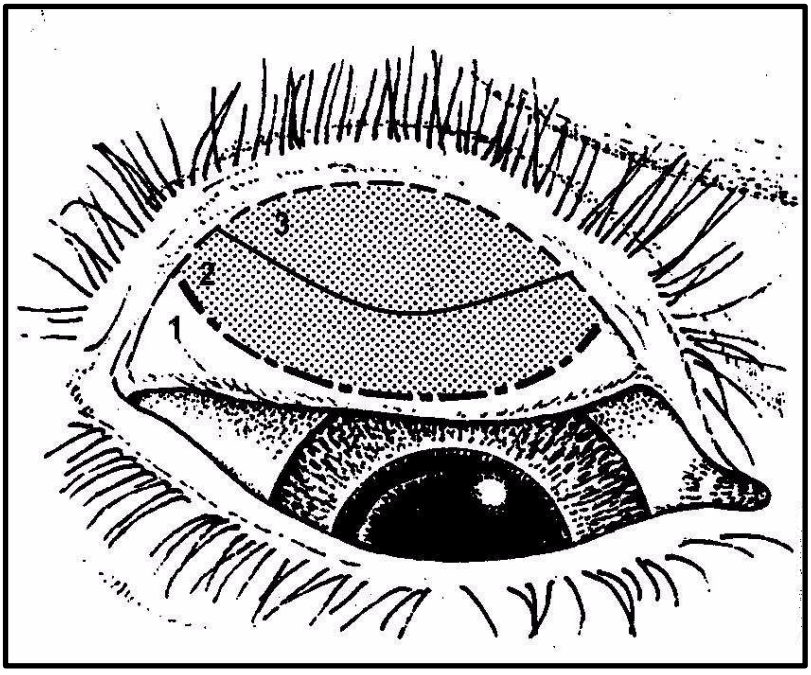


Figure 1.2: The everted upper eyelid. Showing the tarsal conjunctiva with zones from trachoma assessment and grading. The numbered zones refer to those in the FPC Grading System.[26] The shaded area indicates the zone to be examined when using the WHO simplified system.[20]

Table 1.3: WHO 1981 "FPC detailed grading system".[26]

a. Trichiasis and/or entropion (T/E):

Grade	Description
T/E 0	No trichiasis or entropion.
T/E 1	Lashes deviated towards the eye, but not touching the globe.
T/E 2	Lashes touching the globe but not rubbing the cornea.
T/E 3	Lashes constantly rubbing the cornea.

b. Corneal scarring (CC):

Grade	Description
C 0	Absent
C 1	Minimal scarring or opacity but not involving the visual axis, and with clear central cornea.
C 2	Moderate scarring or opacity involving the visual axis, with the papillary margin visible through the opacity.
C 3	Severe central scarring or opacity with the papillary margin not visible through the opacity.

c. Upper Tarsal Follicles (TF):

Grade	Description
F 0	No follicles.
F 1	Follicles present, but no more than 5 in zones 2 and 3 together. (1-4)
F 2	More than 5 follicles in zones 2 and 3 together, but less than 5 in zone 3. (5-9)
F 3	Five or more follicles in each of the three zones. (10+)

d. Upper tarsal papillary hypertrophy and diffuse inflammation (TP):

Grade	Description
P 0	Absent: normal appearance
P 1	Minimal: individual vascular tufts (papillae) prominent, but deep subconjunctival vessels on the tarsus not obscured.
P 2	Moderate: more prominent papillae, and normal vessels appear hazy, even when seen by the naked eye.
P 3	Pronounced: conjunctiva thickened and opaque, normal vessels on the tarsus are hidden over more than half of the surface.

e. Conjunctival scarring (TS):

Grade	Description
C 0	No scarring on the conjunctiva
C 1	Mild: fine scattered scars on the upper tarsal conjunctiva, or scars on other parts of the conjunctiva.
C 2	Moderate: more severe scarring but without shortening or distortion of the upper tarsus.
C 3	Severe: scarring with distortion of the upper tarsus.

However, this revised detailed grading system (1981) was not easily adopted by non-specialist health workers. Therefore, the WHO developed the simplified trachoma grading system, described above, as a tool particularly for use by non-specialist health workers to identify the key features of the disease for programmatic purposes, shown in Table 1.1 above.[20] The grading area is as illustrated in the diagram above (Figure 1.2). The detailed WHO FPC system is mostly used for research studies, as it allows classification of different clinical signs based on their severity. This is the system we generally use in our field studies in Tanzania. The relationship between the detailed FPC grading system and simplified grading system are outlined in table 1.4 below. This links the grading results using two systems for decision purposes such as implementing mass drug administration in a community.

Table 1.4. Correlation between simplified and detailed FPC grading system

Simplified grading system	Detailed FPC grading system
TF	F2/F3
TI	P3
TS	C1/C2/C3

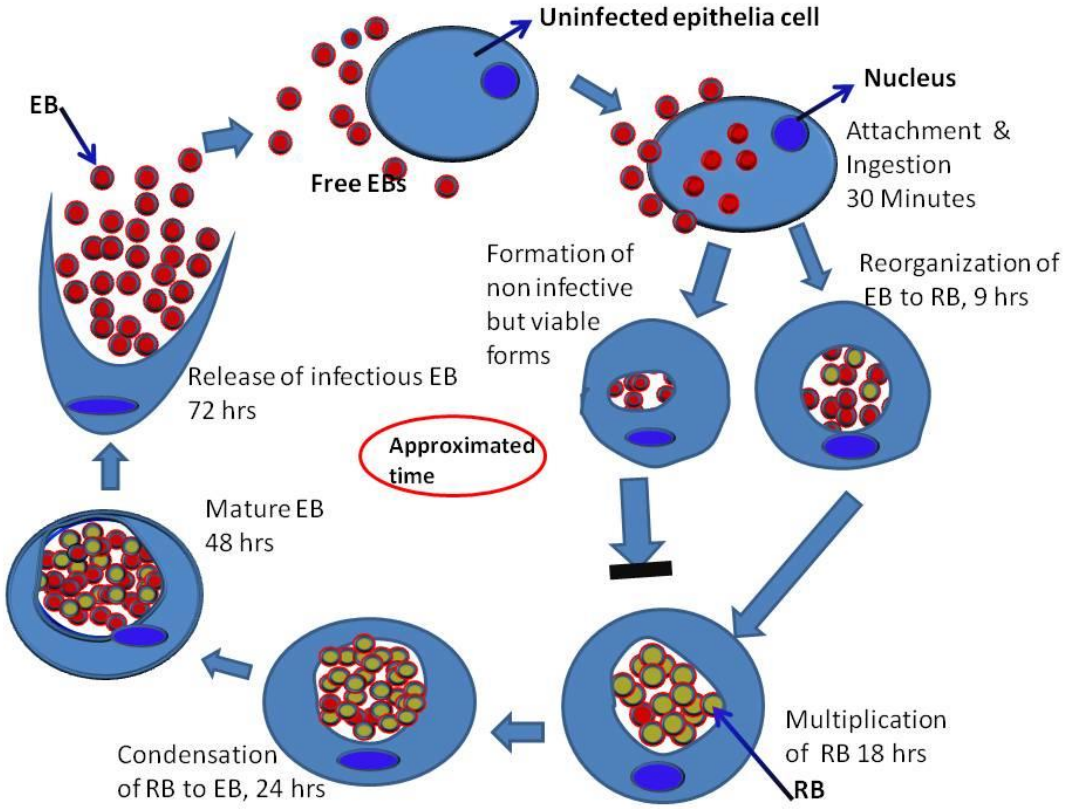
Many studies have reported mismatches between the presence of clinical signs and infection. This is explored in a detailed meta-analysis presented in Chapter 4. These studies tend to find that clinical inflammation persists longer than infection. A study conducted in The Gambia which followed participants every two weeks for 6 months found that younger age was strongly associated with persistence of disease.[27] At the age of 0-4 years the median estimated duration of disease was 13.2 weeks, whereas in 4-14 years olds it was 5.3 weeks, while at the age of 15 years and above the median duration was 1.7 weeks. A similar trend was seen for infection duration, where in children aged 0-4 years, 5-14 years and 15 years and above infection persisted for 3.8 weeks, 2.1 weeks and 1.4 weeks, respectively. Faal et al, also investigated the duration of infection and clinical signs; median duration of disease was 54 days and median duration of infection was 23 days.[28] In a mathematical model, the estimated duration of clinical signs (TF and/or TI) in children aged between 0 and 4 years was 32 weeks, which reduced to 18.8 weeks in children aged 5 to 14 years, and to 7 weeks in older children and adults.[29] In the same mathematical model, the estimated duration of *C. trachomatis* infection in children aged between 0 and 4 years old was 15.4 weeks, reducing to 8.2 weeks in children aged 5 to 14 years old and to 7.6 weeks in older children and adults.[29] In general, the estimated duration of disease was longer than infection duration across all ages, due to the persistence of disease after infection has been cleared. Similar trend of reduced active trachoma following increase in age was also reported by Dawson in 1970's however, infection data were missing.[22]

1.3. *Chlamydia trachomatis* infection

Chlamydia trachomatis is a non-motile, obligate, aerobic intracellular Gram-negative bacterium that infects epithelial cells.[30, 31] *C. trachomatis* was first described in 1942,[32] however, it was not possible to culture until 1957, when it was first cultured in chicken embryo yolk sacs.[33]

C. trachomatis has a biphasic developmental cycle, the metabolically active and non-infectious stage is referred to as a Reticulate Body (RB) and the metabolically inactive infectious phase is known as an Elementary Body (EB) (Figure 1.3).[31] However, recent evidence has challenged this dogma by showing some metabolic activity in the EB.[34] EBs enter host epithelial cells and trigger the formation of a peri-nuclear inclusion body where they transform into RBs and replicate.[35] There is a subsequent transformation back into the EB form, prior to release from the host cell. About 60% of the surface protein is Major Outer Membrane Protein (MOMP), which has epitopes which distinguish the different serovars.

Figure 1.3: Chlamydia developmental cycle.



The EB is a resistant structure covered by a rigid cell wall, which is used for survival under unfavourable conditions. It has the ability to induce its own endocytosis (uptake by the cell)

when it comes into contact with a permissive host cell. This is the main method of entry to host epithelial cells.[36] EBs recognize receptors sites on host epithelial cells, and are taken up by clathrin-mediated endocytosis and the Rho-family GTPase Rac1.[36, 37] EBs enter host epithelial cells and trigger the formation of a peri-nuclear inclusion body where they differentiate into RBs. The RB is an intra-cytoplasmic form with a diameter of 1.0 to 1.2 µm.[38, 39] Inside the inclusion body the RB grows and multiplies, dividing by binary fission. After synthesis of cell walls, the progeny differentiates back into infectious EBs. EB progeny mature for about 2 days and are then released by the host plasma membrane in a process known as exocytosis, extrusion or by cell lysis.[40, 41] The ruptured cell can produce an estimated 100 to 1000 EBs, which have diameters ranging from 0.25 to 0.30 µm.[38, 39] Differentiation between the two phases is controlled by histone-like protein. Histone (Hcta) expression is suppressed during RB late stage development to trigger differentiation into EBs.[42]

C. trachomatis causes conjunctival infection, urogenital infection and lymphogranuloma venereum in men and women, transmitted through contact with infectious secretions. Genital infections cause urethritis, cervicitis, proctitis, epididymitis and pelvic inflammatory disease, which can lead to infertility in women.[43] Lymphogranuloma venereum can cause disseminated infection that affects the lymphatic system.[44]

MOMP of *C. trachomatis* is the largest component of the cell wall surface protein, comprising up to 60%. It's encoded by a single copy gene on the chlamydial chromosome known as *Omp1.* Other surface proteins are PorB (a porin-like molecule), C. *trachomatis* outer membrane complex protein A (OmcA), OmcB and polymorphic membrane proteins (pmp). MOMP maintains rigidity of the wall structure, which facilitates the formation of pores for solute/nutrient diffusion from the extracellular environment. Chlamydia have outer membrane proteins containing lipopolysaccharide but lacking rigid peptidoglycan.[45] The absence of rigid peptidoglycan is important for intracellular adaption and enables bacteria to evade host immune recognition by TLR2. Peptidoglycan, a polymer which constitute sugar (alternating residues of β-(1,4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid) and amino acids (L-alanine, D-glutamate, meso-diaminopimelic acid, and a dipeptide of D-alanine-D-alanine) is only exposed during dividing of RBs but not in EBs.[46] Chlamydia have the necessary genes and synthesis machinery required to make peptidoglycan, which forms the cell wall of many bacteria, however, it is only a minor constituent of the cell wall in chlamydia. MOMP is responsible for adhesion to host cells and therefore facilitates EB entry to host cells. The MOMP type is used to determine different strains of *C. trachomatis* known as serovars. There are 19 serovars of this bacterium.[47] The ocular serovars A, B, Ba and C are responsible for endemic

trachoma.[48] Serovars D-K are responsible for genital infection. They can also infect the conjunctiva; however, they do not cause endemic trachoma. Genital strains are able to infect both ocular and genital sites because they can synthesise tryptophan while ocular serovars cannot.[49] Serovars L1, L2 and L3 cause lymphogranuloma venereum.

C. trachomatis produces heat shock proteins that protect from conditions of stress by stabilizing cellular proteins.[50] Chlamydial GroE operons (GroEL and GroES) are the major heat shock proteins that have been studied.[50, 51] These proteins are implicated in disease pathogenesis and are recognised by Toll-like receptors (TLRs) as part of the innate immune system.[52-55] These proteins are highly conserved among the chlamydial species.

The ability to change between EB and RB facilitates the pathogenesis of *C. trachomatis*. Several factors are reported to be associated with the pathogenesis of this pathogen. Adhesion of EBs to host cells is mediated through sialic acid receptors on non-phagocytic epithelial cells (mucosal epithelial cells and vascular endothelial cells).[56, 57] The chlamydial cell wall prevents fusion of the intra-epithelial phagosome with lysosomes; this prevents exposure to phagolysosomes, protecting *C. trachomatis* and allowing it to replicate. The ability of chlamydia to alter its surface protein (MOMP) prevents host immune cell recognition.[58] The release of enzymes by chlamydia such as chlamydial proteasome/protease-like activity factor (CPAF), helps the bacteria to evade the host immune response.[59-62] These enzymes degrade host transcription factors (RFX5) and upstream stimulation factor 1 (USF-1) which inhibit production of major histocompatibility complex. Similarly chlamydia use ChlaDub1, a protease that inhibits the NF-κB activation and Ik-Bα degradation in its evasion from host immune response. The presence of small needle-like projections, called a type III secretion apparatus, helps chlamydia to enter the host cells and enables it to inject proteins directly into the cytoplasm of the host cell. The chlamydial plasmid is also thought to be involved in pathogenesis through triggering of inflammation.[63-67]

1.4. Differential diagnosis of trachoma

There is some overlap between the clinical signs of trachoma and those of other ocular diseases. Some are infectious while others are auto-immune. Chronic follicular conjunctivitis can be caused by viruses such as Adenovirus, herpes simplex virus and *Molluscum contagiosum*; and bacteria such as *Staphylococcus aureus, Streptococcus pneumoniae*, *Moraxella* spp., *Haemophilus influenzae* and urogenital strains of *C. trachomatis.*[68, 69] Furthermore, topical medication such as for pediculosis palpebrarum (lice infestation) and conjunctivitis or eye cosmetics may also cause chronic follicular conjunctivitis.

Conjunctival scarring and entropion may be related to Stevens-Johnson syndrome, chemical injury, mucus membrane pemphigoid and sarcoidosis.[70] Often the patient's history can help distinguish these conditions. Unaccompanied papillary inflammation without follicles may be caused by other bacteria, viruses, allergy or other physical activities such as prolonged exposure to smoke, pollen and dust. Corneal scarring may also be caused by infection with other bacteria, viruses, fungi and/or traumatic injury.

1.5. Epidemiology

1.5.1. Burden of the disease

The WHO alliance for the global elimination of trachoma aims to eliminate trachoma by the year 2020, however, the burden of trachoma in hyperendemic areas of some of the world's poorest countries is still very substantial.[13] A key step forward in the worldwide drive to eliminate trachoma has been to define the global burden. This was achieved through the Global Trachoma Mapping Project (GTMP), which has mapped the prevalence of TF and TT in previously unmapped districts where trachoma was suspected to be endemic. It was a collaboration between ministries of health of endemic countries and NGO partners. The program was completed in 2016 and was the largest ever infectious disease survey. It estimated that around 190 million people live in areas that require active intervention for trachoma control. The highest burden of disease was found in rural areas of sub-Saharan African countries, some parts of Asia, Australia, Central and South America and the Middle East. The African continent carries about 89% (37 countries out of 41) of trachoma burden reported in 2016.[15] Ethiopia, the country with the greatest burden, had 69.8 million individuals living in areas needing SAFE and Nigeria had 19.9 million.[13] In Tanzania 3.2 million individuals are living in areas that warrant SAFE, with 2.7 million currently receiving MDA treatment.

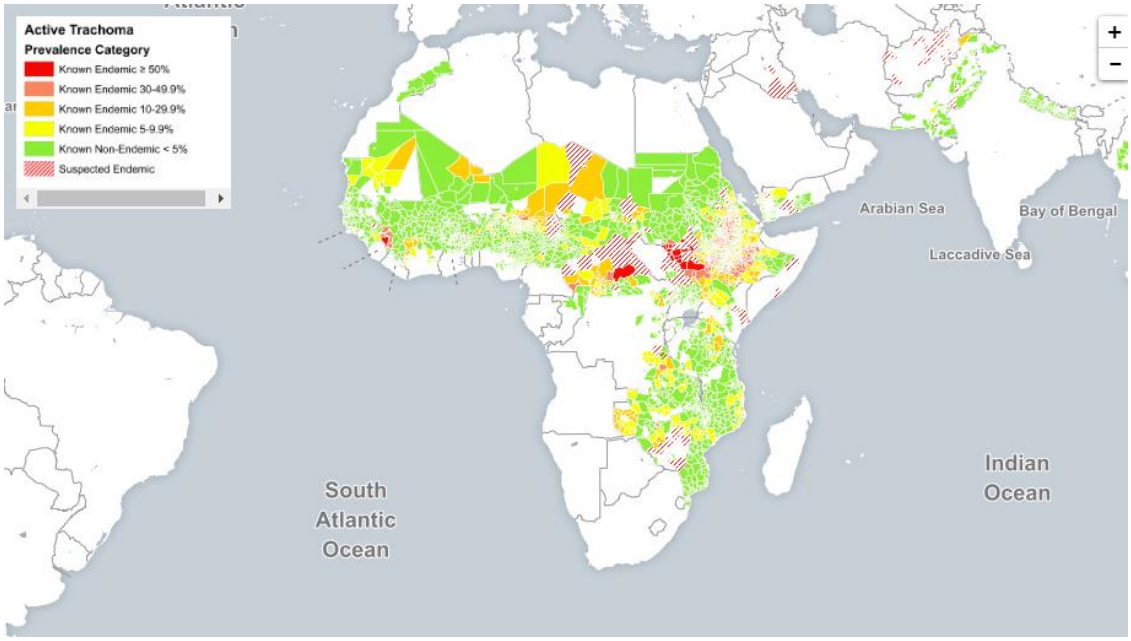
In 2016, 247,000 people were reported as having received trichiasis surgery and about 83 million received antibiotics for trachoma in the African region.[16] The overall worldwide coverage of antibiotic was 44.8% (85million/190million) in 2016 (compared to 29.6% in 2015), of individuals living in areas that warrant SAFE intervention for elimination of trachoma as a public health problem.[16] The highest burden of disease is found in women and children in poor communities, characterised by limited educational opportunities, crowded conditions and limited access to hygiene and sanitation. Recent statistics show that the number of women requiring surgery to correct trichiasis is about four times more than men.[12]

1.5.2. Distribution

In the past few years trachoma has been significantly declining as a result of ongoing efforts to eliminate the disease by the year 2020. For example, the estimated number of people living in endemic districts, at risk of trachoma blindness, has declined from 317 million in 2010 to 157.7 million in 2018. This is over 50% decrease within the last 7 years and rate of decline is increasing with implementation of new control programmes, following GTMP. Several countries have been declared to have achieved the trachoma elimination goals such as Cambodia, Ghana, Islamic Republic of Iran, Lao People's Democratic Republic, Mexico, Morocco and Oman.[13] In addition,

China, The Gambia, Myanmar and Iraq have also claimed to have achieved elimination however, it has not yet been verified by WHO.[13] In Africa, known trachoma endemicity is mostly in the Sahel region and East Africa. The endemic areas are shaped like a *seven* over the continent, crossing from the west to the horn of Africa in the east and then turning south through East Africa (Figure 1.4). Data from the Global Trachoma Mapping Project is being used to guide trachoma control measures by Ministries of Health. However, data is currently lacking from some countries, largely due to inaccessibility from ongoing conflict.

Figure 1.4. A map showing at district level the prevalence of active trachoma in Africa countries. Source: The Trachoma Atlas. Available at: *www.trachomaatlas.org*. Accessed 23rd September 2018.



Trachoma was widespread in Tanzania. From previous reports the prevalence of active trachoma was highest in Central, West and Northern parts, these areas are dry for most of the year.[71-73] However, a recent report showed that there has been a significant reduction in the prevalence of the disease in most parts of the country and the highest burden of disease is now localised in the north of Tanzania, where this study was conducted (Figure 1.5).[13] Areas in Northern Tanzania with a high prevalence of trachoma are mostly occupied by pastoralists. They usually live in compounds with animal enclosures at the centre surrounded by huts with a high density of flies. Access to water is very limited. As a result, unclean faces with many flies surrounding their eyes are commonly seen. This probably facilitates high rates of transmission. The work described in this thesis was conducted on the western side of Mount Kilimanjaro (Figure 1.6).

Figure 1.5: Map showing at district level the prevalence of active trachoma in Tanzania. Source: The Trachoma Atlas. Available: *www.trachomaatlas.org*. Accessed 23rd September 2018.

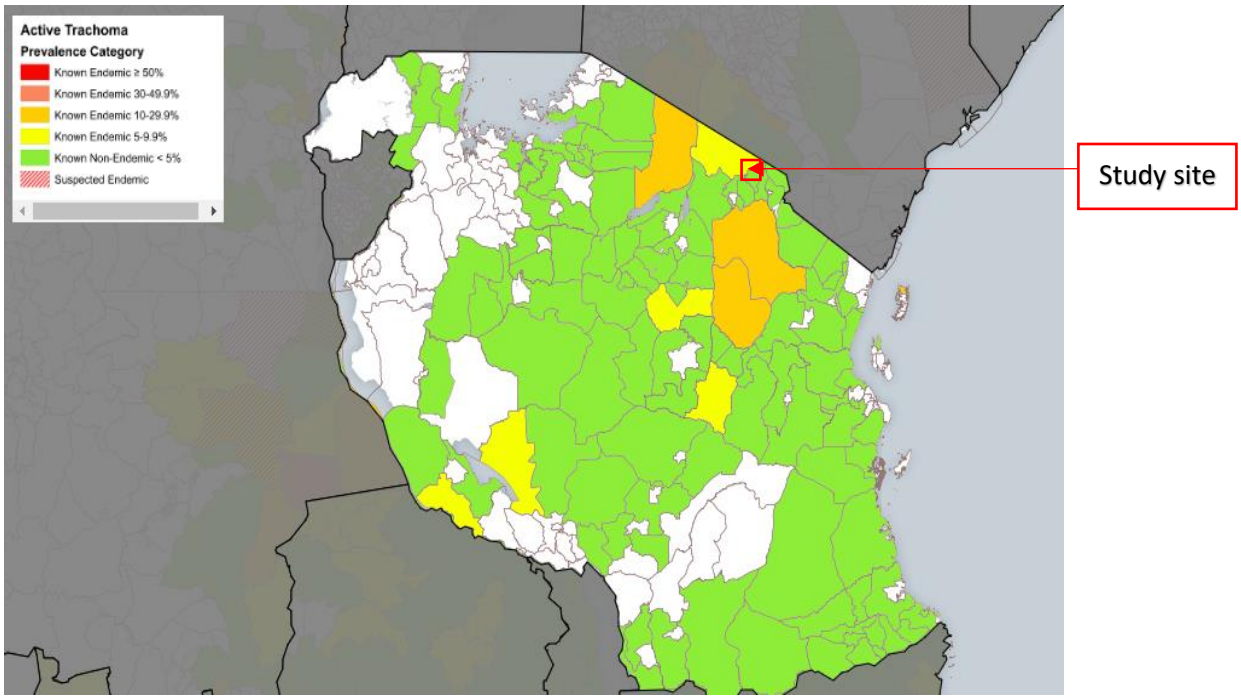
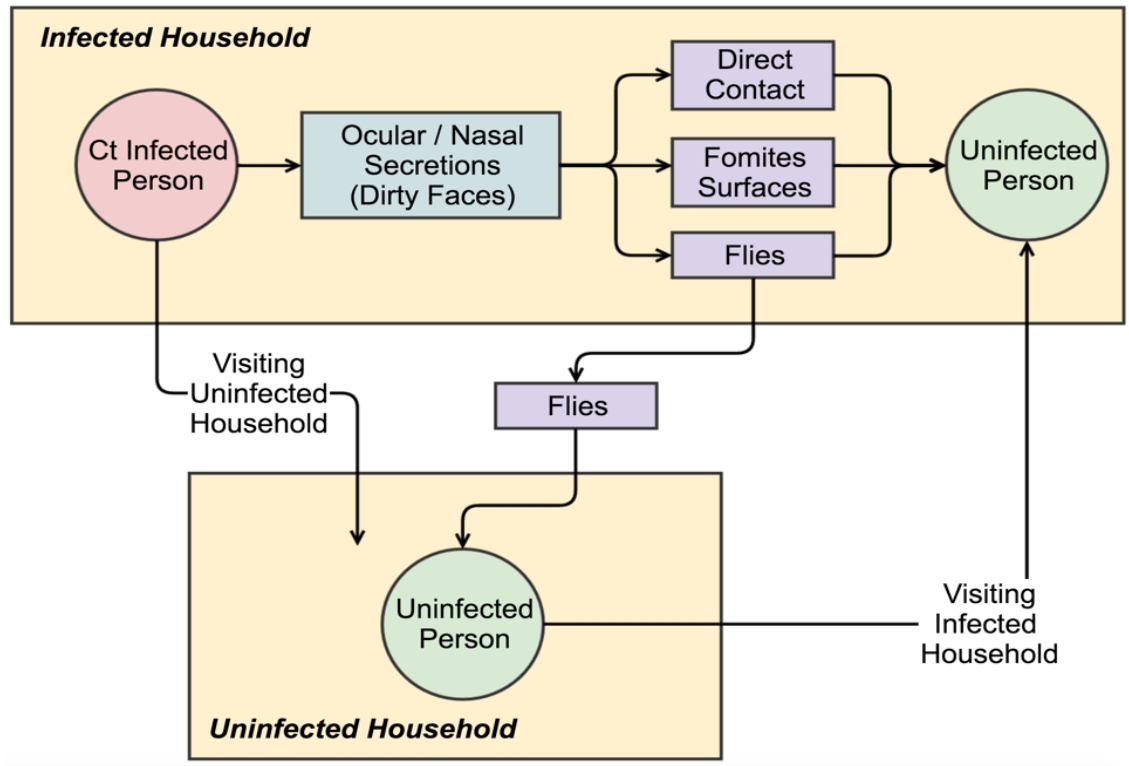


Figure 1.6: Satellite image of part of Kilimanjaro and Arusha regions of Northern Tanzania. The study area has three villages, one in Arusha region and two in Kilimanjaro region. The study villages are located within the black square of the image. Source: https://www.google.com/maps/place/Tanzania/@-2.9616596,37.0059386,67373m/data=!3m1!1e3!4m5!3m4!1s0x184b51314869a111:0x885a17314bc1c430!8m2!3d-6.369028!4d34.888822 Accessed 28th October 2018.

1.5.3. Transmission

The transmission of ocular *C. trachomatis* between people in trachoma endemic settings has not been directly studied in detail. The generally accepted perspective is that transmission occurs through contact with discharge from the eyes, nose or throat secretions of an infected person. *C. trachomatis* may be transmitted on skin, fomites (e.g. clothing and towels) and flies that feed on facial secretions (Figure 1.7). All these routes of transmission in trachoma endemic settings are very common, particularly in children who are the principle reservoir of infection. Because of close contact with children, women are also thought to be at greater risk of being exposed to infection. Trachoma is predominantly a disease of poverty. There are several risk factors, which have been consistently associated with the disease, discussed below in section 1.4.4.

Figure 1.7: Illustration of *C. trachomatis* infection transmission from infected to uninfected persons. This diagram was kindly provided by Professor Matthew Burton, LSHTM



1.5.4. Risk factors of trachoma disease

There are several risk factors for both active trachoma and *Ct* infection, which have been associated fairly consistently in different trachoma endemic populations in different countries. These include:

Age

The prevalence of the disease and infection is highest in younger children. It is likely that this is due to a combination of more frequent exposure to the infection and longer duration. Studies from three different trachoma endemic sites in The Gambia and Tanzania found a strong association between children less than 10 years old and load of infection.[74] A mathematical model using data from a Gambian community examined every two weeks for 6 months found both more frequent and longer duration of infection and active trachoma in children aged less than 5 years when compared to older children and adults.[29] This may reflect a greater exposure of children to chlamydial infection and a less effective immune response to it. Transmission is likely to be more frequent for behavioural reasons: infrequent face washing, playing together and close sleeping conditions with other affected children. Active trachoma is very rare in adults however, it can also be diagnosed in trachoma endemic communities.[75] The trend of disease from infection and active trachoma to scarring complication such as trichiasis to corneal opacification are strongly associated with age.[2]

Sex

Trachoma occurs more frequently in females,[12] with clinical signs more frequent in all stages in the natural history of trachoma, however, this difference between the sexes is only statistically significant at an older age/late stages of disease.[2] In earlier studies from Tanzania active trachoma was diagnosed more frequently in females than males.[2] According to a recent WHO report, females were four times more likely to have trichiasis than males.[12] This is suggested to be due to a greater lifetime exposure to *C. trachomatis* infection from closer contact with children.[76]

Limited water supply

Communities with poor water supply tend to have more trachoma, possibly reflecting general disadvantages in hygiene and sanitation, leading to more transmission.[2, 77, 78] Dirty faces are frequently associated with eye and nose discharge, which may transmit *C. trachomatis* through person to person contact or via flies.[2, 79, 80] Studies done by Bailey and colleagues in The Gambia, and West and colleagues in Central Tanzania found there was a significant relationship between poor accessibility of water, less face washing and active trachoma.[81, 82] Similar findings of association between active trachoma and limited access to water were reported in children from Guinea Bissau.[83] Face washing is thought to reduce transmission to others, by physically removing the infected secretions. However, this relationship has not always been consistently observed, for example, in Niger, Abdou and colleagues found no association between water supply and active trachoma or infection.[84] This study has several shortcomings which the

authors recognised; there was no method to assess whether increased water access was directly associated with face washing as they were only relying on the distance between the houses and water sources. Furthermore, children from both intervention and control villages were given tetracycline eye ointment which could directly interfere with findings and C. trachomatis infection prevalence was higher in intervention villages than control (26% vs 15%, respectively) which might cause some bias due to different infection transmission rates.

Flies and Sanitation

Eye-seeking flies, such as *Musca sorbens,* are hypothesised to carry *C. trachomatis* from infected to uninfected eyes. Multiple studies have found associations between the presence of flies around eyes and active trachoma and *C. trachomatis* infection.[85-88] A study in children aged between 1-5 years found strong association between flies and clinical signs of trachoma but not *C. trachomatis* infection.[78] Facial flies together with less educated household heads in pre-treated communities was associated *with C. trachomatis* infection prevalence at the community level.[89] Societies with little or no access to latrines may have more faecal contamination in the environment. This provides places for flies to breed, resulting in increased fly density in the environment and increased risk of transmitting infection.[90] However, intervention studies have not found consistent results, with some recording an impact on active trachoma and others not.[91, 92]

Crowding

Trachoma tends to cluster within communities, perhaps reflecting the transmission dynamics.[72, 93-95] Humans are the only known biological reservoir of *C. trachomatis* in trachoma endemic settings. Most families affected by trachoma are economically poor with no education. They tend to sleep in the same house/room and probably share clothes, towels, handkerchiefs, beds and many other facilities, which may be contaminated with *C. trachomatis* from secretions of infected individuals. Several studies have found clustering of infection to occur at the bedroom level.[85]

Host Genetics

Individuals living in trachoma endemic communities are thought to have relatively equal exposure to *C. trachomatis* infection, yet they experience different outcomes; while some suffer severely from the disease (progressing to scarring and trichiasis), others suffer less and some not at all.[96] Heritable factors are thought to predispose some people to more severe/scarring disease than others.[73, 97-101] Variation in HLA class I and 2 genes, cell surface receptors, cytokines, transcription factors and extracellular matrix (ECM) enzymes have each been studied for

association with trachoma, using a variety of methods including single nucleotide polymorphism (SNP) typing, variation across loci and family studies (reviewed by Derrick et al).[102] A single genome wide association study did not confirm the genes identified in these previous candidate gene approaches associated with scarring disease but rather confirmed that variation at the pathway level consistent with trachoma transcriptome results was associated with risk of scarring.[101]

Education

Lack of public health education for individuals in trachoma endemic areas may be a risk factor for disease. Communities should be educated about the major risk factors that cause disease and available control measures. Kuper and colleagues reported weak evidence of an association between disease and health education.[103]

1.6. Trachoma Control (SAFE strategy)

Blindness caused by trachoma is a great threat to an individual's quality of life and transforms individuals from being productive into being dependent on others.[104] Due to the public health, social and economic challenges of Trachoma, in 1993 the WHO developed the **SAFE** strategy for trachoma control, designed using prior information from different approaches conducted in different trachoma endemic areas. In 1996 the WHO and other allies including non-governmental organizations and eye health stakeholders initiated a resolution to eliminate the disease as a public health problem. Elimination was defined as reducing the prevalence of trichiasis to less than 0.2% in people aged 15 years and above (or approximately less than 1 case per 1000 people of all ages) and reducing TF prevalence to less than 5% in children aged 1 to 9 years, in formerly endemic districts.[15] The strategy agreed was focused on individuals with trichiasis and infection.[105] There are ongoing efforts in trachoma endemic areas worldwide to eliminate trachoma as a public health problem by 2020 using the SAFE strategy. Four components are included in the strategy as follows:

Surgery to correct trachomatous trichiasis. Prior to the introduction of the SAFE strategy, trichiasis was largely managed through epilation of eyelashes that are touching the eyeball. A similar approach is still in place for some individuals today who have minor trichiasis or decline surgery and in areas where surgery is unavailable or inaccessible. Surgery is the preferred method to manage trichiasis.[106]

In surgery to correct trichiasis, the marginal part of the eyelid is rotated outwards away from the globe, so that the lashes cannot continue to touch the eye.[107] Individuals offered surgery should have one or more eyelashes which turn in and touch the eye.[106] This procedure is performed by trained ophthalmologists, ophthalmic nurses or medical assistants.

Recurrent trichiasis is a major challenge to this control measure; recurrence rates of 60% three years after surgery have been reported.[5] The WHO has recommended two procedures of eyelid surgery; bilamellar tarsal rotation (BLTR) and posterior lamellar tarsal rotation (PLTR). Recently it was reported that more cases of recurrent trichiasis were found in individuals who went for BLTR procedure relative to the PLTR procedure.[9] In the last decade a series of trials have explored alternative approaches to try to improve results, including measures to reduce inflammation after surgery.[3, 7, 108-112] There can be considerable inter-surgeon variation, which has led to developments such as HeadStart, that are used to train surgeons on model eyelids.[113]

In addition to antimicrobial activity, doxycycline was also reported to have both anti-matrix metalloproteinase and anti-inflammatory effects on Trichiasis-Derived Conjunctival Fibroblasts and other body tissues abnormalities.[114-117] As discussed in section 1.7.4 and 1.7.5, TT develops following distortion of normal collagen textures/tarsal plate following prolonged inflammation and abnormal expression of matrix-metalloproteinase in the conjunctiva. Recently, Habtamu and colleagues reported a trial of the effect of oral doxycycline in prevention of recurrence on trichiasis post-operated cases.[108] This was a randomized control trial conducted in individuals aged over 18 years with upper eye lid TT associated with conjunctival scarring who received community-based screening and surgery in an Ethiopian hyperendemic community. They excluded all individuals who had previously received TT surgery. Patients were randomized to doxycycline or placebo in a 1:1 ratio, and given this for 28 days. All participants were examined at 10 days, 1, 6, and 12 months after surgery. At the 12th month, 58 (12%) of individuals who received doxycycline and 62 (12%) of those who received placebo had developed postoperative TT (adjusted OR 0·91, p=0·63). The authors concluded that doxycycline has not associated with reduction of post TT recurrence.

Antibiotics to reduce the reservoir of *C. trachomatis* infection within a community. The most commonly used antibiotic is azithromycin; single oral dose (20 mg/kg up to a maximum dose of 1g). This is not used in children below 6 months of age, who are given tetracycline eye ointment; twice a day for 6 weeks. The WHO has recommended at least three rounds each annually of Mass drug administration (MDA) to all community members if the prevalence of TF is more than 10% in children aged 1-9 years old (TF_{1-9}) in the district and coverage should be at least 80% of the total population. When the prevalence of TF_{1-9} is 5% and above but less than 10%, assessment should be conducted at the community level and treatment should be offered where the TF_{1-9} prevalence is >10%. If the TF_{1-9} prevalence is less than 5% mass treatment is not recommended. One of the key challenges with MDA is that TF is used as a decision factor instead of infection. Due to the delay between the control of infection and the clearance of clinical signs at both individual and population levels, this might lead to treating communities where infection was already brought under control. Currently tests for infection are not being used by trachoma control programmes to guide their use of MDA, with the exception of the Amhara regional control programme where PCR based tests are used within the context of surveillance surveys.

Prior to azithromycin, topical tetracycline eye ointment was recommended to treat trachoma and the dosage was to be applied for at least 6 weeks twice a day, or 5 days consecutively every month for 6 months.[26] There are several challenges of using tetracycline; including the need for supervision during application and the need to apply it multiple times. It is not feasible for

technical personnel to assist in many settings hence the drug has to be left with the caregivers for daily application, introducing doubt about treatment reliability at a community level. Tetracycline may cause discomfort/blurring of vision to the patients. Furthermore, it was not clear if treatment was effective in cases where extraocular sites such as the nasopharynx or nasal secretions were involved. Hence a single oral dose of directly observed azithromycin treatment would be much preferred for effectiveness in control programmes.

A single oral dose of azithromycin was previously reported to be effective in genital *C. trachomatis* infection.[118, 119] Since then several clinical trials were done using azithromycin as an alternative to tetracycline. Bailey and colleagues conducted a clinical trial in The Gambia in the early 1990's comparing azithromycin and tetracycline.[120] Follow up conducted 6 months post treatment found that trachoma had resolved in 76 (78%) of the 97 subjects who received azithromycin, compared with 70 (72%) of 97 who were treated with tetracycline (95% Cl for difference - 6% to 18%). However, tetracycline treatment was delivered by the research team.

Schachter and colleagues led another clinical trial in Egypt, The Gambia, and Tanzania; three doses of oral azithromycin were given in interval of one week to the trachoma endemic villages selected community-wide or treated with 1% topical tetracycline once daily for six weeks.[121] Clinical examination and sample collection were done at baseline and after one year and *C. trachomatis* was detected by ligase chain reaction (LCR). After one year, *C. trachomatis* infection was more reduced in azithromycin treated than tetracycline ((93% versus 77% in Egypt, 78% versus 66% in The Gambia, 64% versus 55% in Tanzania).

Another study conducted in The Gambia included a randomized controlled trial of azithromycin compared to tetracycline without supervision of administration of the ointment.[122] The study included 314 children aged 6 months to 10 years with clinical signs of trachoma who randomly received ether of the two treatments. Six months after treatment children were assessed for clinical signs without testing for *C. trachomatis* infection. A single dose of azithromycin was significantly associated with resolution of intense inflammation ($P = 0.023$, Fisher's exact test). Another study in Tanzania evaluated the effectiveness of a single dose of azithromycin and *C. trachomatis* was detected using PCR.[123] One round of azithromycin in addition to tetracycline was enough to bring down infection from 9.5% at the baseline to 0.1% two years later.

Azithromycin (also known as Zithromax) is one of the three commonly used macrolide antibiotics; the others are erythromyin and clarithromycin. Azithromycin is stable in acid and has a broad range of antibacterial activities. Apart from trachoma, azithromycin is used to treat

a variety of conditions such as sexually transmitted diseases, bronchitis, pneumonia, ear infections and upper respiratory tract infection. It was reported to be effective in treating *Toxoplasma gondii* infection in pregnant women.[124] In patients with cystic fibrosis long term oral azithromycin treatment was associated with a reduced rate of decline in lung function leading to a reduced morbidity and mortality rate.[125] Azithromycin was also reported to be beneficial for individuals with malaria,[126-130] and is used in the elimination program for yaws, which is also planned for eradication by 2020.[131] In combination with sulphadoxine pyrimethamine, azithromycin was reported to prevent low birthweight in Papua New Guinea.[132] In Central Tanzania azithromycin was associated with reduction of diarrhoea in children.[133] A larger 3 country study (in Malawi, Tanzania and Niger) following zithromax MDA specifically conducted to investigate this effect confirmed modest but significant reduction in mortality rate among children aged between 1-59 months.[134]

Azithromycin was further reported to reduce the child mortality rate in Ethiopia.[135] In a mouse model inoculated with *Acinetobacter baumannii*, azithromycin was associated with reduction of lung inflammation.[136] In combination with ampicillin, azithromycin reduced streptococcal sepsis in a murine model.[137] Although widely regarded as safe, azithromycin was reported to be the possible cause of acute and chronic kidney damage in both cystic fibrosis patients and healthy individuals and authors advised that it should not to be used by patients with kidney disease,[138, 139] however, these finding were either case reports or followed prolonged intake of drug. Some studies have reported an association between azithromycin and increased risk of death due to cardiovascular disease,[140] however, other studies did not confirm this association.[141] Known side effects of azithromycin include diarrhoea, nausea, abdominal pain, vomiting, rash, hepatotoxicity, hypersensitivity reactions (for example angioedema, acute generalized exanthematous pustulosis, Stevens-Johnson syndrome, toxic epidermal necrolysis), and *C. difficile* associated diarrhoea.

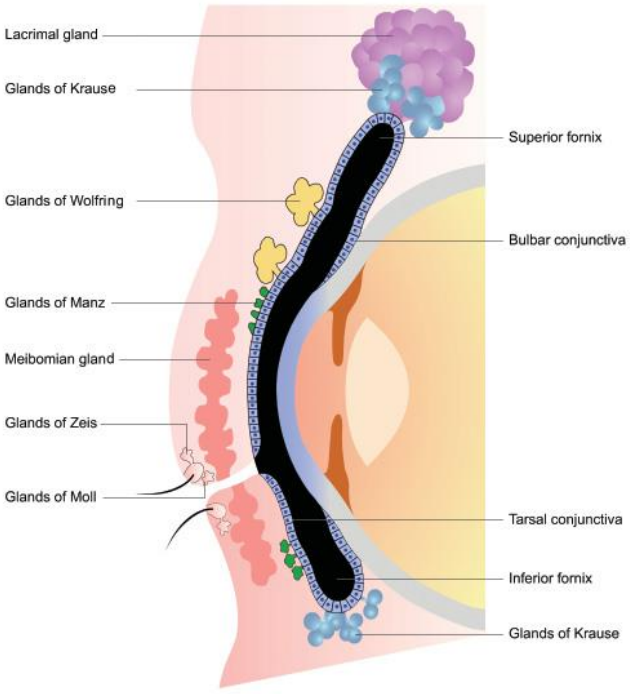
The promotion of **Facial cleanliness** in children and **Environmental improvements (the F and E components of SAFE)** aim to reduce the transmission of trachoma, given the risk factors described above in section 1.4.4. Some epidemiological studies have found an association between dirty faces and active trachoma in children (refer section 1.4.4). Poor sanitation was reported to be associated with breeding sites for eye-seeking flies which can carry eye or nose secretions from infected to uninfected individuals while other finding reported the opposite (refer section 1.4.4). Despite controversial findings about the significance of suppressing transmission of the bacterium, face washing and improving hygiene in the surrounding environment could be generally beneficial beyond trachoma control.

1.7. Immunopathophysiology of trachoma

1.7.1. Physiological barriers

Physiological barriers prevent infection and colonization by many infectious agents at the ocular surface. The eyelids form the primary physical barrier that protects the eye. The ocular surface is coated in the tear film. In the absence of tears the cornea becomes dry and may be subjected to injury and infection.[68, 142] The tear film has three layers: external oily layer, middle aqueous layer and inner mucin layer. Tears are rich in antibodies and antimicrobial peptides and consist of a salty fluid which spreads evenly across the front of the eye to keep it moist and to create an unfavourable environment for pathogens. Evaporation of tears is reduced by an oily outer layer, produced by the Meibomian glands, which lie within the tarsal plate of the eyelid and release secretions from the edge of the lower and upper eyelids (Figure 1.8). Lachrymal glands produce the aqueous layer, which contains many components that contribute to protection.

Figure 1.8: Sagittal view of the eyelids and globe. This figure can be accessed from http://ophthalmologylife.blogspot.com/2012/03/anatomy-of-conjunctiva.html)



Proteins called mucins make up the mucous layer that protects the conjunctiva. Mucins which are produced by corneal surface and conjunctival epithelial cells are an important barrier to pathogens and also act as lubricating agents and clearing molecules.[143] There are several ocular mucins. *MUC7* is derived from the lacrimal gland; *MUC5AC* a gel-forming mucin is among the major mucins expressed by the ocular surface and is reported to be produced by goblet cells; *MUC16* is produced and present in the lacrimal gland and epithelial cells of the nasolacrimal

ducts; *MUC4* and *MUC1* are produced from corneal and conjunctiva epithelia.[144-148] *MUC16* is produced by lacrimal gland ductal epithelium, accessory lacrimal glands and nasolacrimal duct epithelium. The role of *MUC7, MUC5AC, MUC4* and *MUC1* are discussed in more detail in chapter 4.

1.7.2. Innate immune response

Innate immune cells consist of leukocytes (neutrophils, basophils, eosinophils), Natural killer (NK) cells, mast cells, macrophages, and dendritic cells (DCs). Innate immune responses react immediately but do not confer long lasting or protective immunity to the host, however, there is some recent evidence of memory in innate immune cells (trained immunity or innate immune memory), for example, in macrophages and NK cells.[149, 150] Trained immunity was reported to be mediated through epigenetic reprogramming and signals effecting transcription factors.

The innate immune response is initiated by the binding of pathogen-associated molecular patterns (PAMPs) on microorganisms to host cell receptors known as pattern recognition receptors (PRRs). Epithelial cells and circulating cells of the innate immune system (macrophages, dendritic cells (DCs), neutrophils, NK cells) possess PRRs, the most important of which are the TLRs and the nucleotide-binding oligomerization domain (NOD) proteins. These receptors recognise and bind to the PAMPs, enabling cells to recognize microbial structural elements. Upon ligand binding, PRRs induce an intracellular signalling cascade which leads to the activation of transcription factors (e.g. nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)) that bind to nuclear DNA, promoting the production of pro-inflammatory cytokines. For *C. trachomatis* TLR2 and TLR4 are important for recognition. TLR2 is the PRR for the *C. trachomatis* cell wall component peptidoglycan. TLR4 is the PRR for *C. trachomatis* cell membrane component lipopolysaccharide (LPS) and heat shock protein.

In vitro studies have shown that initial innate immune responses to *C. trachomatis* infection occur at the epithelial cell surface, causing epithelial cells to produce pro-inflammatory cytokines and chemokines 20–24 hours after infection has been established and lasted for 2–4 days throughout the chlamydial developmental cycle.[151, 152] These include Chemokine ligand 5 (CCL5) Chemokine ligand 16 (CXCL16), CXCL10, CXCL1, Interleukin 1α (IL-1α), IL-8, IL-12, IL-6, GM-CSF (granulocyte-macrophage colony stimulating factor), TNF (tumour necrosis factor), and GRO (growth-related oncogene). Release of these proinflammatory mediators triggers the rapid recruitment of neutrophils, macrophages, dendritic cells (DCs) and natural killer (NK) cells to the site of infection. T and B lymphocytes are also recruited after activation of the adaptive immune response (discussed further below). Innate immune cells limit the infection through

phagocytosis and destroying infected cells (NK cells). In addition, antimicrobial peptides produced by epithelial cells limit infection through mediating inflammation on epithelial and inflammatory cells, influence diverse processes as proliferation, immune induction, cytokine release, chemotaxis, protease-antiprotease balance.[153-157]

Infected epithelial cells and macrophages are activated during infection which continue to promote the influx of additional inflammatory cells. Epithelial cells, neutrophils, macrophages, DCs and NK cells release different substances such as matrix metalloprotease (MMP) 7, 9, 10, and 12, tissue inhibitor of matrix metalloproteinase 1, and secreted protein acidic cysteine-rich-like 1, which are thought to cause damage to the extracellular matrix. Studies in murine models of genital chlamydial infection have suggested that innate immune responses mediated through TLR2 receptors contribute to the development of fallopian tube scarring.[151, 158] TLRs, dendritic cells and macrophages/monocytes have been linked to wound healing and fibrosis through triggering inflammation during tissue repair in chronic diseases.[159-162]

Findings from human studies support the role of the conjunctival epithelium in the innate immune response to *C. trachomatis* infection. Both immunohistochemistry and gene expression studies of the conjunctival epithelium showed upregulation of proinflammatory and antimicrobial mediators such as IL-1α, IL-1β, TNF-α, PDGF, CXCL5, and S100A7.[163-166] Biopsy and gene expression studies found Major Histocompatibility Complex (MHC) class I which was expressed throughout the epithelial cells and MHC class II on the superficial layers increased in individuals with active trachoma.[167, 168] Presence of markers for Natural Killer (NK) cells and neutrophils indicated that these cells were recruited to the site of infection and activated in both human and animal models of infection and disease.[165, 169, 170] NK cells control chlamydial infection through the production of interferon gamma (IFN-γ) and by killing stressed or infected host cells.[171] In animal models NK cells are found at site of infection within 12 hours. Inflammation can contribute to the development of chronic tissue damage, however, secretory leukocyte protease inhibitors produced by epithelial cells and elafin can contrast the action of matrix metalloproteases, helping to reduce the damage.[172] MHC is a cell surface molecule encoded by a large gene family responsible for recognition of foreign antigens. It is referred to as the human leukocyte antigen (HLA) complex in humans. Each molecule has one binding site that can bind different peptides. Genes in this complex are categorized into three groups: class I, class II, and class III. MHC class I and II proteins bind peptides that belong to a group of molecules known as the Immunoglobulin Supergene Family, which includes immunoglobulins, T cell receptors (TCRs), CD4, CD8, and others. CD4 binding to MHC class II and CD8 binding to MHC class I.

Many studies have reported a strong association between trachomatous scarring and innate immune responses. Scarring was associated with increased inflammatory cell infiltrates on hematoxylin and eosin staining and CD45+, CD8 + and CD56+ cells (but not CD3+ cells) were enriched, leading the authors to suggest that these were NK cell infiltrates.[173] Several genes of the innate immune response have been frequently found to be highly expressed in people with trachomatous scarring. These include psoriasin (S100A7), defensin-β4A, *INDO*, *TNFA*, *IL-1B*, *DEFB4A*, *CXCL5*, *SAA1*, *ARG*, *NOS2*, serum amyloid A1.[174] In addition, scarred conjunctivae were found to have greater infiltrates of NK cells when compared with controls.[173] As *C. trachomatis* infection is very rarely detected in adults with scarring, the factors driving innate immune responses in the conjunctival epithelium are not clear.

1.7.3. Adaptive immune response

The adaptive immune response is mediated by T and B lymphocytes. These cells are antigen-specific and once exposed to their antigen and activated by dendritic cells they generate effector and memory cells. Memory cells enable a faster and amplified response on second exposure to that same antigen. B cells generate antibodies, whilst T cells can be split into two major phenotypic groups, MHC class II restricted CD4+ T helper cells and MHC class I restricted CD8+ cytotoxic T cells. Cytotoxic T cells kill infected cells by recognition of antigen expressed on the cell's MHC class I. Helper T cells help other immune cell types by producing cytokines and are essential for activating intracellular killing in macrophages and high-affinity B cell antibody responses. Subtypes of helper T cells are induced by different cytokine milieus and have different roles and functions. T helper 1 (Th1) cells, induced by Interleukin-12 (IL-12) and IL-18 are primarily associated with intracellular infections and promote the activation of intracellular killing mechanisms in macrophages by release of IFN-γ, IL-6, IL-12 and TNFα.[175, 176] Th2 cells, induced by IL-4 are more associated with extracellular infections and generation of antibody responses.[175-177] T regulator cells (Treg) are a subset of $CD4^+$ T cells that supress inflammation. They are involved in the prevention of autoimmune diseases by maintaining self-tolerance; suppression of allergy, oral tolerance, asthma and feto-maternal tolerance; however, they may limit beneficial responses by suppressing inflammation required for clearing infection and limiting anti-tumour responses.[178, 179] Markers of Tregs (but not exclusive of other T cells) are CD25, CTLA-4, GITR, LAG-3, CD127 and FOXP3. Treg cells secrete the anti-inflammatory cytokines IL10 and TGFβ. Th17 cells are another pro-inflammatory subset of $CD4^+$ T cells. Their development requires the presence of specific factors, TGFβ with IL-6 or IL-21, their growth and stabilization is dependent on IL-21, transcriptional control involves STAT3, RORγt and RORα and survival and maintenance requires IL-23.[180-182] Th1 (CD4+ IFN-γ+), Th2 (CD4+ IL-4+) and Treg (IL-10, TGFβ) cells inhibit the development of Th17 cells.[183] Th17 cells and their associated cytokines

(IL-17) appear to have a major coordinating role in inflammation in many situations, regulating the production of different pro-inflammatory cytokines/chemokines and recruiting neutrophils.[184-193]

C. trachomatis infection triggers adaptive responses and protection from infection that is thought to rely mainly on T cell responses (cell-mediated immunity (CMI)) and B lymphocyte responses. Studies from urogenital infection animal models showed that mice cannot clear infection in the absence of T cells.[194-196] Athymic Mice (without lymphocytes) infected with *C. trachomatis* were not able to clear infection within 20 days, in contrast to the control group, and infection was still detectable after 265 days.[196] However, athymic mice were able to clear infection after adoptive transfer of T cells from wild type mice. IFN-γ is largely responsible for infection clearance. IFN-γ is produced by NK cells, CD4+ T and CD8+ T cells. IFN-γ induces the activation of enzymes such as indoleamine-2,3-dioxygenase (IDO), which inhibits chlamydial growth through depletion of the essential amino acid tryptophan, and nitric oxide synthase (iNOS), which kills bacteria inside phagolysosomes and depletes intracellular iron (also limiting the growth of chlamydia).[197-199] Immunohistochemical staining of conjunctival biopsies from cynomolgus monkeys found infiltrates of CD4+ T-helper cells, CD8+ T cells and B lymphocytes were predominant in the conjunctival inflammatory response to *C. trachomatis* infection in active trachoma relative to a control group.[200] Immunohistochemistry studies from biopsy samples obtained from human subjects with active trachoma found more inflammatory cell infiltrates of CD4+ T-helper cells, CD8+ T cells and B lymphocytes in trachoma specimens than the control group.[163] Gene expression studies following human responses from trachoma endemic areas found expression of IFN-γ was higher in individuals with *C. trachomatis* infection but was reduced in individuals with active trachoma without *C. trachomatis* infection.[164] Studies in children whose active clinical signs persisted for over 6 months showed that they had weaker lymphoproliferative responses to *C. trachomatis* antigens compared to children whose clinical signs resolved over that time.[201]

T cells and particularly IFN-γ are thought to be essential for the clearance of chlamydial infections. Whilst adaptive immune responses are crucial for clearance of infection, their role in the papillary inflammation that drives scarring progression (particularly in adults without *C. trachomatis* infection) is unknown.

1.7.4. Protection from reinfection

In trachoma endemic communities ocular *C. trachomatis* infection frequency and duration decrease with age and infection is rarely detected in adults.[27, 29] This has led to the conclusion

that protective immune responses develop (albeit slowly) following multiple previous infections, enabling adults to quickly clear reinfection. Non-human primates inoculated with serotype E of *C. trachomatis* developed severe but self-limiting conjunctivitis and when re-challenged with the same strain, there were only mild clinical signs that rapidly resolved.[202] Studies from human volunteers in the 1960's found that individuals were protected after reinfection with the same strain but were still susceptible to infection from other strains.[203, 204] This was consistent with a trachoma clinical trial in the 1960's where serovar-specific immunity from a live vaccine was induced in the short-term.[205, 206] Some degree of protective immunity through T-cell mediated memory responses are likely to contribute to the rapid resolution of reinfection. Studies in animal models found a live-attenuated vaccine was safe and effective in preventing *C. trachomatis* infection.[207] In the study, Cynomolgus macaques were infected with a trachoma strain deficient for the 7.5-kb conserved plasmid and were then challenged with plasmid-free strains, this resulted in a short lasting infection without any ocular complications. When the animals were challenged with multiple infections of the same attenuated (plasmid free) strain, no inflammation was induced and infection was associated with induction of anti-inflammatory immune responses. However, when challenged with organisms possessing the plasmid, partial protection was detected and animals had lower infection loads than controls.

Several recent studies from animal models and humans have demonstrated the role of different T lymphocyte subtypes in protection from chlamydial infection. MHC genotyping of Cynomolgus macaques infected with plasmid-free *C. trachomatis* suggested that MHC class II alleles were associated with protection from infection.[207] A study in women aged ≥16 years evaluated T cell phenotypes in PBMCs based on the women's urogenital *C. trachomatis* status.[208] *C. trachomatis* infected women had higher expression of T cell activation markers ($CD38^+HLA\text{-}DR^+$), Th1- and Th2-associated effector phenotypes ($CXCR3^+CCR5^+$ and $CCR4^+$, respectively), and T cell homing marker (CCR7) for both $CD4^+$ and $CD8^+$ T cells relative to uninfected women. During follow up after treatment, expression of these markers associated with CD4+ and CD8+ T cells was reduced. Women who were not re-infected during follow-up had relatively higher expression of $CD8^+$ T cells co-expressing CXCR3 with CCR5 or CCR4 than the re-infected group. A longitudinal study in women at high risk of urogenital *C. trachomatis* infection measured the production of IFN-γ from $CD4^+$ and $CD8^+$ peripheral blood cells. The authors found that women who became infected had a lower frequency of IFN-γ positive CD4+ cells relative to women that remained uninfected, whereas the frequency of CD8+ T cell responses was similar in both groups.[209] These findings show that both CD4+ and CD8+ T cells are involved in protection from *C. trachomatis* infection.

1.7.5. Pathogenesis of scarring

Tissue fibrosis (scarring) is a product of activated mesenchymal and fibrotic cells.[210] Myofibroblasts, the main effectors in tissue fibrosis, produce excessive ECM and cause tissue contraction and distortion, ultimately leading to loss of tissue function.[211-213] Fibrosis has been shown to be driven by chronic infection and inflammation in a number of diseases such as microvascular disease, toxic epithelial injury, diabetes mellitus, myocardial infarction, chronic obstructive pulmonary disease, atherosclerosis, chronic colitis, stroke, and skin diseases such as scleroderma.[159, 210]

Our research group has previously conducted a number of case-control studies of individuals with scarring trachoma (compared to normal matched controls). A study from Ethiopia found several pro-inflammatory cytokines such as IL-1β, CXCL5, and S100A7, and tissue-remodelling proteins MMP-7, MMP-9 and MMP-12 and HAS3 were up-regulated.[166] These individuals also had increased IL-10, which may have a regulatory anti-inflammatory effect in this context. These findings were replicated in a study of a similar design conducted in Tanzania.[174] MMPs are a family of proteolytic enzymes, which have been linked to the development of scar tissue in many diseases.[214-218] MMPs degrade the ECM and promote contraction of scar tissue.

Cytokines and chemokines appear to play a central role on both the initiation and progression of fibrosis through the recruitment of inflammatory cells to the site of tissue injury.[219, 220] During pathological responses, fibrosis is associated with production of proteolytic enzymes, angiogenic factors, fibrogenic cytokines, growth factors and fibrillar extracellular matrix.[221] TGFβ, PDGF, CTGF and FGF are the most prominent growth factors associated with fibrosis.[222-227] Many of these promote the recruitment of leukocytes, local proliferation of fibroblasts and the accumulation of ECM proteins.[210] Several factors that were most strongly associated with scarring trachoma in the studies from Ethiopia and Tanzania (IL-1β, CXCL5, and S100A7) generally arise as part of innate immune responses. In particular they have been shown in both tissue culture experiments and in disease models to be produced by epithelial cells. This suggests that the epithelium may have a central role in producing the chronic inflammatory response that is believed to drive trachomatous scarring. However, data from longitudinal studies are required. *In vitro* studies are consistent with these inflammatory responses, with the release of cytokines and chemokines, provoked by *C. trachomatis* infection of non-immune cells (epithelia and endothelia).[151, 228]

1.7.6. Evidence from histology studies

Despite much effort in recent decades the immunopathogenesis of scarring trachoma remains poorly understood. Several studies were conducted in the past using conjunctival biopsies collected from trachoma patients. In children with active trachoma conjunctival biopsy tissues contained inflammatory cell infiltrates in which macrophages, polymorphonuclear leucocytes, dendritic cells and T lymphocytes (both CD4+ and CD8+) were found.[163, 167] *C. trachomatis* intracellular inclusion bodies were seen in epithelial cells and were strongly associated with inflammatory cell infiltrates. The presence of these infiltrates in the stroma was linked to the presence of follicles, mainly containing B-lymphocytes and surrounded by a T lymphocytic layer. Infiltrates of T and B lymphocytes, macrophages, plasma cells and neutrophils were surrounding the follicles. In the stroma of healthy conjunctival tissue collagen types I, III and IV were found whereas in active trachoma cases there was more collagen type V in the stroma with infiltrates.[229] In individuals with trachomatous conjunctival scarring biopsy tissues had a thin conjunctival epithelium associated with loss of goblet cells and with compact scars replacing the normally loose vascular stroma underneath the epithelium.[230] The thick scar tissue was largely composed of collagen type V. There was an increase in the deposition of collagen type IV in the conjunctival basement membrane of cases, replacing the normal collagen types I and III.[231] The presence of new and different collagens changed the orientation of collagen fibrils from horizontal to vertical arrangement, resulting in distortion of the tarsal plate which eventually causes in-turning of eyelids and eyelashes.[230] The tarsal plate maintained its thickness in trachoma cases however, glands such as the meibomian glands were severely weakened, which was associated with chronic inflammatory infiltrates. Inflammatory infiltrates dominated by T cells were also found in studies on biopsies of scarred individuals with trichiasis.[232] However, the protective and pathological correlates of disease are not well defined, partly because this requires detailed long-term studies. It remains unclear which immunological responses lead to the tissue damage and scarring that causes blindness.

1.7.7. Microbiology and Microbiome

The papillary inflammation associated with scarring and disease progression is often found in the absence of *C. trachomatis*, particularly in adults.[233] The factors driving this inflammation are unknown but might include normally benign things such as dust or components of the normal ocular microflora (which might exacerbate inflammation in an epithelium that has already been compromised by chlamydial infection), or it is possible that other pathogenic infections may be responsible. Changes in mucin expression at the ocular surface might alter the interaction of the epithelium with flora which may lead to more inflammation.

Ocular surface colonization by resident bacteria may serve as a defensive mechanism by inhibiting the growth of pathogenic bacteria.[234] Two recent studies in mice have examined the role of ocular commensal organisms in protection from pathogenic infection. In one study, colonisation of the conjunctiva with *Corynebacterium mastitidis* led to the production of IL-17 by γδT cells, which protected against pathogenic infection with *Candida albicans* and *Pseudomonas aeroginosa*.[235] In the second study, colonisation of germ-free mice with coagulase negative Staphylococcus (CNS) induced resistance against *P. aeruginosa* keratitis.[236] Changes to the ocular surface can also allow some ocular commensal organisms to behave as pathogens and cause disease or allow other pathogens to gain access and colonise the ocular surface.[237] In animal models (monkeys) which were frequently inoculated with *C. trachomatis* or developed clinical signs of trachoma were inoculated with three common human pathogens (*Haemophilus influenzae*, *Haemophilus aegyptius* and *Streptococcus pneumoniae*); they found no difference in susceptibility to infection between animals with trachoma and other animals without trachoma. The authors suggested that *C. trachomatis* is essential in the development of blindness, while other bacteria may only be important in determining the severity of disease through increasing the amount of discharge, hence preventing clearance of infection or increasing chances of *C. trachomatis* reinfection.[238]

In humans there is some evidence that ocular non-chlamydial bacteria are more commonly found in trachoma-diseased individuals than controls. Several studies have examined the relationship between the presence of active trachoma, *C. trachomatis* and other bacterial pathogens. However, these studies tended not to be population based and pre-dated sensitive molecular tests for *C. trachomatis*. Studies conducted in Tunisia, Morocco and Egypt frequently cultured *Haemophilus spp,* pneumococci, *Moraxella sp*, diplobacilli, *Neisseria spp*, *Staphylococcus aureus, Streptococcus viridians, Corynebacterium xerosis, Staphylococcus pyogenes, Escherichia coli* and diphtheroids from the conjunctiva.[22, 239-243] *Haemophilus spp* was the most dominant in all studies done in North Africa. The Tunisian study found an association between ocular bacterial pathogens and seasonal epidemics of purulent conjunctivitis but not normal flora.[22] Both pathogens and commensal flora were significantly reduced after antibiotic treatments,[240-242] however, with the exception of *Haemophilus,* other species were highly affected by environmental changes in the absence of antibiotics.[241] Conjunctivitis was frequently seen in young children and was associated with the prevalence of both pathogens and normal flora.[241, 242] A study in India found no association of bacterial isolates in individuals with different stages of trachoma.[243] The prevalence of pathogenic bacteria in children with trachoma was significantly reduced after a single dose of azithromycin in a longitudinal study done in a trachoma endemic area of Nepal,[244] however, the distribution of specific bacterial species

remained the same. These studies were either cross sectional,[22, 242, 243, 245, 246] case-control [240] or longitudinal with a short follow up duration,[239, 241, 244] and relied on either bacterial culture or staining methods. No convincing link has been identified between intense/follicular inflammation and scarring.

Recent population based studies have also identified the presence of other bacteria in individuals with active trachoma, trachomatous scarring, trichiasis and corneal opacification.[3, 69, 245-247] Species were either strongly associated with disease episodes or were more frequently detected in diseased individuals. In Tanzania, in a cross sectional study of children from an area with a low prevalence of active trachoma, TF was independently associated with the presence of *Streptococcus pneumoniae* and *Haemophilus influenzae* (type B and non–type B).[246] In the same study no association was found between active follicular trachoma and *C. trachomatis* infection. In a cross-sectional study conducted in Northern Tanzania in adults with TS and control subjects, bacteria were cultured from 54% cases and 34% controls.[245] Amongst TS cases the proportion that was culture positive was directly proportional to the severity of scarring. To date there is one published study of the association between scarring progression and non-ocular bacterial infection in which Hu and colleagues conducted a two year longitudinal study in Northern Tanzania investigating progression of scarring and non-chlamydial ocular bacteria.[248] They recruited 800 participants with scarring but without trichiasis and were assessed at baseline, 6, 12, 18, and 24 months. Samples were collected for laboratory bacterial culture and *C. trachomatis* detection. At 24 months data of 452 participants were available for comparison and photographs were assessed directly side-by-side between baseline and 24 months for scarring progression. There was significant association between progression of scarring and non-chlamydial ocular bacteria, particularly those considered pathogens (OR = 2.39; 95% CI: 1.10 to 5.16). However, contrary to this study, a cross sectional study from central Tanzania done in women aged between 18 and 47 years found no association between the presence of scarring and non-chlamydial bacterial infection.[249]

1.8. Current status of Trachoma/C. trachomatis Vaccine Development

The C. trachomatis vaccine strategies that are generally being considered are based on induction of neutralising antibodies, prevention of Ct entry to the host epithelial cells and prevention of bacterial cells replication.[250] For several decades there have been attempts to develop vaccines against trachoma/chlamydia, however these have only induced short-term or partial protection.[251, 252] A study of children in The Gambia who were visited regularly for more than 6-months showed that children who quickly cleared infection or disease had more focused antibody responses,,[253] suggesting that protection is associated with focused antibody responses to a few specific antigens that can be targeted for vaccine design. Currently there are ongoing trials both in animal models and humans in which Chlamydial Major Outer Membrane Protein (MOMP) has emerged as the most suitable target, which can be delivered as a combined systemic and mucosal vaccine. MOMP is the main component of the only C. trachomatis vaccine in current human phase I clinical trials and it is the focus of several candidate vaccines. This clinical trial is sponsored by Statens Serum Institut in collaboration with Imperial College London: "Safety and Immunogenicity of a Chlamydia Vaccine CTH522 (CHLM-02)" with ClinicalTrials.gov Identifier number NCT03926728. Two main designs of CTH522, are CAF01 liposome designed to aid cellular immunity and AL (aluminium hydroxide) to aid antibody production. Another ongoing clinical trial (number NCT02787109) recently published preliminary findings that found that recombinant protein subunit was safe.[254] All vaccinated groups were able to induce anti-CTH522 IgG seroconversion while none of the placebo group experienced seroconversion. Of all evaluated CTH522, CAF01 immunogenicity profile was better which promises further vaccine development.

1.9. Summary

Trachoma remains a significant public health problem which affects the world's most marginalised communities. *C. trachomatis* pathogenesis is challenging to understand because of the complex immunological interaction between host and pathogen and the length of the disease's natural history. There is a gap of understanding of which components play a major role in protection from chlamydial infection and development of scarring. Evidence suggests that innate immune responses, driven by the conjunctival epithelium, play an important role in tissue damage and development of fibrotic complications. Detailed longitudinal studies are required to examine the contribution of *C. trachomatis* infection and components of the host immune response to progressive scarring trachoma.

2. Research Aims and Objectives

2.1 Rationale

Repeated conjunctival infection with *C. trachomatis* during childhood is thought to drive trachomatous inflammation and scarring. However, data supporting the role of repeated infection in driving scarring incidence and progression is limited. This warrants further investigation in order to improve our understanding of trachoma pathogenesis and control. The immunopathological mechanisms underlying how *C. trachomatis* infection and trachomatous inflammation drive scarring progression are also unclear. Understanding these mechanisms could enable development of therapeutic or prophylactic interventions designed to prevent or halt conjunctival scarring. Furthermore, it is not known whether these immune responses are impacted by treatment with the immunomodulatory antibiotic Azithromycin for trachoma control.

The purpose of this study was to investigate *C. trachomatis* infection, clinical signs of inflammation and gene expression in a longitudinal cohort of children, in order to identify the key risk factors and immunological pathways associated with disease and progression. To achieve this, a four-year longitudinal study was conducted in a cohort of 666 children aged 6 to 10 years at baseline from a trachoma endemic community in northern Tanzania. Children were visited every three months for four years and clinical signs and conjunctival swabs were collected for detection of *C. trachomatis* and host gene expression.

2.2 Hypotheses:

1. The relationship between *C. trachomatis* infection and active trachoma is weaker after the initiation of MDA
2. Distinct risk factors are associated with different clinical stages of trachoma
3. Distinct immune responses are associated with *C. trachomatis* infection, conjunctival inflammation which persists after the clearance of infection, and scarring trachoma
4. Azithromycin has an immunomodulatory effect on conjunctival gene expression responses
5. Repeated ocular infection with *C. trachomatis* and clinical signs of inflammation are associated with increased risk of incident and progressive scarring trachoma

2.3. Specific objectives:

1. To perform a literature review and meta-analysis to examine the relationship between *C. trachomatis* infection and active trachoma at a population level before and after MDA for trachoma control
2. To perform a literature review of longitudinal studies to describe the rates and risk factors for trachomatous scarring progression in all clinical stages of the disease
3. To describe clinical signs and infection prevalence at the baseline time-point of the four-year longitudinal study and to investigate associations with conjunctival gene expression responses
4. To analyse the stability of conjunctival gene expression responses prior to MDA and to determine the changes that occur following MDA treatment
5. To describe the rates of scarring incidence and progression in the four-year longitudinal cohort study and to examine risk factors (*C. trachomatis* infection, TF, TP, age and sex) associated with scarring progression.

3. Research Methodology

3.1 Overview

3.1.1 Study country

This study was conducted in the United Republic of Tanzania (Tanzania), one of the five East Africa Countries (Figure 3.1). It has the largest population in East Africa and the lowest population density with a current population estimate of about 54 million.[255] Two third of the population is living in rural areas and youths (<25 years) account for 2/3 of the total population. The domestic growth product (GDP) per capita was estimated to be 1304 USD in 2017, ranked 159 out 187 worldwide. The country's Human Development Index (HDI) value in 2017 was 0.538, one of the lowest HDI in the world ranking 154 out of 189 countries/territories.[256] Tanzania is one of 42 trachoma endemic countries identified by the GTMP from 2012 to 2016.[12] 2018 data from the WHO estimated that about 3,216,046 potentially require intervention with mass antibiotic drug treatments (MDA), facial cleanliness, and environmental hygiene for trachoma control.[13] A total of 2,651,671 (8.2%) had received antibiotic treatment and 2120 were operated on to correct trachomatous trichiasis in 2017. In a recently published report from Tanzania which surveyed 104,959 participants for clinical signs of trachoma in 23,171 households; 44,511 children aged 1 to 9 years old examined for follicular trachoma (TF) and 65,255 aged 15 and above examined for trichiasis.[257] Approximately 1.9% of children were found with TF and 0.3% of adults found with trichiasis. Out of 31 districts surveyed, 3 qualified for mass MDA with azithromycin. To control the disease requires multi-sectoral involvement including health services and water supply. Unfortunately, areas with most trachoma cases are falling under poor health services and long-lasting shortage of clean water.

Figure 3.1. Map of Tanzania. Source: http://ontheworldmap.com/tanzania/tanzania-regions-map.html Accessed 28th October 2018.

3.1.2 Study Site

This study was conducted in between Mount Kilimanjaro (Kilimanjaro region) on the eastern side and Mount Meru (Arusha region) on the western side. The field site is about 100km away from Kilimanjaro Christian Medical centre (KCMC) where this study was coordinated, which is about 2 hours' drive by Landrover. Despite the rocky terrain the roads are passable throughout the year, however, during the rainy season mud can be a challenge in some areas (Figure 3.2). Health services are not easily accessible to this community due to either government facility shortage or nature of the community members being pastoralists hence some temporarily move away from their homes searching for pastures and water. The area is dry for most of the year and has lots of dust, with the exception between February and May when there is rainfall.

Ethical clearance to perform this study was received from the National Institute for Medical Research (NMR) (Reference. NIMR/HQ/R.8a/Vol. IX/1213) and the London School of Hygiene & Tropical Medicine Ethics Committee (Application number 5988). The study was then introduced to Regional Medical Officers, District Medical Officers and the village leaders. From a pilot study carried out in 2012 and previous studies by our group in the neighbouring villages, the prevalence of active trachoma (and *C. trachomatis* infection) in 1-9 year olds was found to be above 10%, this qualified for MDA as per WHO recommendations.

The pilot study was conducted by collecting conjunctival swab samples selected randomly from children aged between 6 and 10 years from the study site which were analysed in the KCMC-KCRI biotechnology laboratory (KCRI-BL) for *C. trachomatis* detection. *C. trachomatis* DNA was isolated from RNAlater stored swabs using the QIAamp DNA minikit (Qiagen, Crawley, United Kingdom) and was detected using the Amplicor CT/NG kit (Roche Molecular Systems, Branchburg, NJ) as previously described.[85] Out of 41 samples tested 16 (39.0%) were positive. From this study was planned to collect samples from this previously untreated community before the first MDA treatment, which was scheduled to be done 6 months (determined by the availability of Azithromycin) after commencing the study and after three time-points of examination.

Figure 3.2. Infrastructure, geographical features and habitat of the study area. Clockwise from top left; challenges of transport during rainy season, children with high density of flies which may be associated with transmission, drought, livestock grazing

3.2 Study designs and sample size

This longitudinal cohort study aimed to investigate factors associated with progression of scarring and host immune responses in individuals with *C. trachomatis* infection, active trachoma (TF/TP) and scarring sequalae in children aged between 6-10 years at the beginning of the study. A total of 666 children were found eligible during registration, however, during recruitment only 616 were available whereas the other 50 either refused to participate or moved away during enrolment. They were visited every three months for four years. There were three potential outcomes to consider: the progression of scarring, the persistence of inflammatory disease between visits, and the number of episodes of *C. trachomatis* infection available for study.

Clinical Scarring: Previous study in the same area but different village found prevalences of TF and *C. trachomatis* were 13.7% and 5.3% respectively in children under 10 years.[246] Furthermore other unreported findings from the same area suggests that some 30% of 10-year olds in

trachoma endemic communities have microscopic scarring detectable by In vivo Confocal Microscopy (IVCM), which is not clinically apparent. Using the data from earlier studies from a neighbouring community with the same patterns of life, we estimated that over a 4-year period it is plausible that 20% (5.4% per annum) would have shown evidence of incident or progressive scarring in a cohort of 6-10-year olds in a community with 30% active trachoma (TF) prevalence in 1-9 year olds. Assuming that we wish to detect a risk factor (exposure) present in one-third of subjects, which increases the relative risk of emergent scarring by 2, we made the following calculations in **Table 3.1.**

Table 3.1. Sample size calculation.

Power	Confidence (equivalent number of comparisons)	Estimated sample size	10% dropout	Total
0.8	95%	291	29	**320**
0.9	95%	384	38	**422**
0.8	99% (5)	417	42	**459**
0.8	99.5% (10)	468	47	**515**
0.8	99.9% (50)	591	59	**650**

To detect fine scarring, side-by-side digital photographs were compared between the baseline (or second time-point for those who were not present during enrolment in the baseline) and final time-point (or 16th time point for those missed in the final time-point) by an experienced ophthalmologist (Matthew Burton) to 448 children (whose were data available).

Inflammatory Trachoma: In a study of 650 6-10 year olds subjects in a stable endemic community with 30% disease prevalence in 1-9 year olds we expect that at each time point, some 20% (130) of subjects aged 6-10 would have disease from previous information. In earlier longitudinal studies from The Gambia, 60% of clinically diseased 6-10 year olds resolved their clinical signs of inflammatory disease at 3 months of follow up.[27, 29] Allowing for annual treatment of the affected communities, loss of independence arising from the fact that the same subjects may become diseased again at another follow up, and dropout, it is reasonable to expect that a cohort of 650 subjects followed for 4 years would yield more than 243 disease (active trachoma) episodes. For gene expression data measured in the cohort, values were normalized relative to the expression of HPRT1 in the same sample, to adjust for variable concentrations and then interpreted by comparing fold change of control versus cases (*C. trachomatis* infection, TF, TP and scarring) adjusted for age and sex.

C. trachomatis Infection: We anticipated that 10% (65) of subjects would show evidence of *C. trachomatis* infection at baseline. A cohort study of 650 6-10-year olds was therefore expected to yield adequate numbers of infected participants for the studies outlined.

SAFE strategy; All community members who were found with trichiasis were offered surgery free of charge at the nearest available health centre. The majority of individuals with TT accepted the offer and most of those surgeries were done at KCMC referral Hospital. All community members were offered free of charge mass drug administration (MDA) once annually for the first three years. This was done together with district eye coordinators who were responsible for dispensing drugs to people while our field team were registering their personal details. The drugs consisted a single-dose of azithromycin i.e. 1g for adults and 20mg/kg for children and tetracycline eye ointment applied twice a day in both eyes for six weeks which was offered to pregnant women and infants aged below six months. Under guidance of village leaders, drugs were given mostly at their homes, where we asked all household members who were around to come back to their homes for treatment. In some cases, children were given drugs at their schools if the time for treatment was due when children were at schools. This was done through guidance of teachers and village leaders. All community members who received drugs were registered in our database. Some people refused the treatment for different reasons, mostly based on previous MDA history, some believed themselves to have healthy eyes and others were away from their homes mostly for livestock activities because the majority of these community members are pastoralists. We had opportunity to promote health education messages in the communities about face washing and general hygiene environments. It was positively taken by most community members, however, some interpreted it in a negative way; telling them about face washing was perceived as insulting them as it was interpreted that we see them as dirty. This could have been rectified through using their leaders and some of educated individuals among themselves.

3.3 Procedures for sample collection and processing

3.3.1 Selecting eligible families for study and Informed Consent

The main factors considered when recruiting participants were age and being a resident of one of three study villages. All children aged between 6 and 10 years were eligible for being included in the study. Individuals who had clinical disease other than trachoma at the beginning of study which could have been confused with trachoma diagnosis were excluded from the study. In collaboration with the regional eye care teams of Kilimanjaro and Arusha Regions, we identified a number of communities with an ongoing problem with active trachoma in children of at least 30%. These communities were contacted, and the nature of the study explained. A census of the

children was conducted, and children aged 6-10 years (both male and female) were enrolled into the study if their parents or guardian gave consent. The children enrolled into the cohort may or may not have had active trachoma at baseline, although overall >30% had clinical signs of active disease. We included all children aged 6-10 years (male and female) and excluded children under 6 years or older than 10 years and/or that had other chronic conjunctival disease, such as vernal conjunctivitis.

The study was conducted in three trachoma endemic villages. Prior to commencing the fieldwork activities in January 2012, the team met with the leaders of each of the three communities to explain the purpose and nature of the study. Following agreement between village leaders and our team, several community meetings were held to sensitise the entire community about the proposed research project and related activities. During the enumeration phase the team visited all households in the study area. At each household they met with parents / legal guardians of the children who would be potentially eligible for enrolment. The parents / guardians of the child were informed about nature of the study and asked if they would be willing for their child to participate. The nature of the study was explained in detail in either Kiswahili or Maasai by the field worker. There was an opportunity to discuss and ask questions. Finally, if the parent or guardian agreed to allow the child to be enrolled into the study this was documented on a consent form in Kiswahili including their signature or thumb print and witnessed by a third party. Following the agreements between two parties, children were visited at either their home or at school where they were examined, samples were collected and treatment was offered if the MDA schedule was due. Collecting samples from schools took less time and cost because the majority of children were available all at once, and setting up facilities for examination were done only once instead of multiple times at different households. In schools the study was introduced at the beginning and teachers were informed about presence of participants at their school. Therefore, during follow up time, the list was presented to teachers and they kindly assisted us to find the children from their classes. However, teachers had no mandate on enrolment of children to the study. After enrolment children were invited for examination as detailed below.

3.3.2 Field team training, travel and working facilities

Field team and field facilities were previously established by Prof. Matthew Burton. Some members of the field team had previously been working in trachoma fieldwork for years; however, before commencing the study a short training was conducted at KCMC to refresh previous procedures and to introduce new ones. Ophthalmic nurses (Patrick Massae and Aiweda Malisa) each had experience of over 10 years in trachoma fieldwork. Fieldworkers (Alex

Pallangyo and Phillipo Mollel) who were assigned the task of informed consent were from the study area and were very familiar with the majority of the society's language; they also recorded participant's personal details and grading scores. Conjunctival photographs were taken by qualified and experienced persons (Henry Marielle and Kelvin Mbuya). An experienced driver (Antipas Massae) was employed who managed driving conditions despite the poor transport infrastructure challenges (**Figure 3.3**).

Figure 3.3. Sample collecting process. Clockwise from left: travelling to the field for children examination, preparation of tent behind the car for examination process, examining eyes and collecting swab samples from the eye.

The following were brought for field work; consent forms (only at beginning of the study), the list of study participants file, a laptop for clinical data collection, clinical data collection paper forms (as backup), boxes containing empty and RNA*later* pre-filled tubes for swab storage, a cool box containing icepacks, examination gloves, preservative-free proxymetacaine hydrochloride placed inside the cool box throughout the field work, 2.5X binocular magnifying loupes with bright torch for clinical examination, a digital camera (Nikon D90 with 105mm Macro lens) for ocular conjunctival photography (including spare batteries) and waste disposal bags, which were returned to KCMC for proper disposal.

Samples were collected by the nurse wearing hand gloves which were changed after each individual examination. Left eyes of the children were anaesthetised with a drop of preservative-free proxymetacaine hydrochloride 0.5%w/v so that participants did not experience any discomfort. The upper conjunctiva of the left eye was examined by the nurse using x2.5 loupes and a bright torch and graded for clinical signs using the 1981 WHO "FPC" detailed grading system.[26] The conjunctiva was photographed using a digital camera and then two swab samples

were taken. The first swab was horizontally rubbed against the conjunctiva four times, with a quarter turn each time, and then stored in labelled RNAlater tubes for gene expression analysis and *C. trachomatis* detection. The same procedure was followed for the second swab collection however, that was stored in dry tubes for future study of non-chlamydial infections. After every 50 clinical swab samples were collected, an air control swab was collected. This was done by waving the swab in the air near to participants everted eye for a few seconds and then the same storage and downstream procedures were followed as for clinical swab samples. Air control swab samples were labelled like clinical swab samples to avoid laboratory bias. There was no loss of specimen. Swab samples stored in RNAlater were labelled CR001, CR002, CR003, etc and those stored dry were labelled CD001, CD002, CD003, etc. All swab samples were kept in a cool box packed with icepacks in the field and were transferred to KCMC later on the same day for further storage (Figure 3.4). The dry swab samples were stored in -80^{0}C freezers on the same day, while the RNAlater stored samples were stored at 2-8^{0}C in the fridge overnight to maximize absorption before prolonged storage in -80^{0}C freezer in the following day.

Figure 3.4. Sample storage and processing facilities. Clockwise from left: KCRI-BL where samples were stored and processed, Biorepository were samples were stored, Sample processing facilities and ViiA7 PCR machine used for both gene expression and *C. trachomatis* detection analyses.

An Access database was created for data collection in the field, with only a very limited number of individuals who accessed it. All data collection forms and consent forms were archived in a secure office locked in a cabinet. Data was entered onto a password-protected computer.

3.3.3 Laboratory team training and working facilities

Most of the laboratory work was done at KCRI-BL, Moshi, Tanzania using the laboratory facility the project had already established. Only *C. trachomatis* detection of baseline and second time-point samples was done in the UK at LSHTM laboratories because the samples were tested using diagnostic droplet digital PCR assay that had been evaluated against Roche CT/NG Amplicor (a ddPCR machine is not available at KCRI-BL). At all remaining time-points including the second time-point, *C. trachomatis* detection and conjunctival gene expression profiling were done at KCRI-BL. *C. trachomatis* detection for the second time point was done both in London and Moshi using different detection methodologies for comparison purposes. The relative diagnostic performance of the *C. trachomatis* qPCR assay used for the majority of this study was also formally evaluated in a separate study.[258] In the laboratory, experiments were performed by myself and two junior laboratory technologists (Karim Mtengai and Elias Mafuru), each at different times, who were recruited to work alongside me under close supervision. A post-doctoral research fellow (Dr Tamsyn Derrick) from LSHTM later joined the team and helped to

oversee the laboratory work. All team members were trained about the ethics of the study as per KCRI regulations which requires all laboratory users to attend Good Clinical Laboratory Practise (GCLP) training and various challenges were solved as required.

3.3.4 Preparation of dry and RNAlater tubes for field work

Sample tubes for swab collection during field work (discussed above) were prepared in the pre-PCR preparation room using gloves. Sterile 2-millilitre screw cap tubes were packed in sample storage boxes (each stores 100 tubes) for dry swab sample collection. The same number of clean 2-millilitre screw cap tubes were filled with 200µl of RNAlater using an automatic dispenser and then packed in the storage boxes. The boxes were then handed to the field team.

3.3.5 Gene expression procedures

Genes of interest were selected following previous findings from Tanzania and The Gambia by Prof. Matthew Burton, Prof. Martin Holland and myself. These genes were selected because they were thought to have a significant role in trachoma. Previous work from The Gambia, Ethiopia and Tanzania reported their differential expression in active trachoma, scarring and trichiasis.[101, 165, 166, 259-261] Primers and probes for each target were pre-printed onto TaqMan Low Density Array cards (Thermo Fisher Scientific), providing a high-throughput platform for gene expression profiling of a large number of samples. Several cards were initially tested and were found to work before commencing the work. At baseline 91 genes were tested on two separate array cards. From analysis of baseline results, 46 genes were chosen as they were found to be most related to infection and/or clinical signs. The selected 46 genes were used for the rest of follow up time-points. At the baseline, expression of three different reference genes: HPRT1, GAPDH, and RPLP0 were measured. However, only HPRT1 was used throughout for normalization as it was expressed at a relatively similar level to the majority of other transcripts of interest, whilst GAPDH and RPLP0 expression was very high. Data were analysed using relative quantification by comparing control versus cases by using the Delta delta CT method.[262] Extraction of RNA and gene expression protocols are presented in appendices 3, 5 and 6.

3.3.6 *C. trachomatis* infection detection procedures

The procedure for *C. trachomatis* detection by qPCR is presented in appendices 3 and 4. Three genes were targeted; *Homo sapiens* RNase P/MRP 30-kDa subunit gene (RPP30) (human endogenous control gene for insuring that sample was successfully extracted), *C. trachomatis* outer membrane complex protein B (OMCB) (single copy gene from *C. trachomatis* chromosome), and Plasmid open reading frame 2 (*pORF2*) gene and primers and probes sequences are displayed in table 3.2. Detection of infection using droplet digital PCR (ddPCR) at

baseline was described by Roberts and colleagues,[263] this was done in London. Following this, a qPCR assay for detection of *C. trachomatis* by qPCR on the ViiA7 thermal cycler at KCRI-BL was optimised for use for all subsequent time-points.

Table 3.2. Oligonucleotides used in *C. trachomatis* detection.

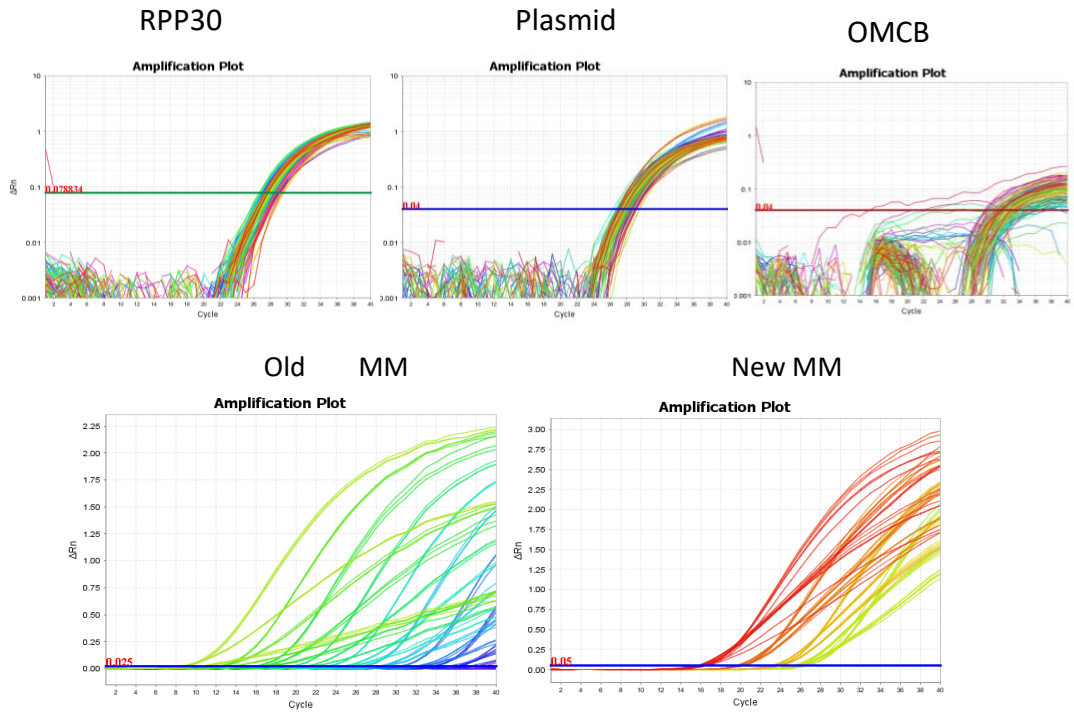
Targets/ Product sizes	primers and probe sequences
RPP30/65 bp	forward: 5' AGATTTGGACCTGCGAGCG 3', reverse: 5' GAGCGGCTGTCTCCACAAGT 3', probe: 5' [HEX] TTCTGACCTGAAGGCTCTGCGCG [BHQ1] 3'
OMCB/106 bp	forward: 5' GACACCAAAGCGAAAGACAACAC 3', reverse: 5' ACTCATGAACCGGAGCAACCT 3', probe: 5' [FAM] CCACAGCAAAGAGACTCCCGTAGACCG [BHQ1] 3'
Plasmid/109 bp	forward: 5' CAG CTT GTA GTC CTG CTT GAG AGA reverse: 5' CAA GAG TAC ATC GGT CAA CGA AGA Probe [NED] 5' CCC CAC CAT TTT TCC GGA GCG A [BHQ1] 3'

3.3.7 Optimization of *C. trachomatis* detection using quantitative PCR (qPCR) in KCRI-BL

One of the most critical parts of this work was optimization of *C. trachomatis* detection at KCRI-BL. The primers and probes used in this work were previously reported,[263] and were developed into a triplex assay for *C. trachomatis* detection by the trachoma research group at LSHTM. ddPCR was used for absolute quantification of nucleic acid with the reaction mixture of 20µl and a concentration of 0.3µM each primer and probe (PLASMID/RPP30), and 4.95µl of unknown quantity sample DNA. PCR thermal cycling conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

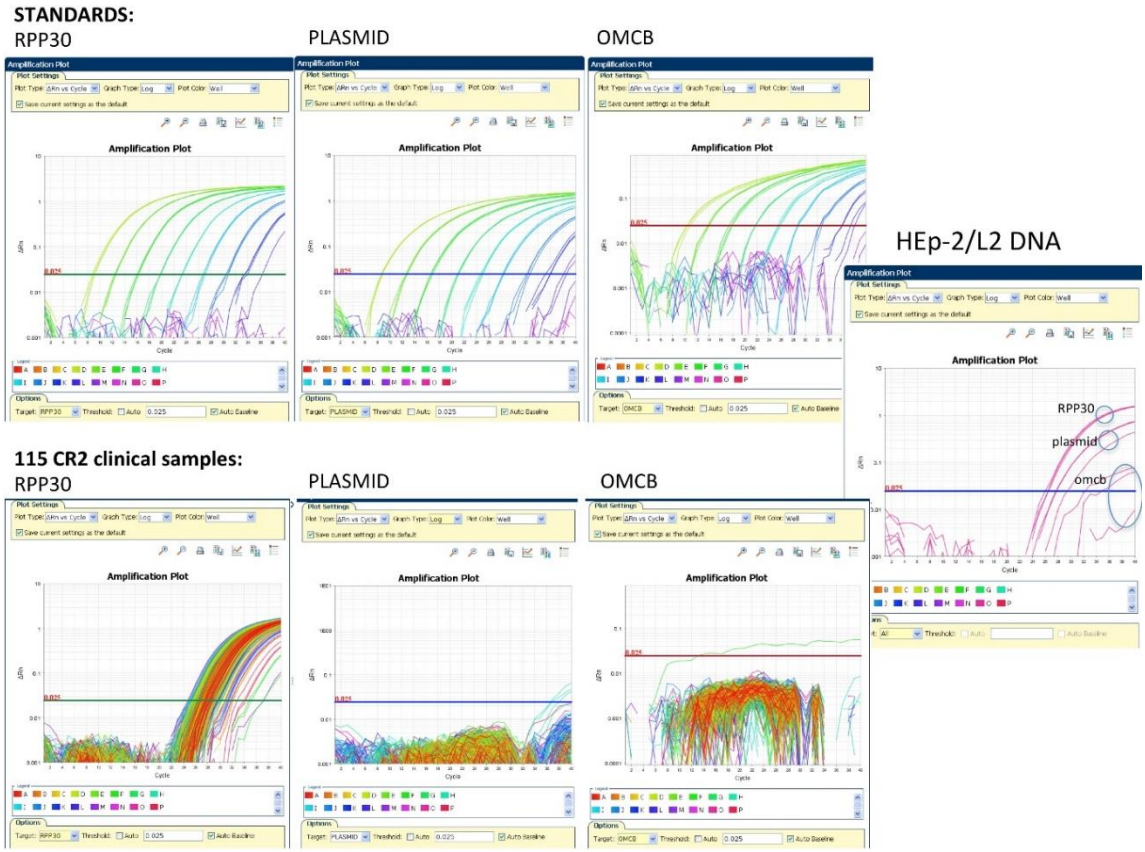
Twenty-four clinical samples were tested using the ViiA7 PCR machine in which all were amplified. However, OMCB produced abnormal curves whereas both Plasmid and RPP30 produced logarithmic curves (Figure 3.5). The status of all 24 samples were known before (by ddPCR) and not all were positive, therefore this was false positivity of the plasmid target. The similar amplification between the plasmid and RPP30 channels suggested that there was fluorescence leakage across the two fluorophores, while the unusual curves of OMCB were thought to be due to the long storage of master mix (retesting using new master mix generated normal OMCB curves) (Figure 3.5).

Figure 3.5. Amplification plots of 24 clinical samples using three targets showing amplification curves



New standards, primers and probes from LSHTM were used and the ATTO fluorophore used on the original plasmid probe was replaced by NED. Four known *C. trachomatis* positive clinical samples, 115 unknown clinical samples, standards and a positive control sample (*C. trachomatis* strain L2 cultured in HeLa cells in London, scraped into RNAlater, sent to KCRI-BL were extracted on site using a DNA/RNA Purification Kit (Norgen Biotek Corp, Canada) were tested. The standards and *C. trachomatis* L2 DNA worked on all 3 channels, although amplification of L2 DNA appeared less efficient than amplification of standards, perhaps indicating some sample inhibition as results of uncleaned nucleic acids from protein and other cells debris or reagents used in the procedure (Figure 3.6). In clinical samples RPP30 was detected (with an expected CT value ~27) but OMCB and Plasmid were not detected. This might have been a result of inhibition in clinical samples (perhaps a result of carry-over of inhibitors during the extraction process, either from the extraction kit, the swab or the sample itself) and a low concentration of chlamydial DNA compared to RPP30.

Figure 3.6. Amplification plots of three targets of *C. trachomatis* detection, showing amplification curves of standards, positive control (L2) and clinical samples



To check that all three targets were present in clinical samples, conventional PCR was run with all three primer pairs separately (RPP30, plasmid and OMCB) data not shown. OMCB and plasmid bands were detected by gel electrophoresis for the standards, L2, *C. trachomatis* positive clinical samples (n= 3), and no OMCB or plasmid bands were detected for *C. trachomatis* negative clinical samples (n=3). The RPP30 band was particularly weak. Chlamydial DNA was therefore present and amplified by the primers, using the same master mix as used in qPCR. This suggested that the problem was due to the probes failed to separate the reporter dyes from quencher dyes resulted into no signal, perhaps in combination with sample inhibition.

The standards, L2 and known *C. trachomatis* positive clinical samples were next tested using SYBR green dye (which is incorporated into double stranded DNA) on a Rotorgene thermal cycler, using the same primers pairs. The standards and L2 amplified RPP30, OMCB and plasmid targets with the expected sigmoidal amplification curves and cycle threshold values <30 cycles in single plex assays using SYBR green Table 3.3. Some of the RPP30/OMCB/plasmid wells did not amplify for the clinical samples, however, this was inconsistent within samples and correlated with a low sample volume. Of the *C. trachomatis* targets that did amplify in clinical samples, CT values were <30, suggesting that target was present. However, the curves were not

sigmoidal but more linear at 45 degrees, suggesting the reaction was less efficient and again indicating the presence of inhibitors or sub-optimal reaction chemistry in clinical samples.

Table 3.3. Cycle threshold values of all three targets (run in separate wells) in standards and *C. trachomatis* positive clinical samples tested using SYBR green on a Rotorgene qPCR machine

SYBR	**Cycle threshold values**		
	RPP30	OMCB	PLASMID
Standard 8	13.62	10.6	11.71
Standard 7	17.16	15.46	15.67
Standard 6	20.76	18	19.46
L2	25.15	18.85	18.44
CR1-041	39.09	26.37	28.55
CR1-250	23.26		
CR1-218		26.41	29.15
CR1-262	23.05	27.94	28.85

In summary, plasmid and OMCB targets failed to reproducibly amplify in *C. trachomatis* positive clinical samples using TaqMan master mix on the qPCR ViiA7 machine. End-point PCR and qPCR using SYBR green showed that the primers amplified the product and the target was present in the clinical samples. The assay worked well with standards and a positive control sample but failed with clinical samples for plasmid and OMCB, whereas RPP30 worked well for both standards and clinical samples. This is possibly due to the high concentration of RPP30 in all clinical samples relative to chlamydial targets. These data suggested that some degree of sample inhibition in clinical samples (but not in the relatively cleaner standards/positive control sample), in combination with inefficient reaction chemistry, led to a lack of amplification of the least abundant targets.

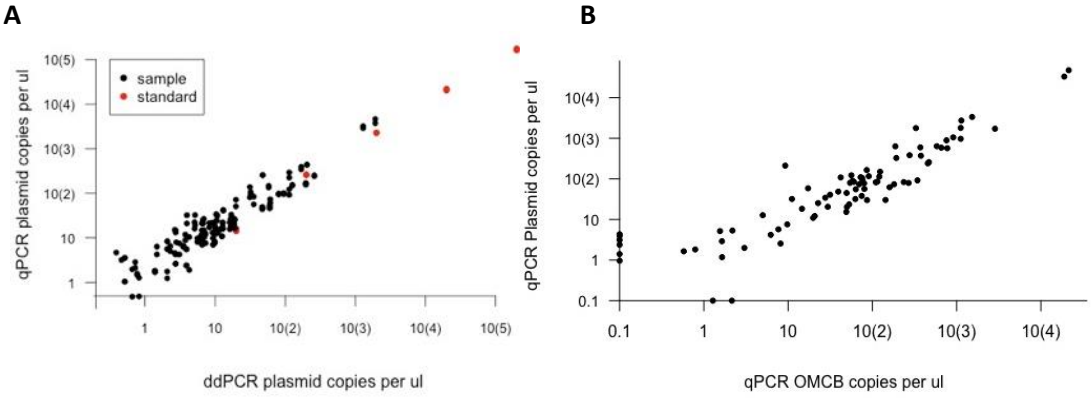
New TaqMan Multiplex Master Mix was ordered and performance was found to be much better than TaqMan Universal Master Mix. TaqMan Multiplex Master Mix is designed for multiplex reactions (whereas Universal is not) and therefore contains a higher concentration of reaction components. In order to evaluate the assays performance in clinical samples, samples from the second cohort time-point were tested by ddPCR at LSHTM (for plasmid and RPP30 targets only, due to sample volume contraints) and by qPCR in KCRI-BL and results were compared. Relative to ddPCR, qPCR had a sensitivity of 100%, specificity of 97.2%, positive predictive value of 83% and negative predictive value of 100% (Table 3.3). There was a good correlation between estimated target concentration by qPCR and ddPCR, and also between OMCB and plasmid

targets by qPCR (figure 3.7). This qPCR assay performance was deemed sufficient and was used to test all subsequent time-points for *C. trachomatis*. The protocol for this assay was attached in the appendix 4B.

Table 3.4. *C. trachomatis* qPCR results in cohort time-point 2 samples relative to ddPCR.

	qPCR negative	qPCR positive
ddPCR negative	445	13
ddPCR positive	0	65

Figure 3.7. Correlation between target concentration estimated by A) qPCR and ddPCR and B) between OMCB and plasmid chlamydial targets, in clinical samples from time-point 2.



3.4 Analyses of data

Clinical data collected in Access were transferred to STATA for analysis. Data were merged with gene expression results and *C. trachomatis* detection results exported from the ViiA7 PCR machine.

TaqMan is a molecular technique that amplifies nucleic acid concurrently with generation of detectable specific signals.[264] It utilises a pair of primers and non-extendable probes. Probes are short specific sequence oligonucleotides which bind within the region delimited by primers. Probes differ from primers because they cannot be extended by Taq polymerase due to lack of free OH group and presence of both reporter (florescent molecule/dye) at 5' end and quencher (quenching fluorescent signal from the reporter) at 3 end'. Primer extension product starts from the 5' end toward 3' end using Taq polymerase. Probe binds to downstream (5' → 3') of primers during PCR reactions, labelled with reporter and quencher is degraded by 5'-3' endonuclease activities of Taq polymerase following hybridisation to specific target for fluorophore base detection.[265, 266] Degradation of the probe releases the fluorophore from it following break of the proximity to the quencher, consequently releasing the quenching effect and allowing

fluorescence of the fluorophore.[267] Energy transfer from reporter to quencher (Fluorescence Resonance Energy Transfer) inhibit fluorescence due to proximity to each other, however following separation of quencher from reporter by the enzyme, reporter emits fluorescence with a dye-specific wavelength. Emitted fluorescence is equivalence to the amount of PCR product accumulated and that the process repeats to every cycle without interference of accumulated PCR product. Fluorescence signals permit quantitative measurements of the accumulated product during exponential stages of the PCR. The technology referred to hydrolysis probes are designed to increase the specificity of quantitative PCR. TaqMan has advantages over SybrGreen following presence of Probes; TaqMan detects only specific PCR product hence used in multiplex reactions and contribute to susceptibility of mutations in the template. However, TaqMan is relatively more expensive.

Interaction terms were tested in some of analyses in this thesis. Interaction occurs when there are three or more variables which are related. Statistically, an interaction term refers to a situation in which the effect of one independent (causal) variable to outcomes depends on or is affected by a second independent (causal) variable specifically when these causes cannot be pooled together. Referring to chapter 4 of this thesis in methodology section in the 4th paragraph the relationship between clinical signs and infection was analysed separately before and after treatment data and then tested for interaction effect of these two variables (pre- and post-treatment) to assess whether the association between clinical signs and infection was similar. Also in the 7th chapter, methodology section (subheading 'Statistical analysis', 3rd paragraph) the change in gene expression was assessed following different time-points and then tested for interaction before and after MDA. Therefore interaction helps to expand understanding of the relationship between different variables and have significant implication on interpreting the results.

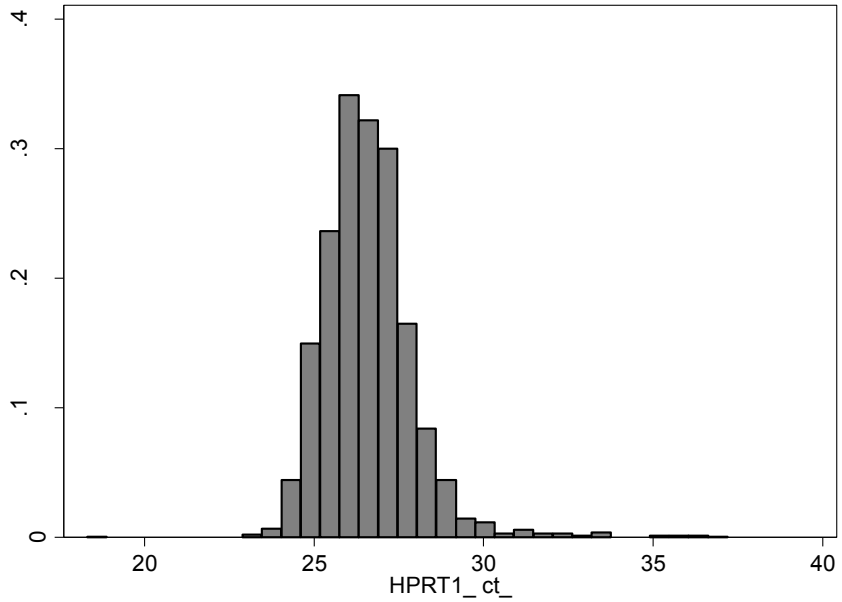
3.5 Summary

Samples were successfully collected every 3 months for 4 years, with three annual MDA-azithromycin treatments. The total number of samples collected at each time-point of examination is summarised below (Table 3.5). Sample collection at every time-point took about three weeks in total. No adverse events, social harms or protocol violations were reported in the study. Overall, the majority of human housekeeping gene (HPRT1) expression CT values were below 30 and were normally distributed, which allowed parametric statistical tests to be used (Figure 3.8).

Table 3.5. Statistics of examination time-points

Time-points	Date Completed	No. children seen
1	25 March 2012	506
2	22 May 2012	536
3	28 September 2012	473
4	23 November 2012	468
5	18 February 2013	478
6	17 May 2013	472
7	30 September 2013	472
8	15 November 2013	459
9	7 February 2014	451
10	12 May 2014	409
11	2 September 2014	433
12	24 November 2014	432

Figure 3.8. Normal distribution of the human housekeeping gene, HPRT1, with the majority of CT values below 30 in all time-points.



4. The relationship between Active Trachoma and ocular *Chlamydia trachomatis* infection before and after mass antibiotic treatment

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

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SECTION A – Student Details

Student	Athumani M. Ramadhani
Principal Supervisor	Prof. Matthew J. Burton
Thesis Title	Studies on the Development of Scarring Trachoma in Tanzania

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	PLOS Neglected Tropical Diseases		
When was the work published?	October 26, 2016		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	N/A		
Have you retained the copyright for the work?*	**No**	Was the work subject to academic peer review?	**Yes**

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	N/A
Please list the paper's authors in the intended authorship order:	N/A
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed the systematic search. I extracted the data. Matthew Burton performed the duplicate data extraction. I wrote a first draft of this paper. Comments from co-authors were considered. I performed the data analysis with guidance from Matthew Burton, David Macleod and Tamsyn Derrick.

tudent Signature: [redacted] Date: 29.10.2018

upervisor Signature: [redacted] Date: 27/10/2018

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RESEARCH ARTICLE

The Relationship between Active Trachoma and Ocular *Chlamydia trachomatis* Infection before and after Mass Antibiotic Treatment

Athumani M. Ramadhani[1,2]*, Tamsyn Derrick[1,2], David Macleod[3], Martin J. Holland[1], Matthew J. Burton[1,4]

1 Clinical Research Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, United Kingdom, **2** Kilimanjaro Christian Medical Centre, Moshi, Tanzania, **3** Tropical Epidemiology Group. London School of Hygiene & Tropical Medicine, United Kingdom, **4** Moorfields Eye Hospital, London, United Kingdom

* athumani.ramadhani@lshtm.ac.uk

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Abstract

Background

Trachoma is a blinding disease, initiated in early childhood by repeated conjunctival infection with the obligate intracellular bacterium *Chlamydia trachomatis*. The population prevalence of the clinical signs of active trachoma; "follicular conjunctivitis" (TF) and/or "intense papillary inflammation" (TI), guide programmatic decisions regarding the initiation and cessation of mass drug administration (MDA). However, the persistence of TF following resolution of infection at both the individual and population level raises concerns over the suitability of this clinical sign as a marker for *C. trachomatis* infection.

Methodology/Principle Findings

We systematically reviewed the literature for population-based studies and those including randomly selected individuals, which reported the prevalence of the clinical signs of active trachoma and ocular *C. trachomatis* infection by nucleic acid amplification test. We performed a meta-analysis to assess the relationship between active trachoma and *C. trachomatis* infection before and after MDA. TF and *C. trachomatis* infection were strongly correlated prior to MDA (r = 0.92, 95%CI 0.83 to 0.96, p<0.0001); the relationship was similar when the analysis was limited to children. A moderate correlation was found between TI and prevalence of infection. Following MDA, the relationship between TF and infection prevalence was weaker (r = 0.60, 95%CI 0.25 to 0.81, p = 0.003) and there was no correlation between TI and *C. trachomatis* infection.

Conclusions/Significance

Prior to MDA, TF is a good indicator of the community prevalence of *C. trachomatis* infection. Following MDA, the prevalence of TF tends to overestimate the underlying infection prevalence. In order to prevent unnecessary additional rounds of MDA and to accurately

ascertain when elimination goals have been reached, a cost-effective test for *C. trachomatis* that can be administered in low-resource settings remains desirable.

Author Summary

Trachoma is the leading infectious cause of blindness worldwide, caused by the bacterium *Chlamydia trachomatis*. Repeated infection of the conjunctiva during childhood can initiate chronic conjunctival inflammation. This can lead to conjunctival scarring, in turning of the eyelashes, abrasion of the eyelashes on the cornea and eventually blindness later in adulthood. The World Health Organization recommends mass drug administration (MDA) for infection control when the prevalence of the clinical sign of Active Trachoma (TF) is ≥10% in 1–9 year olds. This systematic review of the literature examined the relationship between TF and *C. trachomatis* infection before and after MDA in order to evaluate the usefulness of TF for guiding trachoma control programmes. The population prevalence of TF and *C. trachomatis* infection were strongly correlated prior to MDA, however the relationship was weaker after MDA with a greater tendency for TF to overestimate the underlying infection prevalence. A cost effective test for *C. trachomatis* suitable for use in low resource settings could prevent unnecessary additional rounds of MDA in the population and could identify when trachoma elimination goals have been reached at an earlier time point.

Introduction

Sight loss from trachoma is the end result of a scarring disease process that is initiated in early childhood by the obligate intracellular bacterium *Chlamydia trachomatis* [1]. Repeated infection of the conjunctiva by *C. trachomatis* causes a recurrent chronic follicular conjunctivitis (TF) of the upper eyelid mucosal surface (Fig 1) [2]. This can sometimes be particularly severe with intense papillary inflammation (TI). Together, TF and / or TI are collectively referred to

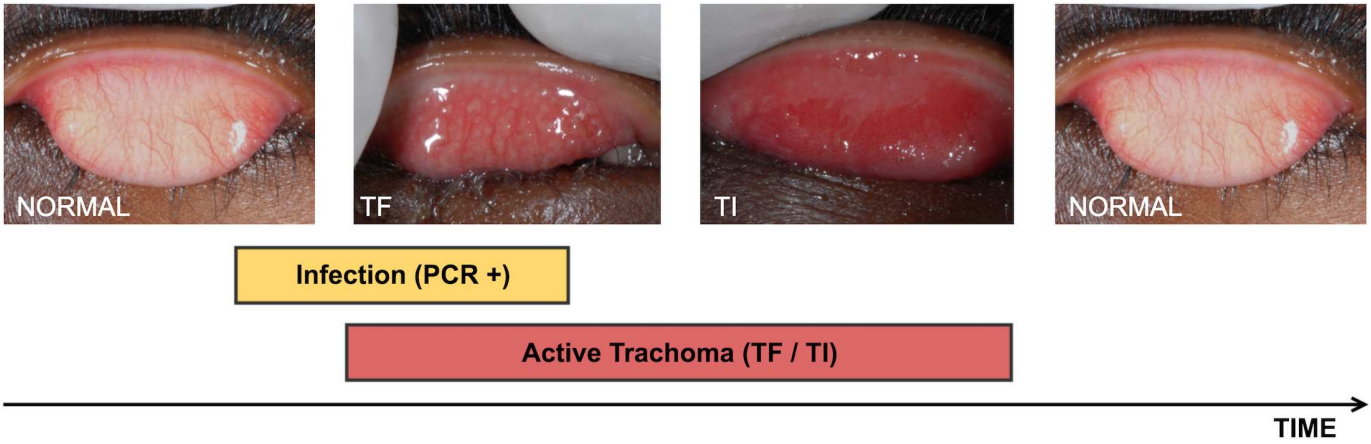

Fig 1. The natural history of an episode of ocular *C. trachomatis* infection and the associated conjunctival inflammatory response. Trachomatous Inflammation–Follicular (TF): the presence of five or more follicles in the upper tarsal conjunctiva. Trachomatous Inflammation–Intense (TI): pronounced inflammatory thickening of the upper tarsal conjunctiva that obscures more than half of the normal deep tarsal vessels [2].

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as "Active Trachoma". Conjunctival scarring gradually develops, usually becoming visible from early adulthood. Eventually the scarring causes the eyelashes to turn in and scratch the surface of the eye. If left uncorrected, trichiasis traumatises the cornea surface, resulting in opacification and sight loss.

There is a variable relationship between the clinical signs of Active Trachoma and the presence of *C. trachomatis* infection. The natural history of an infection episode in children is probably characterised by a brief "pre-clinical" phase, in which there is detectable infection but the clinical signs of the inflammatory response are yet to develop (Fig 1). Human volunteer experiments in which the conjunctiva was inoculated with *C. trachomatis* indicated that the signs of disease typically take about 10 days to develop in previously uninfected individuals [3]. This is followed by a variable period of time in which both infection and disease can be detected at the same time; this may last for several days to many weeks. The immune response brings the infection under control, completely clearing it or reducing it to undetectable levels. However, inflammatory clinical signs persist, typically lasting several weeks after the resolution of the infection. In children aged 4–15 years, data from longitudinal cohort studies estimate the median duration of infection range between 23 days and 8 weeks, and the median duration of disease between 54 days and 18 weeks [4–6]. The duration of disease and infection declines with increasing age. Therefore, at an individual level there is frequently a large mismatch between when infection can be detected and the clinical signs of disease are found [7].

The most recent estimates from the World Health Organization (WHO) Alliance for the Global Elimination of Trachoma by 2020 (GET2020) estimates indicate that about 200 million people live in trachoma endemic areas in 42 countries, 2.2 million have visual impairment or blindness, and about 3.2 million have trichiasis [8]. To meet this large public health challenge, the GET2020 Alliance recommends the implementation of the SAFE Strategy which tackles the disease at different stages: **S**urgery to correct trichiasis, **A**ntibiotics to treat chlamydial infection and **F**acial cleanliness and **E**nvironmental improvements to suppress transmission of the infection [1].

The antibiotic azithromycin is being used in community-wide mass drug administration (MDA); it is given as a single oral dose on an annual basis in endemic districts. Decisions around when to initiate and stop MDA are guided by the prevalence of TF in children in endemic communities. According to the current guidelines, azithromycin MDA is indicated for entire districts where the prevalence of TF in 1–9 year olds is $\geq$10%. Moreover, the determination of whether a program has controlled the active stage of trachoma as a public health problem also rests on the district level prevalence of TF [9].

Therefore, in view of the significance attached to TF for making programmatic decisions, it is important to understand the relationship between active disease and chlamydial infection in endemic communities. It is probable that this relationship changes after the introduction of MDA and will vary with different levels of endemicity. Several studies have specifically investigated what clinical signs can tell us about infection [7, 10–19]. However, in addition there are many other population-based studies, which report on both disease and infection, that can contribute information. Here we systematically review the published literature for reports that can inform our understanding of the relationship between clinical signs of active disease and *C. trachomatis* infection, both before and after the introduction of MDA.

Methods

In this review we included population-based studies and studies involving a random selection of participants that report the relationship between signs of Active Trachoma (TF, TI, TF/TI) and the detection of ocular *C. trachomatis* by nucleic acid amplification tests (NAAT),

including polymerase chain reaction (PCR), polymerase chain reaction/enzyme immunoassay (PCR-EIA), ligase chain reaction (LCR) and transcription-mediated amplification (TMA). We excluded studies which did not test individuals without Active Trachoma for infection. References were identified through searching PubMed for articles using the terms (i) "trachoma" AND "*Chlamydia trachomatis*", (ii) "Trachoma" AND "PCR", (iii) "Trachoma" AND "LCR", (iv) "Trachoma" AND "azithromycin". The search was limited to 1991 onwards, the year of the first report of the use of PCR to test for ocular *C. trachomatis* infection in a trachoma endemic population [20]. The search was last updated on 5th May 2016.

The titles and abstracts of all articles resulting from these searches were screened by two authors (AMR, MJB) for potentially eligible publications. These two authors then independently assessed the potentially eligible articles for inclusion and extracted the data. The bibliographies of publications meeting the inclusion criteria were also reviewed for any additional publications not already identified. Studies were excluded where the participant sample was not population-based. Several studies were identified with multiple related published reports arising from the same data. These are considered as a single study, but for completeness we include all the relevant references.

Core information was extracted using a standardised form. The core information included country, year of publication, study design, study population size, age group, TF prevalence, TI prevalence, TF/TI prevalence (if TF alone was not reported), *C. trachomatis* infection prevalence, diagnostic test used, grading system, use of antibiotic for infection control. The clinical grading system was recorded: the 1981 Detailed WHO FPC System or the 1987 Simplified WHO Trachoma Grading System [2, 21]. For the purpose of this review the prevalence levels of TF in the populations studied were categorised as follows: Hypoendemic $<10\%$, Mesoendemic 10–20% and Hyperendemic $>20\%$ [12].

Due to the heterogeneity in the study designs and the reporting of data, only a limited meta-analysis was performed. Studies report the results for different age groups. Where available we separately present both the "All Age" results and those for children ($\leq$15 years). In the meta-analysis we use the results from children only where these are available; where these are not available, the all age results are used. Summary graphs are presented of the relationship between: (i) the community prevalence of disease signs and *C. trachomatis* infection, (ii) the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of TF for infection, by the community prevalence of disease signs. The degree of correlation between these was tested using Pearson's correlation coefficient, weighted by study size. The relationship between signs and infection was analysed separately for pre-treatment and post-treatment data, then a test for interaction was performed on the two datasets combined to test whether the association between signs and infection was similar in the pre and post-treatment data. Forest plots were generated to illustrate the strength and heterogeneity of the relationship between disease signs and detection of infection; study heterogeneity was estimated using the I^2 statistic. Hierarchical summary receiver operating characteristics (HSROC) curves were plotted to illustrate the relationship between the sensitivity and specificity of signs of TF for the presence of *C. trachomatis* infection and a pooled estimate of sensitivity and specificity was made in both the pre and the post MDA studies. All analyses were performed using Stata 13. A Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist and flow diagram are included in the supporting information (S1 Checklist and S1 Fig).

Results

The search returned a list of 718 publications. The findings from pre and post-MDA studies are presented separately. Thirty-six separate population-based studies reported findings prior

to the introduction of MDA. Several had multiple related publications; yielding a total of 48 publications (Table 1). Twenty-one studies reported findings following the introduction of MDA met inclusion criteria, in a total of 35 publications (Table 2).

Pre-Treatment

Of the 36 pre-treatment study populations, 24 were hyperendemic, 8 mesoendemic and 4 hypoendemic (Table 1). The studies were conducted in populations from Tanzania (12), The Gambia (7), Ethiopia (6), Nepal (4), Niger (3), Cameroon (1), Guinea Bissau (1), Egypt (1) and Australia (1). The Simplified WHO Trachoma Grading System was used in 28 studies and 8 used the Detailed WHO FPC System. The majority of studies (25) used the commercially produced *Amplicor CT/NG* (Roche) PCR assay, five used ligase chain reaction (LCR, Abbott Laboratories), and six used in-house PCR assays. Four of the 36 studies listed in Table 1 did not report the community prevalence of *C. trachomatis* infection, however they report infection on a selected subset of individuals [11, 22–24]. These four studies were therefore retained in Table 1 as they contribute useful information, however they were excluded from the meta-analysis. Of the remaining 32 studies included in the meta-analysis, 29 studies reported data for childhood age groups (variable age ranges), seven of which also reported all age data, and three studies reported only all age data.

Prior to the introduction of MDA there was evidence of a strong positive correlation between the community-level prevalence of Active Trachoma and the community-level prevalence of detected *C. trachomatis*, using data from all 32 studies (r = 0.92, 95%CI 0.83 to 0.96, p<0.0001), Fig 2A. The correlation was similar when the analysis was limited to children only data (29 studies; r = 0.91, 95%CI 0.82 to 0.96, p<0.0001). The correlation was much weaker for all age data (10 studies; r = 0.51, 95%CI -0.18 to 0.86 p = 0.13). Overall, the community prevalence of TF is typically greater than the underlying community prevalence of detected *C. trachomatis*, with TF having a higher prevalence than *C. trachomatis* in 25 out of the 32 studies. The mean difference in prevalence was 10.4% (95% CI 5.9%-15.0%, p = 0.001).

There were 19 studies that reported the prevalence of TI separately from TF. There was evidence of a moderate positive correlation between the community-level prevalence of TI and the community-level prevalence of detected *C. trachomatis* (r = 0.75, 95%CI 0.45 to 0.90 p = 0.0002), Fig 2B. Although there are only a limited number of studies, in all communities where the TI prevalence was >20% the prevalence of *C. trachomatis* was at least 30%. In contrast, where the TI prevalence was below 20% there was a considerable degree of variation in the underlying community prevalence of infection.

There was sufficient data at the individual level presented to estimate the sensitivity, specificity, PPV and NPV for 21 studies. The sensitivity of TF for identifying the presence of *C. trachomatis* infection varied substantially with the prevalence of TF in the community, Fig 3A. There was evidence of a strong positive correlation (r = 0.82, 95%CI 0.59 to 0.92, p<0.0001). In contrast, there was a strong negative correlation between the specificity of TF for infection and the community prevalence of TF (r = -0.92, 95%CI -0.97 to -0.80, p<0.0001), Fig 3B. The Forest plot of the pre-treatment relationship between disease and detection of infection at the individual level showed an overall strong association (OR 6.05, 95% CI 5.49 to 6.67, p<0.0001), although there was marked heterogeneity (I^2 = 87.1%, p<0.001), Fig 4A. A plot of sensitivity against specificity of the pre-treatment studies using TF as a test for *C. trachomatis* infection at the individual level showed that the overall estimated sensitivity was moderate (57.1% (45.4–68.1%) and the overall specificity was good (81.1% (73.4–87.0%), Fig 5A.

The relationship between the community-level prevalence of Active Trachoma and the proportion of people with TF who were infected with *C. trachomatis* (Positive Predictive Value,

Table 1. Active Trachoma and *Chlamydia trachomatis* (Ct) infection prior to mass antibiotic treatment.

Country, Year, Ref.	Study design	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Tanzania, 1991, [20]	Baseline cross-sectional population-based data for a treatment trial. One child aged 1–7 years was randomly selected from 234 households in a village.	1–7 years: 234	1–7 years: • TF/TI 58.5% (137/234 • TI 15.8% (37/234)	1–7 years: 47.9% (112/234)	1–7 years: 65.0% (89/137)	1–7 years: 23.7% (23/97)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: in-house PCR-EIA for *OMP1*.
Gambia, 1994, [29]	Cross-section survey of the entire population of two villages	• All ages: 1332 • 0–15 years: 714	All ages: • TF/TI 15.0% (200/1332)0–15 years: • TF/TI 29.8% (183/714)	• All ages: 17.2% (229/1332) • 0–15 years: 29.8% (183/714)	• All ages: 72.0% (144/200) • 0–15 years: 73.2%% (134/183)	• All ages: 7.5% (85/1132) • 0–15 years: 9.2% (49/531)	• Mesoendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: in-house PCR for plasmid, detected by agarose gel electrophoresis.
Gambia, 1994, [22]	Cross-section survey of the entire population of one village. Only 133 individuals were tested for Ct infection by PCR. All active cases were tested. A sample of 37 normal individuals from two households were tested.	All ages: 844	All ages: • TF/TI 11.4% (96/844)	Data not available	All ages: 51.0% (49/96)	All ages: 5.4% (2/37)	• Mesoendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: in-house PCR for plasmid, detected by agarose gel electrophoresis. • The clinically normal group are not necessarily representative of the whole community as they are drawn from two households only.
Nepal, 1998, [30]	All children under 11 years in primary school and all children from four randomly selected households.	0–10 years: 70	0–10 years: • TF/TI 38.6% (27/70) • TI 7.1% (5/70)	0–10 years: 57.1% (40/70)	0–10 years: 66.7% (18/27)	0–10 years: 51.2% (22/43)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: in-house PCR for *OMP1*. • The sample was not necessarily representative of the child population of this community.
Egypt, 1999, [10, 31]	Baseline cross-sectional, population-based survey; pre-treatment data from a RCT of azithromycin vs tetracycline.	• All ages: 2069 • 1–10 years: 731	All ages: • TF/TI 19.7% (408/2069) • TI 5.8% (120/2069) • 1–10 years: TF/TI 48.3% (353/731) • TI 13.0% (95/731)	• All ages: 35.7% (739/2069) • 1–10 years: 48.6% (355/731)	• All ages: 66.9% (273/408) • 1–10 years: 69.4% (245/353)	• All ages: 31.6% (466/1661) • 1–10 years: 29.1% (110/378)	• Hyperendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: LCR, Abbott Laboratories.
Gambia, 1999, [31]	Baseline cross-sectional, population-based survey; pre-treatment data from a RCT of azithromycin vs tetracycline.	• All ages: 1747 • 0–10 years: 730	All ages: • TF/TI 15.8% (277/1747) • TI 7.0% (122/1747)0–10 years: • TF/TI 33.8% (247/730) • TI 13.7% (100*/730)	• All ages: 35.9% (628/1747) • 0–10 years: 39.3% (287/730)	All ages: 59.9% (166/277)	All ages: 31.4% (462/1470)	• Hyperendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: LCR, Abbott Laboratories. • Main results presented for all ages, possible to derive some of the indicators for the 0–10 year group.

(*Continued*)

Table 1. (*Continued*)

Country, Year, Ref.	Study design	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Tanzania, 1999, [31]	Baseline cross-sectional, population-based pre-treatment survey; data from a RCT of azithromycin vs tetracycline.	• All ages: 2653 • 0–10 years: 940	All ages: • TF/TI 31.8% (844/2653) • TI 16.4% (436/2653) 0–10 years: • TF/TI 60.1% (565/940) • TI 24.7% (233*/940)	• All ages: 18.5% (491/2653) • 0–10 years: 34.6% (325/940)	All ages: 48.3% (408/844)	All ages: 4.6% (83/1809)	• Hyperendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: LCR, Abbott Laboratories. • Main results presented for all ages, possible to derive some of the indicators for the 0–10 year group. • In this study there was some concern about sample degradation, leading to an underestimate of infection
Nepal, 1999, [11]	Cross-sectional survey of all children in six villages. Swabs and LCR test done on all active cases and 1/8 without active disease.	0–10 years: 726	years: • TF/TI 6.3% (46/726)	0–10 years: 0%(0/90)	0–10 years: 0% (0/46)	0–10 years: 0% (0/44)	• Hypoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: LCR, Abbott Laboratories.
Nepal, 2001, [23]	Baseline cross-sectional population-based data from a RCT of mass vs targeted antibiotic treatment. Children recruited from 17 wards. Only the children with active disease and a similar sized random sample of the others were tested by LCR.	1–7 years: 619	1–7 years: • TF/TI 19.0% (118/619)	Data not available	1–7 years: 24.8% (29/117)	1–7 years: 4.2% (5/118)	• Mesoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: LCR, Abbott Laboratories.
Gambia, 2003, [7, 27, 32, 33]	Baseline population-based survey of a longitudinal study of MDA. All residents of 14 villages.	• All ages: 1319 • 1–9 years: 492	All ages: • TF/TI 7.8% (103/1319) • TI 1.4% 19/13191–9 years: • TF 16.3% (80/492) • TI 2.6% (13/492)	• All ages: 7.2% (95/1319) • 1–9 years: 9.3% (46/492)	• All ages: 22.3% (23/103) • 1–9 years: 23.8% (19/80)	• All ages: 5.9% (72/1216) • 1–9 years: 6.6% (27/412)	• Mesoendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: Amplicor PCR, Roche.
Tanzania, 2003, [27, 34, 35]	Baseline population-based survey for a longitudinal study of MDA. All residents of a single sub-village.	All ages: 956	All ages: • TF/TI 18.2% (174/956) • TI 11.6% (111/956)	All ages: 9.5% (91/956)	All ages: 33.3% (58/174)	All ages: 4.2% (33/782)	• Hyperendemic Setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Tanzania, 2003, [27, 36–39]	Baseline population-based survey for a longitudinal study of MDA. All residents of a single village.	• All ages: 871 • 0–10 Years: 325	All ages: • TF/TI 35.8% (312/871) • TI 10.9% (95/871) • 0–10 Years: F/TI 72.6% (236*/325)	• All ages: 56.9% (496/871) • 0–10 Years: 64.9% (211*/325)	All ages: 77.2% (241/312)	All ages: 45.6% (255/559)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2004, [17]	Random selection of 100 children aged 1–6 years from four villages.	1–6 years: 100	1–6 years: • TF 79% (79/100) • TI 55% (55/100)	1–6 years: 63.0% (63/100)	1–6 years: 72.2% (57/79)	1–6 years: 28.6% (6/21)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche

(*Continued*)

Table 1. (*Continued*)

Country, Year, Ref.	Study design	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Gambia, 2006, [40]	Cross-sectional population-based study of school children aged 4–15 years from nine villages.	4–15 years: 331	4–15 years: • TF 18.2% (60/331) • TI 1.5% (5/331)	4–15 years: 21.8% (72/331)	4–15 years: 41.7% (25/60)	4–15 years: 15.3% (38/249)	• Mesoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: in-house RT-PCR assay for chlamydial 16S rRNA
Tanzania, 2006[a], [41]	Cross-sectional population-based study of children aged 1–9 years from two hyperendemic villages.	1–9 years: 464	1–9 years: • TF 44.0% (204/464)	1–9 years: 24.8% (115/464)	1–9 years: 36.3% (74/204)	1–9 years: 13.6% (41/301)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Tanzania, 2006[b], [41]	Cross-sectional population-based study of children aged 1–9 years from one hypoendemic village.	1–9 years: 200	1–9 years: • TF 12.5% (25/200)	1–9 years: 6.5% (13/200)	1–9 years: 32.0% (8/25)	1–9 years: 2.9% (5/175)	• Hypoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Niger, 2007, [42]	Cross-sectional study of randomly selected children from 12 villages.	1–5 years: 651	1–5years: • TF/TI 43.0% (280*/651) • TI 16% (104*/651)	1–5years: 21.0% (137*/651)	1–5years: 37.3% (103*/267)	1–5years: 9.0% (33*/365)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2007, [43]	Cross-sectional population-based study of children aged 0–10 years from two hyperendemic villages.	0–10 years: 56	0–10 years: • TF/TI 78.6% (44/56) • TI 46.4% (26/56)	0–10 years: 39.3% (22/56)	0–10 years: 43.1% (19/44)	0–10 years: 25.0% (3/12)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Nepal, 2008, [44]	Cross-sectional study of 9 randomly selected households from one village	All ages: 127	All ages: • TF 26.8% (34/127) • TI 38.6% (49/127)	All ages: 38.6% (49/127)	All ages: 44.1% (15/34)	All ages: 36.6% (34/93)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Gambia, 2009, [45, 46]	Cross-sectional survey using a two-stage cluster random sampling strategy with probability of selection proportional to size, in 19 enumeration areas in Lower River Region, The Gambia.	1–9 years: 876	1–9 years: • TF 12.3% (108/876) • TI 0.1% (1/876)	1–9 years: 0.3% (3/876)	1–9 years: 0.9% (1/108)	1–9 years: 0.3% (2/768)	• Mesoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2009, [47]	Cross-sectional population-based sample of 8 randomly selected children (1–5 years) per village from eight villages.	1–5 years: 120	1–5 years: • TF/TI 60.8% (73/120) • TI 44.2% (53/120)	1–5 years: 40.8% (49/120)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2010, [18, 48]	Cross-sectional population-based sample of children (1–5 years) living in 24 villages.	1–5 years: 1200	1–5 years: • TF/TI 86.0% (mean village prevalence)	1–5 years: 52.9% (mean village prevalence)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche • NB: The data are reported a mean of the prevalence across all 24 villages.
Gambia, 2010, [25, 49]	Cross-sectional population-based survey of ~100 randomly selected children aged 0–5 years from 48 enumeration areas.	0–5 years: 5033	0–5 years: • TF 6.3% (316/5033) • TI 0.6% (28/5033)	0–5 years: 0.8% (39/5033)	0–5 years: 0.9% (3/316)	0–5 years: 0.7% (36/4717)	• Hypoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche.

(*Continued*)

Table 1. (*Continued*)

Country, Year, Ref.	Study design	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Tanzania, 2010, [49–51]	Cross-sectional population-based survey of ~100 randomly selected children aged 0–5 years from 32 enumeration areas. The villages were purposely selected based on having a preliminary survey prevalence of >20%.	0–5 years: 3122	0–5 years: • TF 30.8% (963/3122) • cTI 7.8% (244/3122)	0–5 years: 21.9% (684/3122)	0–5 years: 48.9% (471/963)	0–5 years: 9.8% (213/2159)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche.
Niger, 2010, [52]	Cross-sectional study of randomly selected children from 12 villages	1–5 years: 557	1–5 years: • TF/TI 41.6% (232/557)	1–5 years: 20.1% (112/557)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Australia, 2011, [14]	Population-based cross-sectional study in five Aboriginal communities.	All ages: 1282	All ages: • TF 8.4% (108/1282)	All ages: 3.6% (46/1282)	All ages: 17.6% (19/108)	All ages: 2.3% (27/1174)	• Hypoendemic setting • Clinical grading: variation of the detailed WHO-FPC system [14] • Chlamydia test: Amplicor PCR, Roche
Tanzania, 2011, [26, 53]	Cross-sectional population-based survey in three villages	0–9 years: 473	0–9 years: • TF 13.7% (65/473) • TI 1.3% (6/473)	0–9 years: 5.3% (25/473)	0–9 years: 6.1% (4/65)	0–9 years: 5.1% (21/408)	• Mesoendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: Amplicor PCR, Roche.
Tanzania, 2011, [54, 55]	Cross-sectional baseline population-based survey in 4 villages.	0–8 years: 1991	0–8 years: • TF 27.8% (553/1991)	0–8 years: 23.7% (463/1956)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Tanzania, 2011, [56]	Cross-sectional baseline, population-based survey of four communities	0–9 years: 2118	0–9 years: • TF/TI 27.7%: (586/2118)	0–9 years: 23.6% (499/2118)	0–9 years: 33.1% (194*/586)	0–9 years: 13.6% (209/1532)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2011, [57]	Cross-sectional population-based survey in 23 communities. Both arms of a cluster RCT at baseline.	0–9 years: 1168	0–9 years: • TF/TI 66.5% (770/1158)	0–9 years: 44.7% (516/1168)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2012, [19, 58, 59]	Cross-sectional baseline, population-based survey of 0–9 year olds in 12 communities	0–9 years: 583	0–9 years: • TF/TI 68.3% (393/575)	0–9 years: 42.4% (248/584)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Cameroon, 2012, [24]	Cross-sectional study, with a random selection of children from 30 villages, with probability proportional to size. Only children with signs of Active Trachoma were tested for infection by PCR.	1–9 years: 2397	0–9 years: • TF/TI 26.2% (628/2397) • TI 5.2% (124/2397)	Data not available	0–9 years: 35.0% (220*/628)	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: in-house PCR assay for chlamydial rRNA gene.
Niger, 2012, [60, 61]	Cross-sectional study for randomly selected children from 48 communities.	0–5 years: 4484	0-5years: • TF 26.0% (1166*/4484)	0-5years: 20.7% (928*/4484)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche

(*Continued*)

Table 1. (*Continued*)

Country, Year, Ref.	Study design	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Guinea Bissau, 2013, [62, 63]	Cross-sectional population-based survey across multiple communities	• All ages: 1508 • 1–9 years: 618	All ages: • TF/TI 11.1% (167/1508) • TI 2.0% (29/1508)1–9 years: • TF/TI 22.0% (136/618) • TI 3.2% (21/618)	• All ages: 17.9% (269/1507) • 1–9 years: 25.4% (157/618)	• All ages: 63.3% (100/158) • 1–9 years: 86/129 (66.7%)	• All ages: 12.1% (164/1351) • 1–9 years: 83/537 (15.5%)	• Mesoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Tanzania, 2013, [64]	Cross-sectional population-based survey of children from one village.	0–9 years: 27	0–9 years: • TF 44.9% (57/127) • TI 16.5% (21/127)	0–9 years: 27.6% (35/127)	0–9 years: 29/57 (50.9%)	0–9 years: 6/70 (8.6%)	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche.
Tanzania, 2014, [65]	Cross-sectional population-based survey of all 1–6 year olds in a single village	1–6 years: 208	1–6 years: • TF/TI 47.0% (98/208)	1–6 years: 25.0% (52/208)	Data not available	Data not available	• .Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche.

* Estimated value inferred from available data in publication.

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PPV) showed a positive correlation (r = 0.80, 95%CI 0.56 to 0.91, p<0.0001), Fig 3C. The pattern of this distribution suggests that when the community-level prevalence of TF is below 20% the PPV of TF for the presence of *C. trachomatis* infection drops substantially. Above a community-level prevalence of TF of 20% the PPV is typically 40–70%, across a wide range of TF prevalence.

The relationship between the community-level prevalence of Active Trachoma and the proportion of people without TF who were not infected with *C. trachomatis* (Negative Predictive Value, NPV) showed a strong negative correlation (r = -0.81, 95%CI -0.92 to -0.57, p<0.0001), Fig 3D. When the community-level TF prevalence was greater than 20%, the NPV was more variable.

Post-Treatment

Of the 21 studies from populations following the introduction of MDA, prior to the first treatment, 15 were hyperendemic, 3 mesoendemic and 3 hypoendemic (Table 2). The studies were conducted in populations from Tanzania (10), Ethiopia (5), The Gambia (3), Nepal (2), and Egypt (1). The Simplified WHO Trachoma Grading System was used in 17 studies and four used the Detailed WHO FPC System. The majority of studies (15) used the Amplicor CT/NG (Roche) PCR assay, four used LCR (Abbott), one used an in-house PCR and one used the Aptima Combo2 (Hologic) TMA assay. There was one study included in Table 2 that did not report the community prevalence of *C. trachomatis* infection, and was therefore not included in the meta-analysis [23]. One of the Gambian studies involved surveys in four separate districts; we have included these as four separate sets of data in the analysis [25]. This resulted in 23 discrete studies included in the meta-analysis: for 20 studies data was available on children only (variable age ranges), two of which also reported all age data, and three other studies reported only all age data.

Table 2. Active Trachoma and *Chlamydia trachomatis* (Ct) infection after the introduction of mass antibiotic treatment.

Country, Year	Study design	Time Point post 1st MDA	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Tanzania, 1993, [20, 66]	Cross-section survey of randomly selected children one month after the completion of a one month tetracycline treatment course, given as MDA to the entire community.	1 month	1–7 years: 227	1–7 years: • TF/TI 41.9% (95/227) • TI 2.6% (6/227)	1–7 years: 23.8% (54/227)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: in-house PCR-EIA for *OMP1*.
Egypt, 2003, [10, 31]	Cross-section population-based survey 14 months after azithromycin MDA.	14 months	1–10 years: 354	1–10 years: • TF/TI 26.0% (92/354)	1–10 years: 5.1% (18/354)	1–10 years: 9.8% (9/92)	1–10 years: 3.4% (9/262)	• Hyperendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: LCR, Abbott Laboratories. • Only the azithromycin arm is included.
Gambia, 1999, [31]	Cross-section population-based survey 12 months after azithromycin MDA.	12 months	All ages: • 675 (exam) • 540 (tested)	All ages: • TF/TI 8.6% (58*/675) • TI 2.2% (15*/675)	All ages: 8.3% (45/540)	Data not available	Data not available	• Hyperendemic setting, pre treatment • Clinical grading: detailed WHO-FPC system • Chlamydia test: LCR, Abbott Laboratories. • Only the azithromycin arm is included.
Tanzania, 1999, [31]	Cross-section population-based survey 12 months after azithromycin MDA.	12 months	All ages: • 1308 (exam) • 1162 (tested)	All ages: • TF/TI 24.6% (322*/1308) • TI 6.3% (83*/1308)	All ages: 7.0% (82/1162)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: detailed WHO-FPC system • Chlamydia test: LCR, Abbott Laboratories. • Only the azithromycin arm is included.
Nepal, 2001, [23]	Cross-section survey of randomly selected normal children and a purposive sample of children with Active Trachoma, 6 months after azithromycin MDA.	6 months	1–7 years: • 5262 (exam) • 394 (tested)	1–7 years: • TF/TI 16% (841*/5262)	Data not available	1–7 years: 11.8% (31/263)	1–7 years: 5.1% (6/118)	• Mesoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: LCR, Abbott Laboratories.
Tanzania, 2004, [34, 35]	Cross-sectional survey of all residents of a sub-village, 24 months after MDA	24 months	All ages: 842	All ages: • TF/TI 5.8% (49/842)	All ages: 0.1% (1/842)	All ages: 2.0% (1/49)	All ages: 0.0% (0/793)	• Hyperendemic setting, pre-treatment • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Gambia, 2005, [7, 27, 32, 33]	Cross-sectional survey of all residents of 14 small villages, 12 months after MDA	12 months	• All ages: 1210 • 1–9 years: 440	All ages: • TF/TI 3.9% (47/1210)1–9 years: • TF 6.8% 30/440) • TI 0.9% (4/440)	• All ages: 2.3% (28/1210) • 1–9 years: 5.4% (24/440)	• All ages: 29.8% (14/47) • 1–9 years: 36.7% (11/30)	• All ages: 1.2% (14/1163) • 1–9 years: 3.2% (13/410)	• Mesoendemic setting, pre-treatment • Clinical grading: detailed WHO-FPC system • Chlamydia test: Amplicor PCR, Roche.
Tanzania, 2005, [27, 36–39]	Cross-sectional survey of residents of a village, 12 months after MDA	12 months	0–7 years: 287	0–7 years: • TF/TI 46.8% (134*/287)	0–7 years: 12.8% (37*/287)	Data not available	Data not available	• Hyperendemic • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche

(*Continued*)

Table 2. (*Continued*)

Country, Year	Study design	Time Point post 1st MDA	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Tanzania, 2007, [39]	Cross-sectional of population-based survey 5 years after baseline MDA and 3.5 years after a second MDA.	5 years	0–10 years: 464	0–10 years: • TF 30.2% (140/464) • TI 10.6% (49/464)	0–10 years: 25.9% (120/464)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2008, [67]	Cross-sectional population based sample of children from 32 communities. These had received between 1 and 3 rounds of MDA, with the most recent does less than 6 months in about half the communities.	Variable	3–9 years: 1459	3–9 years: • TF 23.6% (345/1459)	3–9 years: 3.0% (44/1459)	3–9 years: 6.1% (21/345)	3–9 years: 6.1% (23/1114)	• Hyperendemic setting • Heterogeneous treatment history • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2009, [47]	Cross-sectional population-based sample of 8 randomly selected children (1–5 years) per village from eight villages. The villages had received MDA biannually for 2 years, with the last dose 18 months prior to the survey.	42 months	0–5 years: 120	0–5 years: • TF/TI 47.5%: (57/120) • TI 30.0% (36/120)	0–5 years: 15% (18/120)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Ethiopia, 2010, [18, 48]	Cross-sectional population-based sample of children (1–5 years) living in 24 villages. Communities had received 4 biannual MDA, the most recent 6 months before survey	24 months	1–5 years: 1234	1–5 years: • TF/TI 39.2% (mean village prevalence)	1–5 years: 2.0% (mean village prevalence)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche • NB: The data are reported a mean of the prevalence across all 24 villages.
Tanzania, 2011, [15]	Cross-sectional population-based sample of children from 71 communities. All communities had received 3 to 7 rounds of MDA.	Variable	0–5 years: 7817	0–5 years: • TF 10.0% (784/7817) • TI 3.2% (252/7817)	0–5 years: 5.5%% (429/7817)	0–5 years: 23.5% (184/784)	0–5 years: 3.5% (245/7033)	• Mesoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche Heterogeneous MDA history. Wide range of community level prevalence of infection and disease. Summary data here pools these communities
Ethiopia, 2011, [57]	Cross-sectional population-based survey in 24 communities. Both arms of a cluster RCT of latrine provision received a single round of MDA at baseline.	24 months	0–9 years: 1211	0–9 years: • TF/TI 47.0% (567/1207)	0–9 years: 14.6% (177/1211)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche • Data from both arms combined.
Ethiopia, 2012, [19, 58, 59, 68, 69]	Cross-sectional survey of 50 children per village under 10 years in 12 villages. Three annual rounds of MDA were given. The final survey was months after the last MDA.	36 months	0–9 years: 577	0–9 years: • TF 43.5% (251/577)	0–9 years: 4.3% (25/577)	Data not available	Data not available	• Hyperendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche

(*Continued*)

Table 2. (*Continued*)

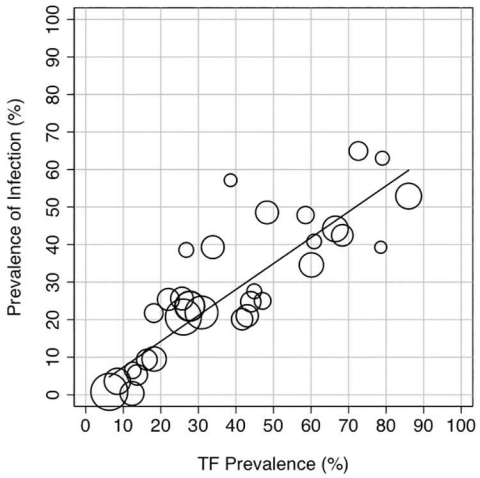
Country, Year	Study design	Time Point post 1st MDA	Participants	Active Trachoma %	Ct %	Ct+/TF+	Ct+/TF-	Comments
Gambia, 2013, [25, 70]	Cross-sectional population-based sample of children from 48 communities enrolled in an cluster RCT from four districts. 24 communities had 3 annual MDA and 24 communities had a single round of MDA at baseline. Survey was 3 years after the first round of MDA.	36 months	0–5 years: • District 1: 1128 • District 2: 1199 • District 3: 1243 • District 4: 1246	0–5 years: • TF 0.2% (2/1128) • TF 0.3% (3/1199) • TF 3.8% (47/1243) • TF 6.4% (80/1246)	0–5 years: • 0.5% (6/1128) • 0.1% (1/1198) • 1.1% (13/1241) • 0.3% (4/1235)	0–5 years: • 0.0% (0/2) • 0.0% (0/3) • 6.4% (3/47) • 0.0% (0/80)	0–5 years: • 0.5% (6/1126) • 0.1% (1/1196) • 0.8% (10/1196) • 0.3% (4/1166)	• Hypoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche. • Data from both trial arms were combined, and presented separately for each district.
Tanzania, 2014, [51, 71]	Cross-sectional survey of all children under 10 years in four villages. Four annual rounds of MDA were given. The final survey was 6 months after the last MDA.	42 months	0–9 years: 2234	0–9 years: • TF/TI 7.9% (176/2234)	0–9 years: 5.1% (114/2234)	Data not available	Data not available	• Hyperendemic setting, pre-treatment • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Tanzania, 2014, [71, 72]	Cross-sectional population-based sample of children aged 0–5 years in 32 villages, 100 children per village. Survey was done 12 months after the third MDA.	36 months	0–5 years: 3136	0–5 years: • TF 7.6% (237/3136)	0–5 years: 4.5% (142*/3136)	0–5 years: 29.5% (70/237)	0–5 years: 2.5% (72*/2899)	• Hyperendemic setting, pre-treatment • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Tanzania,: 2015,[73]	Cross-sectional population-based of residents of a village, which had received 2 rounds of MDA 12 and 10 years previously.	12 years	• All ages: 571 • 1–9 years: 200	All ages: • TF 2.5% (14/571)1–9 years: • TF 6.5% (13/200) • TI 1.5% (3/200)	All ages: 0% (0/571)1–9 years: 0% (0/200)	Data not available	Data not available	• Hyperendemic setting, pre-treatment • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Nepal, 2016, [74]	Cross-section survey of 1–9 year olds in 24 randomly selected communities. Four rounds of MDA. The survey was conducted 5 years after the first round.	5 years	1–9 years: 1124	1–9 years: • TF/TI 0.3% (3/1124)	1–9 years: 0% (0/1124)	Data not available	Data not available	• Hypoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Amplicor PCR, Roche
Tanzania, 2016, [75]	Random sample of children aged 1–9 years, from 30 hamlets. 50 children were sampled per hamlet. Communities had received variable rounds of MDA, between 4 and seven years previously.	7 years	1–9 years: 1474	1–9 years: • TF 0.4% (6*/1474)	1–9 years: 1.1% (16/1474)	Data not available	Data not available	• Hypoendemic setting • Clinical grading: simplified WHO system • Chlamydia test: Aptima Combo2, Hologic

* Estimated value inferred from available data in publication.

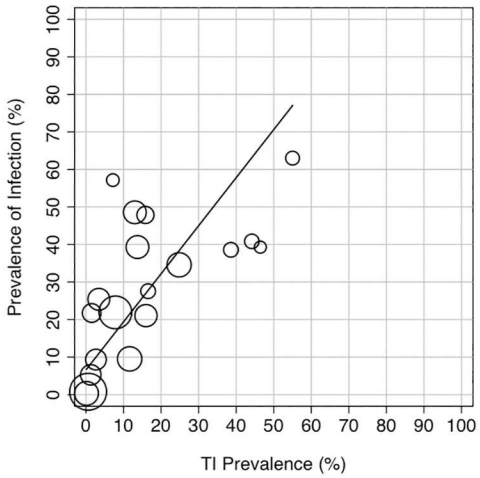
[a] Additional data sub-divided by district were provided by the authors of this study.

doi:10.1371/journal.pntd.0005080.t002

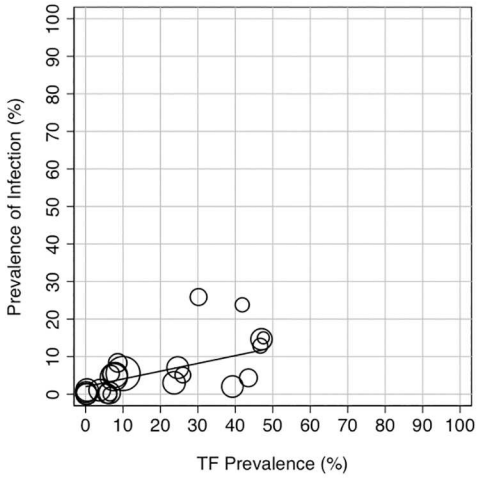
a) TF Prevalence and infection before MDA



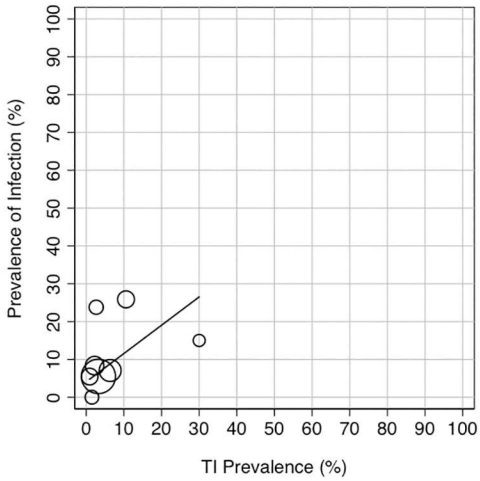
b) TI Prevalence and infection before MDA



c) TF Prevalence and infection after MDA



d) TI Prevalence and infection after MDA



e) TF Prevalence and infection after MDA

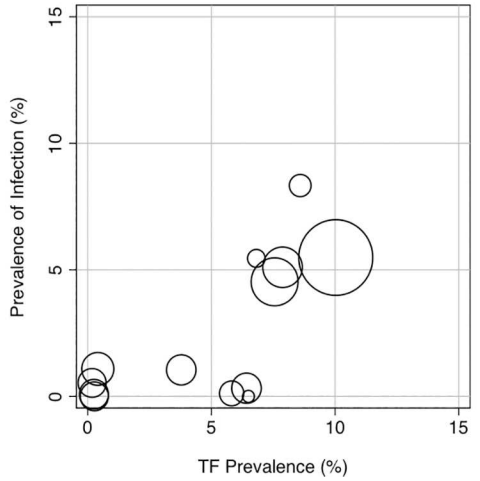


Fig 2. The relationship between the prevalence of disease signs and infection before and after the introduction of MDA. (a) Community prevalence of TF (or TF/TI) vs. the community prevalence of *C. trachomatis* infection before the introduction of MDA. (b) Community prevalence of TI vs. the community prevalence of *C. trachomatis* infection before the introduction of MDA. (c) Community prevalence of TF (or TF/TI) vs. the community prevalence of *C. trachomatis* infection after the introduction of MDA. (d) Community prevalence of TI vs. the community prevalence of *C. trachomatis* infection after the introduction of MDA. (e) Community prevalence of TF (or TF/TI) vs. the community prevalence of *C. trachomatis* infection after the introduction of MDA, showing only the communities with less than 15% TF. Data from population-based studies, summarised in Tables 1 and 2. The size of the circles reflects the sample size. Line fitted by linear regression, weighted by the size of the studies.

doi:10.1371/journal.pntd.0005080.g002

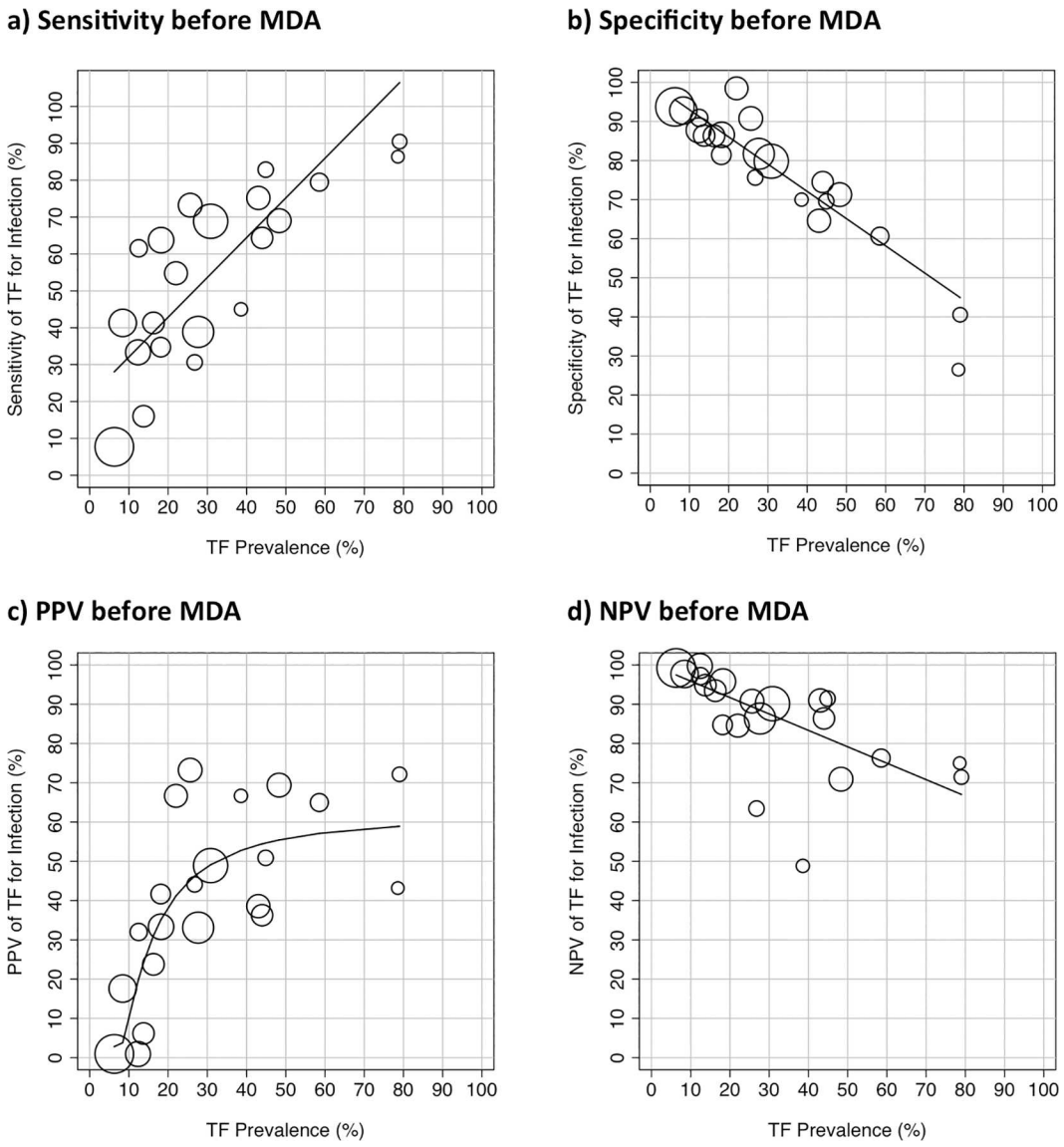
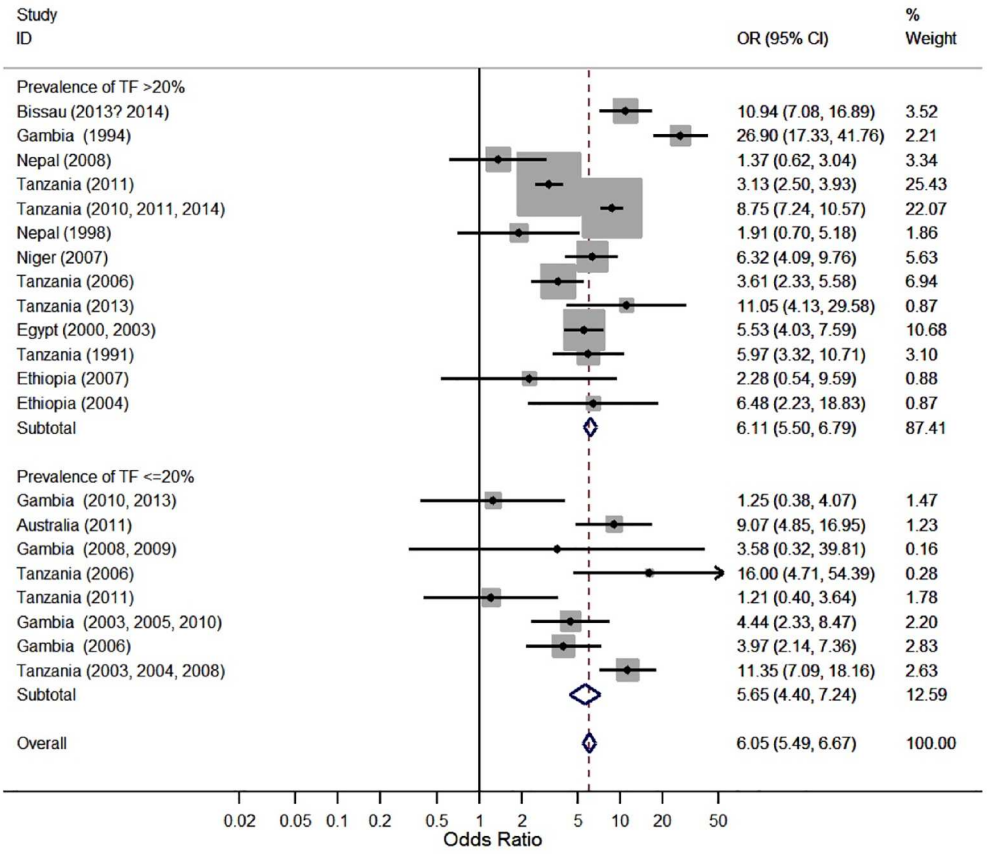


Fig 3. The relationship between the individual level presence of Active Trachoma (TF or TF/TI) and the detection of *C. trachomatis* infection by community TF prevalence before the introduction of MDA. (a) Sensitivity of TF for infection. (b) Specificity of TF for infection. (c) Positive Predictive Value (PPV) of TF for *C. trachomatis* infection. (d) Negative Predictive Value (NPV) of TF for *C. trachomatis* infection. Data from population-based studies, summarised in Table 1. The size of the circles reflects the sample size. Line fitted by linear regression, weighted by the size of the studies, except for the PPV which was fitted by polynomial.

doi:10.1371/journal.pntd.0005080.g003

(a) Before MDA



(b) After introduction of MDA

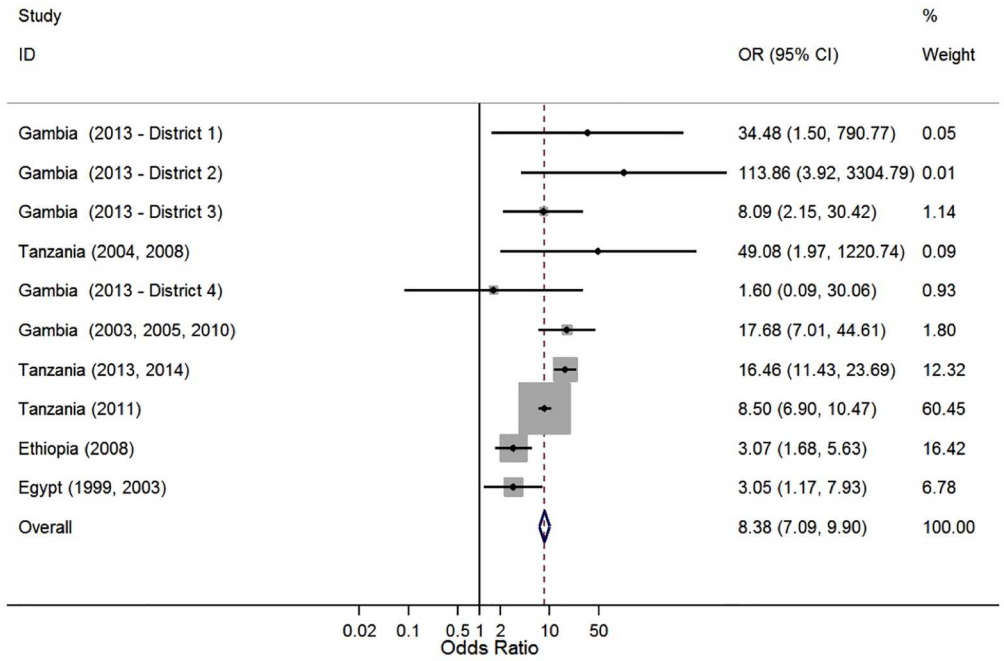


Fig 4. A Forest plot showing the relationship between Active Trachoma (TF or TF/TI) and the detection of *C. trachomatis* infection at the individual level (a) before and (b) after the introduction of MDA, grouped by community TF prevalence level. Studies are ordered by increasing prevalence of TF, the size of the grey boxes represent the how much weight each study contributes to the overall estimate, the blue diamonds represent the subtotal and overall pooled odds ratio estimates. The odds ratios are for ocular *C. trachomatis* infection as the outcome and TF as the explanatory variable.

doi:10.1371/journal.pntd.0005080.g004

Following the introduction of MDA there was evidence of a moderate positive correlation between the community-level prevalence of Active Trachoma and the community-level prevalence of detected *C. trachomatis*, using data from all 23 studies (r = 0.60, 95%CI 0.25 to 0.81, p = 0.003), Fig 2C. The relationship was similar when the analysis was limited to the 20 studies of children only (r = 0.60, 95%CI 0.21 to 0.82, p = 0.005). However, for the five studies reporting results for all ages there was no significant correlation (r = 0.72, 95%CI -0.18 to 0.86 p = 0.18). The overall impression is that the relationship between disease and infection is more uncertain post-MDA, such that the community-level prevalence of TF can substantially overestimate the underlying community-level prevalence of *C. trachomatis*; the community prevalence of TF can remain high (>20%) even when the prevalence of infection has declined (<10%), Fig 2C and 2E.

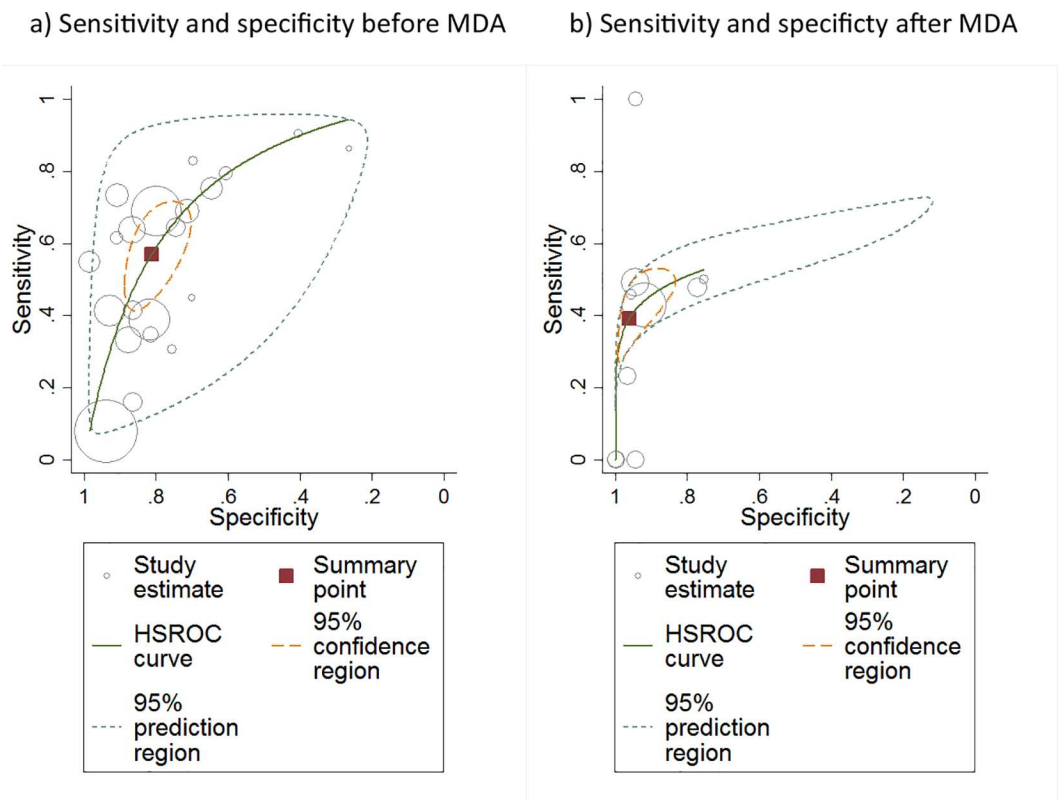


Fig 5. Sensitivity versus specificity of each study, using TF to diagnose *C. trachomatis* infection at the individual level (a) before and (b) after the introduction of MDA. Each circle represents the estimate for a single study, with the size of the circle representing the size of the study. The red square is the estimated pooled sensitivity and specificity for all studies (Pre- or Post-MDA). The orange dashed line represents the 95% CI (or confidence region in 2 dimensions) for the sensitivity and specificity. The grey curve (hierarchical summary receiver operating characteristic (HSROC) curve) represents the estimated relationship between sensitivity and specificity in these studies, with the grey dashed line indicating the region in which we would expect 95% of studies to fall.

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We found evidence that the relationship between the prevalence of TF and the prevalence of *C. trachomatis* infection differs between pre and post-MDA. Assuming a linear relationship between *C. trachomatis* and TF, the prevalence of *C. trachomatis* was associated with an expected 6.5% increase for every 10% increase in TF in pre-MDA studies, compared with an increase of 2.8% in post-MDA (test for interaction p = 0.004).This demonstrates that as the prevalence of TF increases, the expected increase in *C. trachomatis* is higher in pre-MDA communities than in post-MDA communities.

There were only eight studies that reported the prevalence of TI separately from TF after the introduction of MDA. There was no evidence of a correlation between the community-level prevalence of TI and the community-level prevalence of detected *C. trachomatis* (r = 0.50, 95% CI -0.31 to 0.89 p = 0.20), Fig 2D. However, it is noteworthy that the prevalence of TI was low (<10%) in all but two communities.

There was sufficient data presented to estimate the sensitivity, specificity, PPV and NPV for only 10 studies at the individual level after introduction of MDA. Therefore, only limited inference can be drawn. The sensitivity of TF for identifying the presence of *C. trachomatis* infection after the introduction of MDA varied widely, across a range of community-level TF prevalence levels, Fig 6A. In contrast, there was evidence of a strong negative correlation between the specificity of TF for infection and the community prevalence of TF (r = -0.99, 95% CI -0.99 to -0.96, p<0.0001), Fig 6B. The Forest plot of the post-treatment relationship between disease and detection of infection at the individual level showed an overall strong association (OR 8.38, 95% CI 7.09 to 9.90, p<0.0001), although there was marked heterogeneity (I^2 = 75%, p<0.001), Fig 4B. Although this overall OR is slightly higher post-MDA compared to pre-MDA, it should be noted that this result is calculated from individual-level data, whereas the correlations between TF and *C. trachomatis* infection prevalence (Fig 2) are calculated from population-level data. Compared to the pre-MDA situation (Fig 5A), after the introduction of MDA the individual level sensitivity was weaker (39.2% (30.9%, 48.2%) while specificity was stronger (96.1% (89.6%, 98.6%), Fig 5B.

The community-level prevalence of Active Trachoma and the proportion of people with TF who were infected with *C. trachomatis* (PPV) did not appear to be correlated (r = 0.16, 95%CI -0.52 to 0.72, p = 0.65), Fig 6C. Finally, the proportion of people without TF who were not infected with *C. trachomatis* (NPV) was high (>90%), across the limited range of community-level prevalence of Active Trachoma in these studies, Fig 6D.

Discussion

It has long been observed that the relationship between the signs of Active Trachoma and the detection of *C. trachomatis* infection at the individual level is not highly concordant [12]. Surveys, including those in this review, consistently find that within endemic populations there are many individuals with signs of disease who do not have detectable infection and conversely there are people who do not meet the diagnostic criteria for Active Trachoma (TF or TI) who do have detectable infection. Therefore, at the individual level, signs of infection cannot be depended upon to determine which members of an endemic community have ocular *C. trachomatis* infection.

In a trachoma endemic population, the main reason for this mismatch between active disease and infection is probably the different time courses of the typical infection and disease episodes, outlined in the introduction and illustrated in Fig 1. In addition, it is also possible that other factors contribute to this mismatch. Some individuals who have detectable infection but do not meet the diagnostic criteria for TF may have a mild trachomatous follicular conjunctivitis. Others may have previously acquired immunity and are able clear the infection without

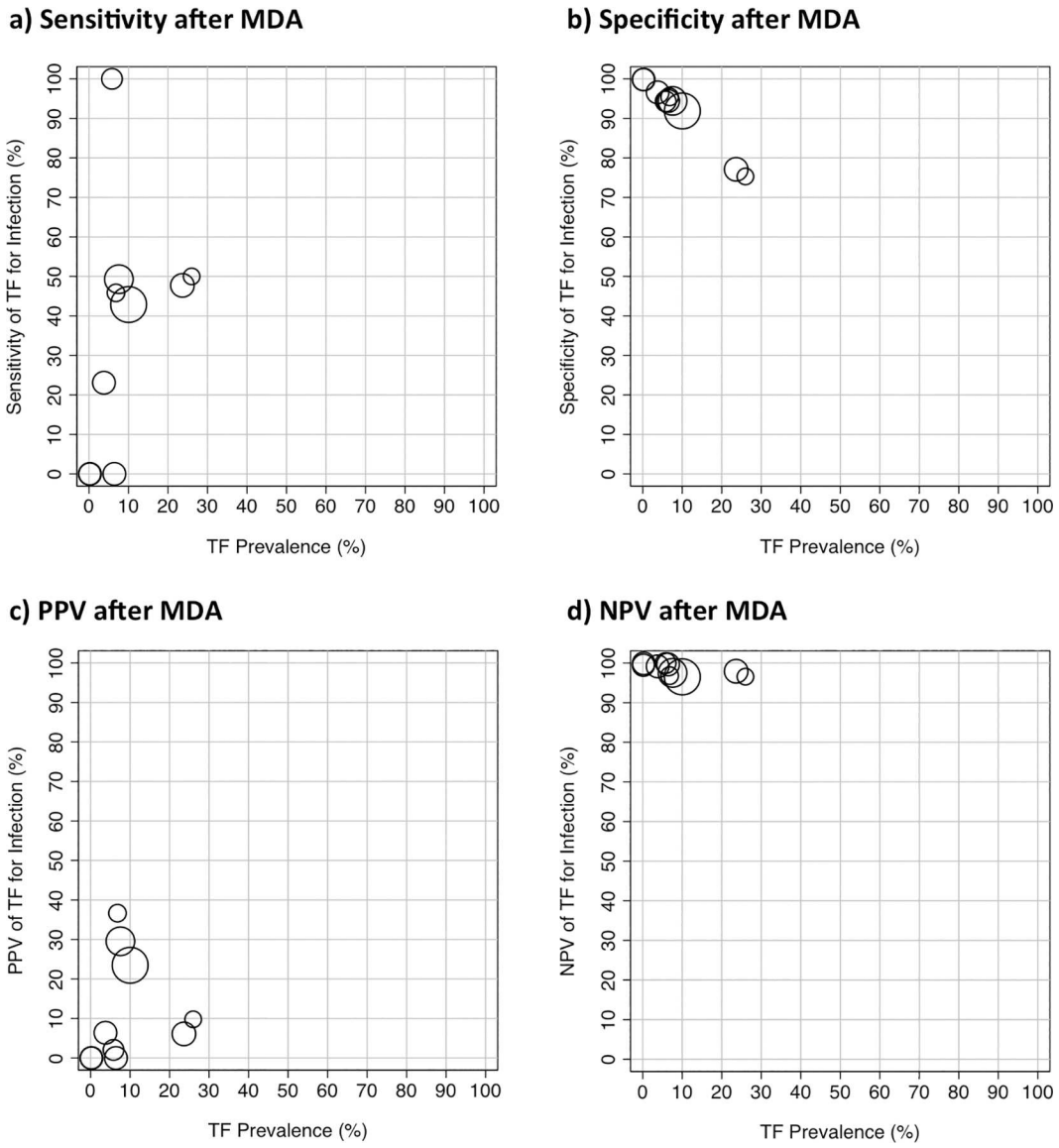

Fig 6. The relationship between the individual level presence of Active Trachoma (TF or TF/TI) and the detection of *C. trachomatis* infection by community TF prevalence after the introduction of MDA. (a) Sensitivity of TF for infection. (b) Specificity of TF for infection. (c) Positive Predictive Value (PPV) of TF for *C. trachomatis* infection. (d) Negative Predictive Value (NPV) of TF for *C. trachomatis* infection. Data from 6 population-based studies, summarised in Table 2. The size of the circles reflects the sample size.

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developing any detectable inflammatory signs. Alternatively, a positive NAAT test for *C. trachomatis* in a clinically normal individual could arise through cross-contamination during sample collection or processing. Clinical signs similar to those of Active Trachoma can also arise for other reasons, such as viral or bacterial infections, vernal conjunctivitis and hypersensitivity reactions [26].

It is clear that clinical signs are not a reliable indicator for *C. trachomatis* at the individual level. There is no point-of-care diagnostic test available for programmes to use to determine which individual members of a community are infected, and who would therefore benefit from targeted antibiotic treatment. Moreover, a strategy of testing everybody is not considered a

practical option. Therefore, the WHO guidance and the standard practice is to conduct community-wide antibiotic distribution of the entire population of endemic districts.

Decisions around the initiation and cessation of trachoma control measures are based on the prevalence of TF in children aged 1–9 years, determined through district level surveys, such as those conducted by the Global Trachoma Mapping Project. District-wide antibiotic treatment programmes and F&E measures to suppress transmission are initiated where the initial prevalence of TF is $\geq$10%. Below 10% TF the advice is to conduct sub-district level surveys. If a sub-district has $\geq$10% TF MDA and F&E are implemented. For sub-districts between 5% and 9.9% TF the guidance is to consider targeted MDA and F&E measures. For sub-districts $<$5% no MDA is needed and implementation of F&E can be considered.

The dependence on the clinical signs of disease to guide programmatic decisions raises the important question of how accurate these clinical signs are as a proxy measure for *C. trachomatis* infection at the population level. This question is particularly relevant after the introduction of MDA, when the association between clinical signs and infection prevalence at the population level is weaker, and as we try to determine when elimination targets have been reached.

In this systematic review, we found that prior to the introduction of antibiotic treatment the relationship between the community-level prevalence of TF in children correlated well with that of infection. The prevalence of TF was usually slightly greater than that of infection. Therefore, over a wide prevalence range, the community-level prevalence of TF in children is a reliable indicator that broadly reflects the underlying population burden of infection. The association between disease and infection for all ages was much weaker than that for children only; this supports the rationale for measuring TF in 1–9 year olds as the key indicator group for determining the need of antibiotic. The prevalence of TI was less well correlated than TF with infection before MDA, with the disease prevalence generally underestimating the level of infection.

At the individual level the utility of TF as a marker for infection is highly sensitive to the underlying prevalence of TF. Both the sensitivity and PPV rise substantially with increasing TF prevalence, and the specificity and NPV both drop. This sensitivity increase is therefore offset by a corresponding decrease in the specificity of this test in high TF prevalence communities. The usefulness of TF as an indicator of an individual's infection status is dependent on both the sensitivity and the specificity of the test; too low a sensitivity leads to *C. trachomatis* infected individuals not receiving treatment, whereas too low a specificity leads to wasted resources treating uninfected individuals. The PPV possibly gives the clearest indication of where TF is useful as an indicator of an individual's infection status; when the prevalence of TF is low (~5–10%), TF will only indicate an estimated 10% probability that the individual has *C. trachomatis* infection. This probability increases steadily with prevalence and once the community prevalence reaches 30%, the estimated PPV is in the order of 50–70%. Thus, where the population TF prevalence is above 30%, a positive TF diagnosis gives a 50–70% probability that an individual will be *C. trachomatis* infected.

After the introduction of MDA the relationship between the community prevalence of TF and chlamydial infection is less certain. Although the relationship between Active Trachoma and *C. trachomatis* infection appears to remain strong at the individual level, the population-level data suggests that post-MDA, Active Trachoma has a greater tendency to overestimate the underlying population prevalence of *C. trachomatis* infection. Below the 10% TF level the prevalence of infection was consistently low, and therefore below this level, TF prevalence appears to be a good marker for infection having being brought under control. However, in the studies where the prevalence of TF was above 10% after the introduction of MDA, the underlying prevalence of *C. trachomatis* infection was much less predictable. In some settings TF prevalence persists at high levels despite relatively little infection being detected. There might be several reasons for this observation. For example, if the loads of infection are substantially

lower following the introduction of MDA these may not be so readily detected by diagnostic tests. Other bacterial species might also provoke a follicular conjunctivitis more readily in individuals previously infected with *C. trachomatis* [26]. However, whatever the explanation, it is likely that in some settings, the prevalence of TF will underestimate the impact MDA has had on the prevalence of *C. trachomatis*. This might lead to the on-going use of MDA after the infection has been adequately controlled.

There is much less published data on the relationship between TI and infection following the introduction of MDA. In general, the prevalence of TI appears to reduce more readily than TF. A number of studies have investigated the relationship between the load of infection and disease signs. These suggest that TI is particularly associated with high loads of infection [27]. Therefore, the decline in TI may reflect a shift to less intense infections. However, there were a few studies in which the prevalence of TI was low, but the prevalence of infection remained substantial.

We identified a reasonable number of studies reporting the community-level relationship between disease and infection prior to treatment, over a wide TF prevalence range. There were, however, fewer studies documenting the situation following the introduction of MDA, potentially limiting the conclusions that can be drawn after MDA. However, there was generally less detail in these reports about the individual-level relationship between clinical signs and infection. The studies came mostly from several East and West African countries, providing reasonable geographical coverage of the regions with the greatest trachoma burden. Standard WHO definitions of disease were generally used and the large majority of studies used the same commercially produced PCR assay, providing consistency across studies. However, there was some methodological heterogeneity. The age groups reported varied; where possible we use the data for children only in the meta-analysis to try to provide greater consistency between studies. The sample sizes varied considerably (from 56 to 7817); we weighted our analyses to adjust for size. The sampling methodology also varied considerably. The studies included were generally population-based samples or surveys of the entire resident population of a defined area.

Overall, the use of TF prevalence to guide the decision to initiate MDA in previously untreated districts appears to be reliable. In contrast, the situation following treatment is more uncertain, calling into question the reliability of clinical signs for monitoring progress towards the achievement of the elimination targets [28]. There are reports from hyperendemic regions that have received many rounds of high-coverage MDA that suggest that the prevalence of TF can be recalcitrant, even when *C. trachomatis* infection appears to have been brought under control. Therefore, contextually appropriate, cost-effective tests for *C. trachomatis* infection that can be administered in low-resource settings, and used to estimate the infection prevalence in a population-based sample, would be very helpful in guiding decisions around the cessation of MDA. Such tests are anticipated to reduce the number of annual rounds of MDA required and lead to the confirmation of trachoma control at an earlier stage.

Supporting Information

S1 Checklist. PRISMA Checklist
(DOC)

S1 Fig. PRISMA Flow Diagram
(DOC)

Author Contributions

Conceptualization: MJB MJH.

Data curation: AMR TD DM MJB.

Formal analysis: MJB DM.

Funding acquisition: MJB MJH.

Investigation: AMR MJB.

Methodology: MJB DM.

Validation: AMR MJB.

Visualization: AMR TD DM MJH MJB.

Writing – original draft: AMR MJB.

Writing – review & editing: AMR TD DM MJH MJB.

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5. Blinding Trachoma: systematic review of rates and risk factors for progressive Disease

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

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Student	Athumani M. Ramadhani
Principal Supervisor	Prof. Matthew J. Burton
Thesis Title	Studies on the Development of Scarring Trachoma in Tanzania

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	PLOS Neglected Tropical Diseases		
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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed the systematic search. I extracted the data. Matthew Burton performed the duplicate data extraction. I wrote a first draft of this paper. Comments from co-authors were considered. I performed the data analysis with guidance from Matthew Burton, David Macleod and Tamsyn Derrick.

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RESEARCH ARTICLE

Blinding Trachoma: Systematic Review of Rates and Risk Factors for Progressive Disease

Athumani M. Ramadhani[1,2]*, Tamsyn Derrick[1,2], Martin J. Holland[1], Matthew J. Burton[1,3]

1 London School of Hygiene & Tropical Medicine, London, United Kingdom, **2** Kilimanjaro Christian Medical Centre, Moshi, Tanzania, **3** Moorfields Eye Hospital, London, United Kingdom

* athumani.ramadhani@lshtm.ac.uk

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Abstract

Background

Sight loss from trachoma is the end result of a scarring disease process starting in early childhood and characterised by repeated episodes of conjunctival inflammation (active trachoma). Subsequently, the conjunctiva becomes scarred, causing the eyelashes to turn inwards and scratch the cornea (trichiasis), damaging the corneal surface and leading to corneal opacification and visual impairment. It is thought that this process is initiated and driven by repeated infection with *Chlamydia trachomatis*. We review published longitudinal studies to re-examine the disease process, its progression rates and risk factors.

Methodology/Principal Findings

We searched PubMed for studies presenting incidence and progression data for the different stages of trachoma natural history. We only included studies reporting longitudinal data and identified 11 publications meeting this criterion. The studies were very heterogeneous in design, disease stage, duration, size and location, precluding meta-analysis. Severe conjunctival inflammation was consistently associated with incident and progressive scarring in five studies in which this was examined. One study reported an association between *C*. *trachomatis* infection and incident scarring. No studies have yet demonstrated an association between *C*. *trachomatis* infection and progressive scarring. Several studies conducted in regions with low prevalence active disease and *C*. *trachomatis* infection found evidence of on-going scarring progression.

Conclusions/Significance

Overall, there are few longitudinal studies that provide estimates of progression rates and risk factors, reflecting the challenges of conducting such studies. Our understanding of this disease process and the long-term impact of control measures is partial. Intense conjunctival inflammation was consistently associated with scarring, however, direct evidence demonstrating an association between *C*. *trachomatis* and progression is limited. This suggests that on-going chlamydial reinfection may not be mandatory for progression of established

scarring, indicating that sight threatening trichiasis may continue to evolve in older people in formerly endemic populations, that will require service provision for years after active disease is controlled.

Introduction

Sight loss from trachoma is the end result of a scarring disease process. The widely accepted view is that it follows the natural history illustrated in Fig 1 [1]. Trachoma is caused by the obligate intracellular bacterium *Chlamydia trachomatis*. In a typical endemic setting, repeated chlamydial infection of the conjunctiva starts early in life. This can initiate recurrent episodes of chronic conjunctival inflammation, characterised by the formation of lymphoid follicles most easily seen in the upper tarsal conjunctival surface. In the Simplified WHO grading system this is referred to as Trachoma Inflammation—Follicular (TF), which is equivalent to F2 and F3 of the detailed WHO-FPC grading system [2, 3]. In some cases the inflammation can be more marked, with severe papillary hypertrophy, referred to as Trachoma Inflammation—Intense (TI) in the simplified system and P3 in the detailed system [2, 3]. These signs of "Active Trachoma", TF and TI, are most frequently found in younger children, becoming less prevalent with increasing age. They are characterised by a cell-mediated adaptive immune response to *C.*

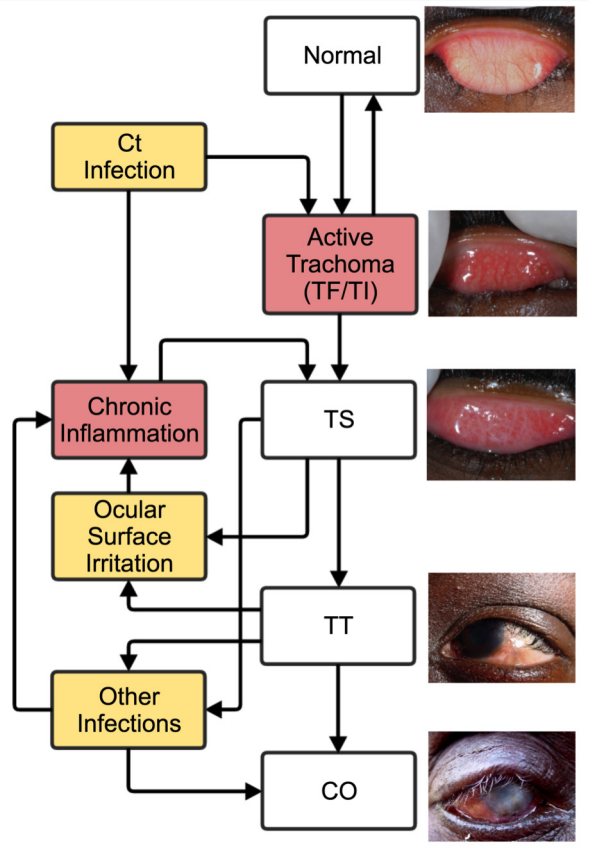

Fig 1. Natural history of trachoma: Normal healthy tarsal conjunctiva without inflammation, follicular trachoma (TF), intense inflammatory trachoma (TI), scarring trachoma (TS), trichiasis (TT) and corneal opacification (CO). Ct: *Chlamydia trachomatis*.

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trachomatis [4]. With time trachomatous conjunctival scarring (TS) gradually develops as a result of inflammation-induced tissue damage [2, 4]. The Simplified WHO grading of TS is taken to be equivalent to the grades C1-C3 of the Detailed WHO grading system [2, 3]. In highly endemic regions TS can develop in childhood, whereas in less endemic regions it usually becomes visible from early adulthood.

Chronic conjunctival inflammation is also frequently found in adults with established conjunctival scarring, Fig 1. This later stage conjunctival inflammation is usually not follicular in nature, *C. trachomatis* infection is rarely detected and it is characterised by a prominent innate epithelial immune response [4–6]. It is possible that non-chlamydial infections, dryness and irritation of the scarred conjunctiva also contribute to driving inflammation and progression in the context of established scarring, Fig 1 [4].

Eventually the conjunctival scarring causes the eyelashes to turn in, such that they now scratch the surface of the eye, which is referred to as trachomatous trichiasis (TT) [2]. If the trichiasis is left uncorrected it traumatises the surface of the cornea resulting in sight loss from corneal opacification (CO). Again other pathogens (bacteria and fungi) may contribute to the development of corneal scarring through secondary infections, Fig 1.

The most recent World Health Organization (WHO) estimates suggest that about 232 million people live in trachoma endemic areas in 51 countries [7]. Approximately 1.2 million people are irreversibly blind from trachoma. A further 40 million are thought to be affected by active trachoma, and are at risk of developing scarring and blindness [8]. To meet this large public health challenge, the WHO-led Global Alliance for the Elimination of Trachoma by 2020 recommends the implementation of the SAFE strategy which tackles the disease at different stages: Surgery to correct trichiasis, Antibiotics to treat chlamydial infection and Facial cleanliness and Environmental improvements to suppress transmission of infection [1].

Despite many years of research, there are relatively few detailed longitudinal studies that investigate the rates and determinants of disease progression through these stages. An understanding of this process is relevant to disease control measures and planning. In this review we draw together and re-examine the literature on the natural history of trachoma, to identify the rates and risk factors for progression through the stages of this disease process.

Methods

References were identified through searches of PubMed for articles published at any date, by use of the terms (i) "trachoma" AND (ii) "scarring", "trichiasis", "cornea opacity", "*Chlamydia trachomatis*", "visual impairment" OR "blindness". Articles resulting from these searches and relevant publications cited in these articles were reviewed. We included longitudinal studies which: (1) reported the incidence and/or progression of conjunctival scarring; (2) the incidence and/or progression of trachomatous trichiasis; (3) the incidence and/or progression of corneal opacification; (4) the incidence and/or progression of visual impairment and blindness from trachoma. For the purposes of this review, we define incident disease (conjunctival scarring, trichiasis or corneal opacification) to be the development of these signs in individuals who did not previously have evidence of them. We reviewed the bibliographies of publications meeting these inclusion criteria for any additional publications that might also meet them. This search yielded 1595 articles and one author screened the titles and abstracts of these for potentially eligible publications. Two authors (AR and MB) jointly reviewed potential articles for eligibility and extracted the data.

For each disease progression stage we extracted and recorded core information using a standardised form and present this in the Tables. The core information included country, year of publication, population active disease level, study design, study population, duration of follow-

up, numbers of participants, number and proportion progressing, grading system, use of antibiotic for infection control. Additional information on specific associations with disease progression and clinical inflammation, *C. trachomatis* infection, gender and age were collected if reported. For the purpose of this review the prevalence levels of TF in the populations or regions studied were categorised as follows: Hypoendemic <10%, Mesoendemic 10–20% and Hyperendemic >20% [9]. For some reports data on active disease prevalence was not presented, therefore, we have categorised the active disease prevalence level for such studies based on other contemporary sources of information about disease prevalence for the same regions. Clinical signs are reported using the specific trachoma grading systems used in the study (details provided in Tables 1–5), these were usually either the Simplified or the Detailed WHO Trachoma Grading Systems [2, 3]. Due to the heterogeneity of the study designs and reports a meta-analysis was not performed.

Results/Discussion

Progression from Active to Scarring Trachoma

We identified four prospective cohort studies that reported the relationship between active trachoma and the subsequent development of incident scarring (new conjunctival scarring in those with no prior visible conjunctival scarring), Table 1 [10–13]. Three of these were from Tanzania and one from Tunisia. In addition, we identified a report of a mathematical model of the five year incidence of TS, based on cross-sectional age-stratified data from Tanzania [14]. The study designs were heterogeneous in terms of sample size, clinical grading system used, MDA treatment background and study duration. All four were conducted in hyperendemic communities, which had not previously received mass drug administration (MDA) for trachoma control. After enrolment all the communities received some form of antibiotic treatment, although this was variable in nature and frequency (Table 1). The duration of the follow-up varied from 5 to 14 years. The assessment of clinical signs used either the simplified or detailed WHO Trachoma Grading Systems [2, 3]. In two studies the development of incident scarring was identified by a change in the clinical field grading [10, 11]. In the other two studies the development of incident scarring was identified through the grading of photographs, using a more detailed scar grading system [12, 13].

The earliest study, from Tunisia, found that 17% of individuals (n = 82, children and young adults) without conjunctival scarring at baseline developed severe scarring with distortion of the tarsal plate (C3) over a 14 year period (Table 1) [10]. This study also reported on scarring progression and incident TT, which are discussed below. It did not report the proportion of individuals who developed milder degrees of scarring during the same period, nor did it report chlamydial infection data. A strong association was found between the presence of TI at baseline and the subsequent development of severe scarring (RR = 18), and a weaker association for the presence of TF (RR = 2.8). Unfortunately, the reported details are fairly limited, and the scarring incidence rates are not disaggregated by gender.

The first Tanzanian study was a 7-year cohort of children (baseline age 1–7 years) with and without "constant severe trachoma" (defined by the presence of TI on at least 3 out of 4 examinations during the baseline year) [11]. At 7 years, 29.2% of children with "constant severe trachoma" (n = 96) developed incident TS, compared to only 9.6% of the comparison group (n = 94). Incident scarring was independently associated with "constant severe trachoma", increasing age and female gender. Incident scarring was associated with the presence of TI at the 7-year time-point. The cohort was tested for *C. trachomatis* infection at the 7-year time-point only; those that had incident scarring were more likely to be infected at that point (OR 2.48, p = 0.02).

Table 1. Progression from active to scarring trachoma.

Country / Year	Study design	Participants	Progression to TS	Comments
Tunisia, 1990 [10]	Prospective 14-year study of the resident population of a trachoma-endemic Tunisian village. Conducted to identify clinical signs and environmental factors associated with development of scarring. A random sample of people seen at baseline were re-examined at 14-years.	Baseline, 1969–72: 2000 people, of all ages. Follow-up, 1986–87: 213 people who were aged 1 month to 32 years at baseline. Loss to follow-up: Information not provided. Baseline Scarring: • C0 82 (38.7%) • C1 51 (24.0%) • C2 58 (27.4%) • C3 21 (9.9%)	• Baseline: 82 had no scarring (C0). • At 14 years 14/82 (17.1%) had developed severe scarring (C3) • Incident TS rate: 1.2%/ year	• Hyperendemic setting (Regional survey data) • No previous MDA, however during the 1980's systematic antibiotic treatment was carried out which dramatically reduced active trachoma.[35] • Clinical grading: detailed WHO-FPC system.[3] • The data presentation and analysis in this report are relatively limited. However, it represents the first long term study of the relationship between inflammation and subsequent scarring. The report only includes the rate of transition from no scarring (C0) to C3 (the most severe grade with distortion), it is likely that many other individuals without scarring at baseline would have gone on to develop some lesser degree of scarring (C1 or C2). Data not disaggregated by gender. • Predictors of severe scarring (C3) at 14 years (whole group of 213): ○ Baseline TF (F2/F3): RR 2.8 ○ Baseline TI (P3): RR 18 ○ Household density (closeness of houses): RR 1.3
Tanzania, 1997 [14]	Mathematical model of the 5-year incidence of TS in women. Using age-stratified cross-sectional data on the different clinical stages.	• 4898 women in survey. • Loss to follow-up: N/A	• Incident TS (model) by age group: 15–19 years: 3.1% / 5 yrs; 55–59 age: 14.3% / 5 yrs • Incident TS rate: 15–19 years: 0.6%/year; 55–59 years: 4.9%/year	• Hyperendemic setting • No previous MDA. • Clinical grading: simplified WHO system.[2]
Tanzania, 2001 [11]	Prospective cohort study of "constant severe trachoma" in the development of incident trachoma scarring in children. Cases of "constant severe trachoma" were defined by the presence of severe inflammatory trachoma (TI) on at least 3 out of 4 examinations during the baseline year. The comparison group was defined as children "without constant severe trachoma" (up to 2 episodes of TI out of 4). The two groups were matched by age, gender and neighbourhood. Swabs were collected for *C. trachomatis* infection by PCR at 7 years only.	• Baseline, 1989: Age 1–7 years; Cases 118; Comparison 118. • Follow-up, 1996: Age 8–14 years; Cases 96; Comparison 94 • Loss to follow-up: 42	• 7 year Incident TS: Cases: 28/96 (29.2%); Comparison 9/94 (9.6%). • Incident TS rate: Cases: 4.2%/year; Comparison: 1.4%/year	• Hyperendemic setting. (Regional survey data) • No previous MDA. After baseline MDA with topical tetracycline eye ointment was administered for 30 days.[36] • Clinical grading: simplified WHO system.[2] • Predictors of incident scarring at 7 years: ○ Age (per year): OR 1.32, 95% CI 1.07–1.62 ○ Female: OR 2.49, 95% CI 1.02–6.08 ○ Constant severe trachoma: OR 4.85, 95% CI 2.05–11.4 • Incident scarring was highest in those who had TI and lowest in those that had TF at the 7-year follow-up (1996): ○ Cases: TF 1/30 (3.3%), TI 22/40 (55.0%), None 5/26 (19.2%) ○ Comparison: TF 1/30 (3.3%), TI 5/13 (38.5%), None 3/51 (5.9%) • *C. trachomatis* infection at the 7-year time point was more frequently detected in individuals that developed scarring: TS 12/26 (42.9%) vs no TS 20/67 (29.9%), OR 2.48, p = 0.02. The clinical / infection status of individuals from the two groups was unknown during the 7 year interval.

(*Continued*)

Table 1. (*Continued*)

Country / Year	Study design	Participants	Progression to TS	Comments
Tanzania, 2009 [12]	Prospective cohort study of the impact of *C. trachomatis* infection and severe trachoma on development of scarring. Participants were all children under 10 years (at baseline) living in a single village; examined at baseline, 2, 6, 12, 18 months and 5 years. Definitions: • "Constant infection": infection on at least 3 of 5 visits during the initial 18 months. • "Constant severe trachoma": severe trachoma (10+ follicles or TI or both) on at least 3 of 5 visits during the initial 18 months. • "Constant infection and constant severe trachoma": combination of the above.	• Cohort of 189 children, aged 0–9 years at baseline (2000). • Loss to follow-up: 6	• Baseline TS prevalence: 6/189 (3.0%) • Incident TS by 5 years: 32/183 (17.4%) • Incident TS rate: 3.5%/year	• Hyperendemic setting (70% TF at baseline) • Clinical grading: simplified WHO system with additional more detailed grading of scarring on photographs.[2, 13] • MDA was administered at baseline and at 18 months. • Chlamydia test: Amplicor PCR, Roche • Baseline: ○ Scarring: male; 2/90 (2.1%), female; 4/99 (4.0%) ○ *C. trachomatis* infection: male; 35/90 (38.8%), female 43/99 (43.4%) • Incident TS at 5 years by disease/infection group: ○ No infection/disease: 4/59 (6.8%) ○ Sporadic infection/disease: 12/79 (15.2%) ○ Constant severe trachoma only: 7/20 (35.0%) ○ Constant infection only: 4/9 (44.4%) ○ Constant severe trachoma and constant infection: 5/16 (31.2%) • Significant predictors of incident scarring at 5 years (multivariable logistic regression model): ○ Age (per year) OR 1.26, 95% CI 1.08–1.47 ○ Gender (female) OR 2.55, 95% CI 1.13–5.75 ○ Sporadic infection/disease (relative to no infection/disease): OR 1.76, 95% CI 0.48–6.50 ○ Constant infection and/or severe disease (relative to no infection/disease): OR 5.74, 95% CI 2.39–13.77
Tanzania, 2009 [13]	Prospective cohort study of incident and progressive scarring. Participants were individuals of all ages, examined at baseline and 5-years.	• Baseline (2000): 990 people of all ages. • Follow-up (2005): 487 people of which 453 had gradable images from all time points. • Loss to follow-up: 437	• Baseline TS prevalence: 86/453 (18.9%) • Incident TS by 5 years: 75/367 (20.4%) • Incident TS rate: 4.1%/year	•Hyperendemic setting (70% TF at baseline) • Clinical grading: simplified WHO system with additional more detailed grading of scarring on photographs.[2, 13] • MDA was administered at baseline and at 18 months. • There was a trend of increasing incidence with age, p = 0.038

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In a separate cohort of Tanzanian children (n = 183) who were aged <10 years at baseline, the 5-year cumulative incidence of TS was 17.4%. In a logistic regression model, the development of TS was strongly associated with increasing age, female gender, "constant severe trachoma" (10+ follicles or TI or both, on at least 3 of 5 visits during the initial 18 months) and/or "constant *C. trachomatis* infection" (infection on at least 3 of 5 visits during the initial 18 months), compared to those without constant severe trachoma or constant infection, Table 1 [12]. The infection and disease states were not analysed as separate variables.

In the fourth prospective study to investigate incident conjunctival scarring in previously unaffected individuals, a cohort of Tanzanians of all ages (n = 367) was recruited and followed for 5 years (Table 1) [13]. The overall cumulative incidence of TS was 20.4% in 5 years. The

Table 2. Progression of scarring trachoma.

Country / Year	Study design	Participants	Progression of TS to TS+	Comments
Tunisia, 1990 [10]	Prospective 14-year study of the resident population of a trachoma-endemic Tunisian village. Conducted to identify clinical signs and environmental factors associated with development of scarring. A random sample of people seen at baseline were re-examined at 14-years.	Baseline, 1969–72: 2000 people of all ages. Follow-up, 1986–87: 213 people who were aged 1 month to 32 years at baseline. Loss to follow-up: Information not provided. Baseline Scarring:• C0 82 (38.7%) • C1 51 (24.0%) • C2 58 (27.4%) • C3 21 (9.9%)	Progressive Scarring, by 14 years: "worse scarring" reported in 146/213 (68.5%):• C0 to C3: 14/82 (17.1%) • C1 to C3: 10/51 (19.2%) • C2 to C3: 40/58 (69.0%) Progressive TS rate: • C0 to C3: 1.2%/year • C1 to C3: 1.4%/year • C2 to C3: 4.9%/year	• Hyperendemic setting (Regional survey data) • No previous MDA, however during the 1980's systematic antibiotic treatment was carried out which dramatically reduced active trachoma.[35] • Clinical grading: detailed WHO-FPC system.[3] • The data presentation and analysis in this report are relatively limited. The "worse scarring" analysis appears to include incident scarring cases as well as deterioration of established scarring. Not possible to sub-divide the presented data. Data not disaggregated by gender. • Predictors of severe scarring (C3) at 14 years (whole group of 213): ○ Baseline TF (F2/F3): RR 2.8 ○ Baseline TI (P3): RR 18 ○ Household density (closeness of houses): RR 1.3
Tanzania, 2009 [13]	Prospective cohort study of incident and progressive scarring. Participants were people of all ages, examined at baseline and 5-years.	• Baseline, 2000: 990 people of all ages. • Follow-up, 2005: 487 people of which 453 had gradable images from all time points. • Loss to follow-up: 437	• Baseline TS prevalence: 86/453 (18.9%). • Progressive TS by 5 years: 40/85 (47.1%) • Progressive TS rate: 9.4%/year	• Hyperendemic setting (70% TF at baseline) • Clinical grading: simplified WHO system with additional more detailed grading of scarring on photographs. [2] • MDA was administered at baseline and at 18 months. • There was no evidence for a difference in the proportion showing progression with age
Ethiopia, 2015 [17]	Prospective cohort study of progressive scarring in adults with established scarring and minor trichiasis (<6 lashes touching the eye). Examined and swabs collected every 6 months for two years. Swabs were analysed for *C. trachomatis* infection and expression of several genes potentially involved with inflammation and scarring. Progressive scarring was determined by direct comparison of baseline and two year photographs.	• Baseline, 2008: 650 participants. • 585 people had paired photographs from baseline and 24 months. • Loss to follow-up: 65	• Progressive scarring by 2 years: • 135/585 (23.1%) • Progressive TS rate: 11.6%/year.	• Hyperendemic setting (Regional survey data) • Clinical grading: detailed WHO-FPC system, with more detailed grading of scarring on photographs.[3] • MDA had been delivered in this region of Ethiopia several times before the start of the study and during the two year period. • Progressive scarring was strongly associated with and increasing number of inflammatory (P2/P3) episodes: OR 5.93, 95%CI 3.3–10.6, p<0.0001. • There was no association between scarring progression and age, gender or body mass index. No episode of *C. trachomatis* infection were detected. • Gene expression analysis (106 progressors vs 106 non-progressors): clinical inflammation (not scarring progression) was associated with increased expression of *S100A7*, *IL1B*, *IL17A*, *CXCL5*, *CTGF*, *CEACAM5*, *MMP7*, *CD83* and reduced *SPARCL1*.

(*Continued*)

Table 2. (*Continued*)

Country / Year	Study design	Participants	Progression of TS to TS+	Comments
Tanzania, 2015 [17]	Prospective cohort study of progressive scarring in adults with established scarring. Examined and swabs collected every 6 months for two years. Swabs were analysed for *C. trachomatis* infection and expression of several genes potentially involved with inflammation and scarring. Progressive scarring was determined by direct comparison of baseline and two year photographs.	• Baseline, 2009: 804 participants • 577 people had paired photographs from baseline and 24 months	• Progressive scarring by 2 years: • 173/577 (30.0%) • Progressive TS rate: 15.0%/year.	• Hypoendemic setting (Regional survey data) • Clinical grading: detailed WHO-FPC system, with more detailed grading of scarring on photographs.[3] • No MDA has been delivered to this region. • Progressive scarring was strongly associated with and increasing number of inflammatory (P2/P3) episodes: OR 5.76, 95% CI 2.6–12.7, p<0.0001. • No association between scarring progression and gender or body mass index. • Progressors were a bit older: 50.9 years vs 43.8 years, p<0.0001. *C. trachomatis* infection was very rare and not associated with progression. • Gene expression analysis (97 progressors vs 97 non-progressors): clinical inflammation (not progressive scarring) was associated with increased expression of *S100A7*, *IL17A*, *CXCL5*, *MMP7 and CEACAM5*. Only *IL1B* (Fold Change 1.54, p = 0.0067) and *S100A7* (Fold Change 1.43, p = 0.027) had associations with progressive scarring of marginal significance.

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cumulative incidence of scarring generally increased with age, although it was noted it was particularly rapid in younger children, for reasons that could not be determined. In this study the relationship between incident TS and the signs of active trachoma or the presence of infection were not reported. The study found that at baseline and at five years, across all age groups, the cross-sectional prevalence of TS was higher in females than males. However, data disaggregated by gender were not presented on the 5-year incidence of new scarring in this cohort.

The data from a mathematical model supports these longitudinal studies. The 5-year cumulative incidence of new scarring in females, based on cross-sectional data, indicated increasing incidence with increasing age: 3.1% in 15–19 year olds and 14.3% in 55–59 year olds [14].

Overall, there are surprisingly few prospective studies and relatively limited data investigating the link between active trachoma and/or chlamydial infection and the development of incident TS. Three studies present consistent evidence that severe conjunctival inflammation (TI) is associated with substantially increased risk of new scarring several years later [10–12]. The evidence from these studies for an association between TF, in the absence of TI, and the development of scarring is less clear. In the Tunisian study there was an increase in risk associated with TF (RR = 2.8), however, no test statistics (95%CI) were provided to assess the strength of this evidence [10]. In the Tanzanian populations TF was common and affected most of the children at some point in the observation periods [11, 12]. However, those that were found to have only sporadic disease or infection were not at a significantly higher risk of incident TS than those with no episodes of disease or infection [12].

The evidence from these prospective studies demonstrating a link between repeated or persistent *C. trachomatis* infection and subsequent incident TS is limited to only one study from

Table 3. Progression to trachomatous trichiasis.

Country / Year	Study design	Participants	Progression of TS to TT	Comments
Tunisia, 1990 [10]	Prospective 14-year study of the resident population of a trachoma-endemic Tunisian village. Conducted to identify clinical signs and environmental factors associated with development of scarring. A random sample of people seen at baseline were re-examined at 14-years.	Baseline, 1969–72: 2000 people of all ages. Follow-up, 1986–87: 213 people who were aged 1 month to 32 years at baseline. Loss to follow-up: Information not provided. Baseline Scarring: • C0 82 (38.7%) • C1 51 (24.0%) • C2 58 (27.4%) • C3 21 (9.9%)	Progression to TT occurred in 17/213 (8.0%). The risk of progression to TT was related to the baseline scarring severity: • C0 1/82 (1.2%) • C1 0/51 (0%) • C2 8/58 (13.8%) • C3 8/21 (38.1%) Progression from TS to TT: 0.6%/year • C0: 0.1%/year • C1: 0.0%/year • C2: 1.1%/year • C3: 2.7%/year	• Hyperendemic setting (Regional survey data) • No previous MDA, however 1980s systematic antibiotic treatment was carried out which dramatically reduced active trachoma[35] • Clinical grading: detailed WHO-FPC system.[3] • The data presentation and analysis in this report are relatively limited. The risk of developing TT was greater with increasing baseline scarring and inflammation.
Tanzania, 1997 [14]	Mathematical model of the 5 year incidence of TT in women. Using age-stratified cross-sectional data on the different clinical stages.	• 4898 women in survey. • Loss to follow-up: N/A	• Incidence of TT by age in all women: 15–19 age 0.3% / 5 years 55–59 age 6.4% / 5 years • Incidence of TT by age in women with TS at baseline: 15–19 age 3.2% / 5 years; 55–59 age 15.1% / 5 years • Incident TT rate in all women: 15–19 years: 0.06%/ year; 55–59 years: 1.3%/ year. • Incident TT rate in women with TS: 15–19 years: 0.6%/ year; 55–59 years: 3.0%/year	• Hyperendemic setting • No previous MDA. • Clinical grading: simplified WHO system.[2]
Tanzania, 1999 [19]	Prospective 7 year cohort study to measure the incidence of TT in women with and without baseline conjunctival scarring. The cohort was recruited from six villages. All women were examined for TS and TT at baseline (1989). Seven years later all available women who had TS (without TT) at baseline and a similar sized random sample of those without any TS were re-examined.	• Cohort participants who completed the follow-up at 7 years: 523 with TS at baseline. 503 without TS at baseline. • Loss to follow-up: 468	• Incidence of trichiasis at 7-years by baseline TS status: TS: 9.2% / 7 years No TS: 0.6% / 7 years. • Incident TT rate: 1.3%/year in women with pre-existing scarring. 0.1%/year with no TS	• Hyperendemic setting (Regional survey data) • No previous MDA • Clinical grading: simplified WHO system.[2] • Predictors of trichiasis: ○ Age (increase per year): OR 1.03, 95%CI: 1.01–1.06 ○ Infection at follow up: OR 2.51, 95%CI: 1.1–5.69
Gambia, 2001 [20]	The 1986 Gambian National Blindness and Eye Disease Survey was a 1% sample of the total population. In this 8174 people were examined. 12 years later individuals who were found to have TS in 1986 were retraced and assessed for the development of TT and CO.	• Baseline, 1986: 639/8174 people ≥18 years were found to have TS • Follow-up, 1998: 326/639 (51%) were re-examined • Loss to follow-up: 313	• Progressed from scarring to trichiasis: 19/297, 6.4% / 12 years. • Incident TT rate in people with TS: 0.5%/year	• Hypoendemic setting (Regional survey data) • No previous MDA • Clinical grading: simplified WHO system.[2] • Risk factor for trichiasis: ○ Old age: OR 1.07, 95% CI 1.01–1.12
Gambia, 2010 [21]	Five year prospective study of the population of 14 adjacent Gambian villages.	• Baseline, 2001: 592 people >15 years • 5-years, 2006: 456 people >15 years • Loss to follow-up: 136	• Baseline TT: 9/592 (1.5%). • 5-year TT: 6/456 (1.3%) • Incident TT Cases: 2/456, 0.4% / 5 years • Incident TT rate in all adults: 0.1%/year	• Mesoendemic setting (15% TF at baseline) • No previous MDA • Clinical grading: detailed WHO-FPC system.[3] • At 5-years, 3/6 case had TT at baseline and 1/6 was a new resident

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Table 4. Progression of trachomatous trichiasis.

Country / Year	Study design	Participants	Progression of TT to TT+	Comments
Gambia, 2002 [22]	One year longitudinal study of individuals with TT in at least one eye.	• Baseline, 1996: 190 people. • Major trichiasis 135; Minor trichiasis 55. • Follow-up, 1997: 169 people were re-examined at 12 months. • Loss to follow-up: 21	• Progression of Minor to Major trichiasis: 18/55 (33%) / 1 year • Progression of unilateral to bilateral TT: 21/46 (46%) / 1 year	• Hypoendemic setting (Regional survey data) • No previous MDA. • Clinical grading: simplified WHO system.[2]
Gambia, 2006 [23]	Four year longitudinal study of individuals with TT in at least one eye, who had declined surgery. Examined at baseline and 4 years.	• Baseline, 1996: 220 people • Follow-up, 2000: 153 people were re-examined • Loss to follow-up: 67	• Progression of Minor to Major trichiasis: 28/75 (37.3%) eyes. • Progression Rate: 9.3%/year • Progression of unilateral to bilateral trichiasis: 12/42 (29%) eyes • Progression rate: 7.3%/yr	• Hypoendemic setting (Regional survey data) • No previous MDA • Clinical grading: detailed WHO-FPC system.[3] • Univariate association between TT progression and conjunctival inflammation (P2 or P3) at 4-years: OR 3.07, 95%CI 1.23–7.70, p = 0.017. This was not significant in a logistic regression model. • *C. trachomatis* was detected in 2/146 (1.4%) tested at 4-years and was not associated with TT progression.
Ethiopia 2011 [24, 25]	Two year prospective randomised controlled trial of epilation vs surgery for Minor trichiasis (<6 lashes). 650 individuals were randomised to the epilation arm at baseline and followed every six months for two years. Outcome measure was presence of 5 + lashes. At two years all were offered TT surgery, 383 chose to continue epilating and were followed up for an additional two years.	• Baseline, 2008: 650 people • Primary outcome data available for 637 people at 2 years (2010). • Follow-up, 2012: 383 people who continued epilating were re-examined. Loss to follow-up: at 2 years: 13; at 4 years: 267	• At 2 years: progression of Minor to Major trichiasis: 84/637 (13.2%) eyes. • Progression rate: 6.6% / year. • At 4 years, comparing baseline to four years, 82 /383 (21.4%) had more eyelashes touching and 200/383 (52.2%) had fewer.	• Hyperendemic setting (Regional survey data) • Clinical grading: detailed WHO-FPC system, with more detailed grading of scarring on photographs.[3] • MDA had been delivered in this region of Ethiopia several times before the start of the study and during the two year period.

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Tanzania [12]. In this study, data on disease and infection were combined for the analysis, so it is not possible to determine the independence of the effect of infection from disease. Notably, although a small proportion of individuals with only sporadic disease and/or infection did develop TS, they were not at significantly greater risk of scarring than individuals who had no disease or infection episodes documented. This might suggest that in a programmatic context, reducing the pressure of infection and associated TI through MDA might reduce disease in the majority of individuals below a threshold required for the development of scarring.

As would be expected from the known cross-sectional age-specific prevalence of TS from population based surveys, the three prospective Tanzanian studies and the mathematical model found clear and consistent evidence of increasing risk of incident TS with increasing age [11–14]. However, it is noteworthy that some incident scarring was developing during childhood in these populations. Similarly, the prospective Tanzanian studies found that females were at consistently higher risk of incident TS than males, probably because of a greater lifetime exposure to chlamydial infection [11–13].

West and colleagues suggested that there might be two distinct routes to the development of TS [11]. Firstly, in the "classical" model, which may account for the majority of TS cases, are individuals who are repeatedly exposed to *C. trachomatis*. In this group the risk of TS is primarily determined by the cumulative number of infection episodes experienced. This is

Table 5. Progression to corneal opacification, visual impairment and blindness.

Country / Year	Study design	Participants	Progression of TT to CO	Comments
Tanzania, 1997 [14]	Mathematical model of the 10 year incidence of CO in women. Using age-stratified cross-sectional data on the different clinical stages.	• 4898 women in survey. • Loss to follow-up: N/A	Incidence of CO, attributable to trachoma: All women: • 15–24 yrs 0.16% / 10 years • 45–54 yrs 2.80% / 10 years Progression rate: • 15–24 yrs 0.02%/year • 45–54 yrs 0.3%/year Women with TT: • 15–24 yrs 27.2% / 10 years • 45–54 yrs 53.5% / 10 years Progression rate: • 15–24 yrs 2.72%/year • 45–54 yrs 5.35%/year	• Hyperendemic setting • No previous MDA • Clinical grading: simplified WHO system.[2] • In this model around half of all corneal opacity was due to causes other than trachoma. In women under 35 years these other causes dominated. In older ages trachoma was the main cause.
Gambia, 2001 [20]	The 1986 Gambian National Blindness and Eye Disease Survey was a 1% sample of the total population. In this 8174 people were examined. 12 years later the people who were found to have TS in 1986 were retraced to assess them for the development of TT and CO.	• Baseline, 1986: 639/8174 people ≥18 years were found to have TS • Follow-up, 1998: 326/639 (51%) were re-examined • Loss to follow-up: 313	• Progressed from TS to CO: 18/302 (5.9%) / 12 years. • Progression rate: 0.5%/ year • Progressed from TT to CO: 4/20 (20%) / 12 years. • Progression rate: 1.7%/ year	• Hypoendemic setting (Regional survey data) • No previous MDA • Clinical grading: simplified WHO system.[2] • Risk factor for corneal opacity: ○ Trichiasis at baseline: OR 8.4, 95% CI 1.8–39.2 • Old age: OR 1.07, 95% CI 1.01–1.12 • Progressed from TS to incident visual impairment / blindness: ○ 53/321 (16.5%), all causes ○ 8/321 (2.5%), attributed to cornea scarring • Progressed from TT to incident visual impairment / blindness: ○ 4/26 (15.4%), all causes ○ 2/26 (7.7%), attributed to cornea scarring
Gambia, 2002 [22]	One year longitudinal study of individuals with TT in at least one eye. Progression was considered significant if the baseline cornea grading had been CC0 / CC1 and the 1-year grade was CC2 / CC3.	• Baseline, 1996: 190 people. Major TT 135. Minor TT 55. • Follow-up, 1997: 169 people were re-examined at 12 months • Loss to follow-up: 21	• Incident CO in individuals with TT: 10/104 (10%) / 1 year • Progressive CO in individuals with un-operated Major TT: 33/96 (34%) / 1 year	• Hypoendemic setting (Regional survey data) • No previous MDA • Clinical grading: detailed WHO-FPC system used for cornea grading.[3] • Change in vision over one year: ○ 8/88 (9%) had incident visual impairment or blindness. • There was a non-significant trend to more visual deterioration with major TT (9%) compared to minor TT (4%) at baseline.
Gambia, 2006 [23]	Four year longitudinal study of individuals with TT in at least one eye, who had declined surgery. Examined at baseline and 4 years.	• Baseline, 1996: 220 people • Follow-up, 2000: 153 people were re-examined, with 241 eyes that had not been surgically treated. • Loss to follow-up: 67	• Incident CO in eyes with TT at baseline: 16/211 (7.6%) / 4 years. • Progression rate: 1.9%/ year	• Hypoendemic setting (Regional survey data) • No previous MDA • Clinical grading: detailed WHO-FPC system.[3] • At baseline 30/241 eyes had CO. • CO was only found in eyes with TT. New CO was associated with the presence of Major TT at 4-years (14/16 had major TT). Incident CO by 4 years was more frequent in eyes that had Major TT at baseline: ○ Minor or no TT at baseline: 6/117 (5.1%) ○ Major TT at baseline: 10/99 (10.1%). • There was an overall deterioration in visual acuity over the 4 years, of 0.22 LogMAR unit. This change was more marked (non-significant) for eyes with TT (0.30) than for those without TT (0.15). 29/221 eyes had newly deteriorated to <3/60. However, only 6/29 were due to CO, the large majority were due to cataract.

(*Continued*)

Table 5. (*Continued*)

Country / Year	Study design	Participants	Progression of TT to CO	Comments
Ethiopia, 2011 [17]	Two year prospective randomised controlled trial of epilation vs surgery for Minor trichiasis (<6 lashes). 650 individuals were randomised to the epilation arm at baseline and followed every six months for two years. The change in CO was assessed by direct comparison on digital photographs.	• Baseline, 2008: 650 people • Primary outcome data available for 637 people. • Loss to follow-up: 13	• Change in CO, in people with Minor trichiasis who were epilating at 2 years: • Increased CO: 33/603 (5.5%) / 2 years; 2.75%/ year • Reduced CO: 7/603 (1.2%) / 2 years; 0.6%/ year	• Hyperendemic setting (Regional survey data) • Clinical grading: detailed WHO-FPC system, with more detailed grading of corneal scarring on photographs.[3] • MDA had been delivered in this region of Ethiopia several times before the start of the study and during the two year period. • One eye per person analysed. • 87/603 (14.5%) had a deterioration in visual acuity of >0.3 LogMAR units by 2 years. Most of this was not associated with a deterioration in CO, suggesting that other causes such as cataract were responsible.

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consistent with the observation that TS incidence is higher in females and with increasing age. This model has since been incorporated into the design of mathematical models of the scarring sequelae of trachoma [15, 16]. The second route, which may account for a minority of people who develop TS, is characterised by the development of a severe and persistent inflammatory phenotype, "constant severe trachoma". Data from several of these prospective studies is consistent with this hypothesis [10–12]. Whether these individuals are responding unfavourably to *C. trachomatis*, to other bacteria, or are genetically prone to develop a severe persistent inflammatory phenotype is unclear [4].

Progression of Scarring Trachoma

We identified four prospective cohort studies which reported the rates and risk factors for the progression of established trachomatous conjunctival scarring (Table 2) [10, 13, 17]. One was from Tunisia, one from Ethiopia and two from Tanzania. There was some heterogeneity in the study designs, although the two parallel studies we conducted in Ethiopia and Tanzania used similar designs and were reported together [17]. Three studies were conducted in hyperendemic communities [10, 13, 17] and one in a hypoendemic community [17]. The Tunisian and both Tanzanian studies were in areas which had not previously received MDA [10, 13, 17]. After enrolment, three of the study populations received some form of antibiotic treatment for trachoma control, although this was variable in nature and frequency (Table 2). The Ethiopian study was conducted in an area that had previously received MDA on several occasions and continued to do so during the study period [17]. The second Tanzanian study was conducted in an area where the current prevalence of active trachoma is below treatment thresholds [17]. The duration of follow-up varied from 2 to 14 years. The assessment of clinical signs used either the simplified or detailed WHO Trachoma Grading Systems [2, 3].

The identification of progressive scarring used different methodologies. In the Tunisian study the development of scarring was based on a change in the clinical field grading score [10]. In the other three studies the progression of scarring was identified through the grading of photographs. For one study this was done by the comparison of an independent grading of the baseline and five-year photographs [11]. In the other two studies the baseline and two-year photographs were graded using a detailed grading system for scarring [18]. The photographs were directly compared side-by-side for assessment of progression, possibly allowing for more subtle changes in TS to be detected [17].

The Tunisian study (described above) reported scarring progression in 68.5% of individuals (n = 120) over 14 years (Table 2) [10]. However, this report is limited in the level of detail

provided and it appears that the total progression estimate may include some individuals who were incident scarring cases. There is some partial information on progression from mild or moderate scarring (C1 / C2) to the most severe grade (C3), Table 2. The specific risk factors for progression to C3 are not presented. However, as noted above, the development of new C3 in those who had no scarring at baseline was associated with TI at baseline.

In the first Tanzanian study progression of conjunctival scarring occurred in 47.1% of individuals (n = 85, all ages) who had some established TS at baseline, over a five year period (Table 2) [13]. There was no difference by age in the proportion with evidence of progression. No data on the relationship between scarring progression and gender, clinical inflammation or *C. trachomatis* infection were reported.

In our study in Ethiopia, progression of conjunctival scarring developed in 23.1% of participants (n = 585) over a 2 year period (Table 2) [17]. This was a prospective cohort of adults who had minor trichiasis at baseline that was managed by epilation. They were examined and photographed every six months, with swab samples collected for *C. trachomatis* PCR and conjunctival gene expression analysis. There was a strong relationship between progressive scarring and increasing number of observed episodes of clinical inflammation, defined as P2 or P3 in the detailed WHO grading system (OR 5.93, 95%CI 3.3–10.6, $p<0.0001$). There was no evidence of an association between scarring progression and gender, age or body mass index (BMI). The samples from individuals with progressive scarring and a frequency matched sample of individuals who did not show progressive scarring were tested for *C. trachomatis* by PCR on 4 occasions; all samples were negative for *C. trachomatis*. Conjunctival inflammation, but not scarring progression, was associated with increased gene expression of a mixture of pro-inflammatory cytokines (*IL1B*, *IL17A*, *CXCL5*), anti-microbial effectors (*S100A7*) and factors associated with tissue remodelling (*CTGF*, *MMP7*, reduced *SPARCL1*).

In the parallel cohort study we conducted in Tanzania we used the same protocol, including sample collection, as the Ethiopian study; all individuals had conjunctival scarring at baseline, were aged 18 years or more and no individuals had baseline trichiasis [17]. Progression of conjunctival scarring was observed in 30.0% of participants (n = 577) over a 2-year period (Table 2). Again, there was a strong relationship between progressive scarring and an increasing number of episodes of clinical inflammation (P2/P3), of a similar magnitude to that found in the Ethiopian study (OR 5.76, 95% CI 2.6–12.7, $p<0.0001$). There was no evidence of an association between scarring progression and gender, age or BMI. In this Tanzanian cohort there were 804 people with TS recruited and tested for *C. trachomatis* by PCR at baseline, of which only 4 samples were positive. At 6, 12 and 18 months after baseline, the samples from the individuals with progressive scarring and a frequency matched sample of individuals in the cohort who did not show progressive scarring were also tested for *C. trachomatis* and only one sample was positive. In this study progressive scarring was associated with a modest increase in expression of *IL1B* and S100A7. In addition, increased *S100A7*, *IL17A*, *CXCL5*, *MMP7* and *CEACAM5* expression were associated with clinical inflammation (P2/P3).

A consistent finding across these studies was that trachomatous conjunctival scarring continues to progress over time. The studies are probably too heterogeneous to provide an overall estimate range for disease progression. However, it is noticeable that in the two studies using the same protocol the rates of scarring progression were similar [17]. Moreover, it might have been anticipated that the Ethiopian cohort, which was conducted in a hyperendemic region, would have experienced a greater proportion of progression than that observed in a region of Tanzania that had a low level of active trachoma, and was therefore not included in the national antibiotic distribution programme.

There was a strong association between conjunctival inflammation and progressive scarring, with individuals experiencing more inflammatory episodes being at greater risk of progression

[10, 17]. In two studies the immunofibrogenic correlates of clinically apparent inflammation were investigated using gene expression analysis, and this demonstrated that its presence was associated with increased expression of a range of pro-inflammatory factors [17]. These observations are consistent with the findings for incident scarring outlined above; children who experienced more episodes of active trachomatous inflammation were more likely to develop incident scarring (Table 1) [11, 12].

There are three noteworthy findings from these four cohort studies of progressive scarring. Firstly, we found no published evidence that the progression of established scarring was associated with the detection of *C. trachomatis* infection [17]. Indeed, infection was detected in only very few individuals with TS. This contrasts with the observation, albeit from limited prospective data, of an association between the development of incident scarring and episodes of chlamydial infection [12]. Secondly, we found no published evidence of a difference between males and females in the proportions showing signs of scarring progression. Again, this contrasts with the findings for incident scarring outlined above, which developed more frequently in females (Table 1). Thirdly, amongst people with established scarring the proportion showing scarring progression did not vary with age. This also contrasts with the findings for incident scarring, which developed more frequently with increasing age.

Therefore, once TS is established the risk of scarring progression does not appear to be associated with exactly the same set of risk factors that were associated with the development of incident scarring. This could be interpreted as suggesting that progression of established scarring is less dependent than incident scarring on recurrent *C. trachomatis* infection, episodes of which are more frequent in females and accrue with increasing age. It may also indicate that other factors such as non-chlamydial bacterial infection, a dry ocular surface or altered tissue inflammatory or scarring responses may also contribute to a pro-inflammatory / pro-fibrotic state. This might be anticipated as scarred ocular surface tissue probably has altered physiology and compromised defence mechanisms. However, prospective data on these factors has not been reported.

Progression to Trachomatous Trichiasis

We identified four prospective cohort studies which report rates and risk factors for the development of trachomatous trichiasis (Table 3) [10, 19–21]. One was from Tunisia, one from Tanzania and two from The Gambia. In addition, we identified a mathematical model based on cross-sectional data from Tanzania [14]. Two studies were conducted in hyperendemic areas and two in hypoendemic areas. None of the study populations had previously received systematic MDA for trachoma control. After enrolment, two of the study populations received some form of antibiotic treatment for trachoma control, although this was variable in nature and frequency (Table 3) [10, 21]. The duration of follow-up varied from 5 to 14 years.

The Tunisian study (described above) reported incident TT in 8.0% over a 14 year period [10]. The risk of developing TT was related to the baseline conjunctival scarring severity, being 1.2% in people with no scarring and rising to 38.1% in those with severe baseline scarring (C3), Table 3. The Tanzanian study was a 7 year cohort of females with and without TS at baseline [19]. In those with TS at baseline 9.2% developed TT, and of those without TS at baseline 0.6% developed TT by 7-years. The development of TT was associated with increasing age and the detection of *C. trachomatis* infection at the 7-year time-point. The number of individuals with *C. trachomatis* infection at follow up was 60/513 (11.7%) in the group with TS at baseline and 36/501 (7.5%) in the group with no TS at baseline. The first of the Gambian studies reported the 12-year incidence of TT in people with TS at baseline to be 6.4% [20]. The development of TT was associated with increasing age. The second Gambian study was a five year study of all

the residents of 14 villages, most (94%) of whom did not have scarring at baseline. The five year cumulative incidence of TT in those >15 years was 0.4% [21].

The mathematical model estimated the 5 year cumulative incidence of TT in women from a hyperendemic region of Tanzania using age-stratified cross sectional data from different clinical stages [14]. The cumulative incidence of TT was highest in older women (6.4% versus 0.3% in young women) and in women with TS at baseline (15.1% in 55–59 year olds, 3.2% in 15–19 year olds).

Progression of Trachomatous Trichiasis

We identified three prospective cohort studies that reported the rates and risk factors for the progression of trachomatous trichiasis from unilateral to bilateral disease, or from minor trichiasis (<6 lashes touching the eye) to major trichiasis (6+ lashes touching the eye), Table 4 [22–25]. Two of these were from The Gambia and one from Ethiopia. The Gambian studies were both conducted in hypoendemic areas. The Ethiopian study was in a hyperendemic setting with on-going MDA. The duration of follow-up varied from 1 to 4 years.

In the first Gambian study, 46% of people with unilateral trichiasis (n = 46) had developed bilateral disease in one year [22]. Of those with minor trichiasis (n = 55), 33% progressed to major trichiasis. In the second Gambian study 29% of people with unilateral trichiasis (n = 42) progressed to bilateral disease in four years and 37% of minor trichiasis (n = 75) progressed to major trichiasis [23]. In this second study several factors were analysed in relation to the progression of trichiasis over four years: age, gender, conjunctival inflammation (at four years), chlamydial infection and pathological bacterial infection (at four years). In univariate analysis only conjunctival inflammation at 4-years was associated with TT progression. However, no factor was significant in a multivariable model. It is noteworthy that at 4-years both conjunctival inflammation and bacterial infection were independently associated with the presence of major trichiasis. Only 2/146 samples tested were positive for *C. trachomatis* at the 4-year timepoint.

The Ethiopian study was of a group of people with minor trichiasis (n = 650) who were in the epilation arm of a randomised controlled trial of epilation vs. surgery, with regular follow up over a two year period [24]. Individuals were equipped with high quality forceps and a relative was trained in how to perform epilation. During the first two years 13% of study eyes progressed from minor to major trichiasis. After two years all participants were offered surgery for their trichiasis. About a third accepted surgery at two years, while the majority (n = 383) chose to continue with epilation and were followed up for an additional two years [25]. Comparing the amount of trichiasis by counting the absolute number of lashes touching the eye at baseline with that at four years, 21.4% of these 383 individuals were found to have had some degree of increase in the number of lashes touching the eye, however this was difficult to assess in the context of regular epilation. In addition, 36.6% had only a modest increase in the degree of entropion, although it should be noted that this is a variable and difficult clinical sign to grade. In a multivariable model, over the four years, progression to major trichiasis was associated with older age and having ≥3 lashes touching the eye at baseline.

Progression to Corneal Opacification, Visual Impairment and Blindness

We identified four prospective cohort studies that reported the rates and risk factors for the development of corneal opacification (CO) and visual impairment (Table 5) [20, 22–25]. Three were from The Gambia, conducted in hypoendemic areas. One study was from a hyperendemic area of Ethiopia, with on-going MDA. The duration of follow-up varied from 1 to 12 years. In addition, the mathematical model based on Tanzanian cross-sectional data provided 10 year

estimates for the development of CO [14]. We have not included prospective data on CO change from clinical trials where TT was treated with surgery.

The first Gambian study found the 12-year cumulative progression from TS to CO to be 5.9% and for TT to CO to be 20% [20]. Risk factors for incident CO were increasing age and TT at baseline. Visual impairment developed in 16.5%, but only 2.5% of this was attributed to CO; most was due to cataract (Table 5). The second Gambian study found that in one year incident CO developed in 10% of people with TT and that CO progressed in 34% with major trichiasis [22]. New visual impairment or blindness developed in 9%. The third study from The Gambia found incident CO developed in 7.6% of eyes with TT at baseline over the course of four years, with the risk being greater for those with major TT (Table 5) [23]. Again, the risk of visual impairment was increased with TT, however, the large majority of incident cases of visual impairment were due to cataract.

The four year Ethiopian study of people with minor trichiasis has been outlined above [24, 25]. Incident or progressive CO was determined by the comparison of photographs in this study. At 2 years, 5.5% of the 650 individuals with minor trichiasis had some increase in CO. When modelled at 4-years, incident/progressive CO was associated with being $\geq$50 years of age and having moderate CO at baseline. Visual acuity deteriorated in 14.5% by >0.3LogMAR by 2 years, however, most of this was not attributable to changes in CO.

The mathematical modelling study, described above, found that the overall incidence of CO was higher in older women: 2.8% in 45–54 years versus 0.16% in 15–24 years [14]. The cumulative incidence rates were estimated to be very much higher in those with TT at baseline: 53.5% in 45–54 year olds and 27.2% in 15–24 year olds. This model estimated around half of all CO was due to causes other than trachoma. In women <35 years these other causes dominated, whereas in older women trachoma was the main cause.

Conclusions

Conducting long-term prospective studies of the rates and risk factors of progressive trachoma from active disease through to blindness is complex and expensive. Here we have reviewed the longitudinal studies that contribute to our understanding of this disease process. In addition to these longitudinal studies, there are many cross-sectional studies that provide indirect evidence about risk factors for progression. The prospective studies are relatively few in number, of variable design and frequently small in size. The determination of the presence of progression in conjunctival scarring can be challenging, particularly where there is also extensive inflammation masking scar tissue. Despite these limitations, collectively they provide evidence that supports much of the widely accepted description of the natural history of trachoma, illustrated in Fig 1.

The studies of incident and progressive conjunctival scarring were consistent in showing a strong association with repeated or persistent conjunctival inflammation. In contrast, the evidence linking the development of incident conjunctival scarring with *C. trachomatis* infection is limited to only one study. Moreover, in two large cohorts with scarring progression rates of 23.1% and 30% over 2 years only very few episodes of chlamydial infection were detected.

The model proposed by West and colleagues, and outlined above, suggests that the majority of individuals who develop TS do so after repeated rounds of infection by *C. trachomatis*. In addition, there appears to be a subgroup of individuals who experience repeated or protracted intense inflammation, who are at increased risk of developing scarring. It is anticipated that the introduction of control programmes involving MDA and F and E interventions to limit transmission, will reduce the number of episodes of infection experienced and therefore the risk of developing incident scarring. However, the potential impact of control measures on halting

progression of previously established scarring is less certain. A treatment intervention that could reduce chronic conjunctival inflammation in adults in trachoma-endemic areas might be desirable to prevent scarring progression.

The lack of a clear association between chlamydial infection and scarring progression in adults could have a number of explanations [17]. It is possible that the number and frequency of clinical observations and tests for chlamydial infection in these studies were too few to capture infection events. It is also possible that the detection of infection in adults is more difficult than in children, because of lower infection loads or shorter infection duration, due to the acquisition of some protective immunity [26–28]. This potential explanation might imply that only brief bursts of infection with *C. trachomatis* is needed to trigger chronic inflammation and an associated scarring response.

Alternatively, it is possible that conjunctival inflammation observed in adults with established conjunctival scarring may have additional causes besides *C. trachomatis*. A number of studies have found cross-sectional associations between non-chlamydial bacteria cultured from the conjunctival surface and the presence of inflammation at all stages of the natural history of trachoma [23, 29–33]. Currently however there are no longitudinal data to determine whether this inflammation associated with other infections represents a pro-fibrotic state that contributes additional scarring, or whether it is a by-product of the altered physiological environment of a scarred conjunctiva. Longitudinal studies that include tests for *C. trachomatis* and non-chlamydial bacteria are needed to determine their relative contributions to scarring progression.

These observations raise important issues of programmatic significance for trachoma control, particularly over whether conjunctival scarring can continue to develop and progress in individuals with intermittent or chronic inflammation, but in the absence of on-going regular chlamydial infection. Several long-term population based studies of the impact of MDA have demonstrated that even after *C. trachomatis* has been cleared or brought down to very low prevalence levels, the prevalence of signs of active conjunctival inflammation persist for some years [21, 34]. While it is possible that the cross-sectional nature of these studies may have missed recent infection episodes, it is striking that the inflammatory phenotype was still present after the prevalence of *C. trachomatis* infection had been reduced to a low level, and this may have significance for disease progression. A question worth consideration is whether the clinical sign TI should be included in the monitoring of trachoma control in addition to TF, because of the consistently strong association between TI and incident and progressive scarring. Finally, these data support the recommendation that trachoma control programmes maintain mechanisms to detect and treat trichiasis for many years to come.

Author Contributions

Conceived and designed the experiments: MJB AMR MJH. Performed the experiments: AMR MJB. Analyzed the data: AMR MJB MJH TD. Contributed reagents/materials/analysis tools: MJB. Wrote the paper: AMR MJB MJH TD.

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6. Immunofibrogenic Gene Expression Patterns in Tanzanian Children with Ocular *Chlamydia trachomatis* Infection, Active Trachoma and Scarring: Baseline Results of a 4-Year Longitudinal Study.

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
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Student Signature: [redacted] Date: 29.10.2018

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Immunofibrogenic Gene Expression Patterns in Tanzanian Children with Ocular *Chlamydia trachomatis* Infection, Active Trachoma and Scarring: Baseline Results of a 4-Year Longitudinal Study

*Athumani M. Ramadhani[1,2], Tamsyn Derrick[1,2], David Macleod[3], Patrick Massae[2], Tara Mtuy[1,2], David Jeffries[4], Chrissy H. Roberts[1], Robin L. Bailey[1], David C. W. Mabey[1], Martin J. Holland[1] and Matthew J. Burton[1]**

[1] Clinical Research Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, [2] Kilimanjaro Christian Medical Centre, Moshi, Tanzania, [3] Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, United Kingdom, [4] Support Services (Statistics), Medical Research Council Unit The Gambia, Fajara, Gambia

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Peter Timms, University of the Sunshine Coast, Australia

***Correspondence:**
Matthew J. Burton
matthew.burton@lshtm.ac.uk

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Trachoma, caused by *Chlamydia trachomatis,* is the world's leading infectious cause of blindness and remains a significant public health problem. Much of trachomatous disease pathology is thought to be caused indirectly by host cellular and immune responses, however the immune response during active trachoma and how this initiates progressive scarring is not clearly understood. Defining protective vs. pathogenic immune response to *C. trachomatis* is important for vaccine design and evaluation. This study reports the baseline results of a longitudinal cohort of Tanzanian children, who were monitored for 4 years in order to determine the immunofibrogenic and infectious correlates of progressive scarring trachoma. In this cohort baseline, 506 children aged 6–10 years were assessed for clinical signs, infection status and the expression of 91 genes of interest prior to mass azithromycin administration for trachoma control. *C. trachomatis* was detected using droplet digital PCR and gene expression was measured using quantitative real-time PCR. The prevalence of follicles, papillary inflammation and scarring were 33.6, 31.6, and 28.5%, respectively. *C. trachomatis* was detected in 78/506 (15.4%) individuals, 62/78 of whom also had follicles. *C. trachomatis* infection was associated with a strong upregulation of *IFNG* and *IL22,* the enrichment of Th1 and NK cell pathways and Th17 cell-associated cytokines. In individuals with inflammation in the absence of infection the *IFNG/IL22* and NK cell response was reduced, however, pro-inflammatory, growth and matrix factors remained upregulated and mucins were downregulated. Our data suggest that, strong *IFNG/IL22* responses, probably related to Th1 and NK cell involvement, is important for clearance of *C. trachomatis* and that the residual pro-inflammatory and pro-fibrotic phenotype that persists after infection might contribute to pathological scarring. Interestingly, females appear more susceptible to developing papillary inflammation and scarring

than males, even at this young age, despite comparable levels of *C. trachomatis* infection. Females also had increased expression of a number of IFNγ pathway related genes relative to males, suggesting that overexpression of this pathway in response to infection might contribute to more severe scarring. Longitudinal investigation of these factors will reveal their relative contributions to protection from *C. trachomatis* infection and development of scarring complications.

Keywords: trachoma, *Chlamydia trachomatis*, gene expression, mass azithromycin administration, longitudinal study, active trachoma, conjunctival scarring

INTRODUCTION

Trachoma, a Neglected Tropical Disease caused by the bacterium *Chlamydia trachomatis,* remains the leading infectious cause of blindness worldwide. It is characterized by repeated conjunctival infection initiated early in childhood, triggering inflammation (*trachomatous inflammation—intense* and *trachomatous inflammation—follicular*) that drives scarring and trichiasis (in-turned eyelashes) and eventually blinding corneal opacification (Hu et al., 2013a; Taylor et al., 2014; Ramadhani et al., 2016a). The burden of this disease is high; current estimates indicate 200 million people live in trachoma endemic areas in 42 countries (World Health Organization, 2016). Approximately 1.9 million people are visually impaired or irreversibly blind from trachoma (Bourne et al., 2013). To meet this public health challenge, the WHO-led Global Alliance for the Elimination of Trachoma recommends the implementation of the SAFE strategy which tackles the disease at different stages: **S**urgery to correct trichiasis, **A**ntibiotics to treat chlamydial infection and **F**acial cleanliness and **E**nvironmental improvements to suppress transmission of infection (Taylor et al., 2014).

Our understanding of this disease process is only partial (Hu et al., 2013a). There are few detailed long-term longitudinal studies that investigate the risk factors for and pathophysiology of scarring trachoma (Dawson et al., 1990; West et al., 2001; Wolle et al., 2009a,b; Burton et al., 2015). These studies have consistently found incident and progressive scarring to be strongly associated with clinically apparent conjunctival inflammation (Ramadhani et al., 2016a). However, only one study has prospectively examined the relationship between *C. trachomatis* infection and the development of incident scarring in younger people; this found that the detection of infection and/or severe inflammation on multiple occasions was associated with increased risk of new scarring (Wolle et al., 2009b). The relationship between chlamydial infection and progression of previously established scarring in adults has been prospectively examined in two longitudinal cohorts; neither found an association (Burton et al., 2015). This raises the possibility that progressive scarring may not be entirely dependent on continual re-exposure to *C. trachomatis*. It is possible that repeated chlamydial infection results in long-term physiological changes in conjunctival tissue responsiveness, such that other pro-inflammatory factors may stimulate on-going fibrotic responses (Kechagia et al., 2016). For trachoma control programmes, this raises the possibility that scarring may continue to progress after ocular chlamydial infection has been eliminated from the population. The implication of this is that services for managing incident trichiasis may be needed for many years.

Previously, we and others have explored the immunological correlates of the different stages of trachoma mostly through cross-sectional studies using a range of methodologies, including gene expression and protein analysis, immunohistochemistry and genome wide association studies (Bobo et al., 1996; Burton et al., 2004, 2011b, 2015; Skwor et al., 2008; Holland et al., 2010; Natividad et al., 2010; Derrick et al., 2013, 2016a,b; Hu et al., 2013a, 2016; Roberts et al., 2015). These studies have shown an increase in pro-inflammatory and matrix factors (*IL1B* (interleukin 1 beta), *TNF* (tumor necrosis factor), *S100A7* (psoriasin), *IL17A*, *IFNG* (interferon gamma), perforin, *IL12, IL10, CXCL5, CTGF* (connective tissue growth factor) and *MMP9* (matrix metalloproteinase 9) in individuals with active trachoma and/or chlamydial infection, indicating the involvement of a type 1 T helper (Th1) cell response, NK cell cytotoxicity and potentially Th17 cells. In individuals with trachomatous scarring and/or trichiasis, factors involved in innate pro-inflammatory responses and matrix remodeling (*IL1B, CXCL5, S100A7, CTGF, MMP7,* and *MMP9*) were upregulated.

To understand the immunofibrogenic correlates of progressive conjunctival scarring we conducted a long-term cohort study. Here we present the baseline findings for a large panel of factors, to define those to be examined in the prospective study.

METHODS

Ethics Statement

This study was reviewed and approved by the Tanzanian National Institute for Medical Research Ethics Committee, the Kilimanjaro Christian Medical Centre Ethics Committee, and the London School of Hygiene and Tropical Medicine Ethics Committee. It adhered to the tenets of the Declaration of Helsinki.

Study Population

The study was conducted in three adjacent trachoma endemic villages in Kilimanjaro and Arusha regions, Northern Tanzania. The villages are relatively remote, geographically neighbors and have similar patterns of life and traditions. This area is predominantly inhabited by people of the Maasai tribe. Pastoral activities are the main occupation. The area is dry for much of the year, except for the rainy season (February to May). Water supply is therefore limited, and largely depends on a long-distance

water pipe scheme from Mount Kilimanjaro. Family units are organized in Bomas, with living huts arranged in a circle around a central animal enclosure, which is often characterized by a high density of flies.

In January 2012, we recruited a cohort of children aged 6–10 years from these villages. The cohort has subsequently been followed-up every 3 months for 4 years to investigate the pathogenesis of conjunctival scarring. All children, aged 6–10 years, who were normally resident in one of the three villages, were eligible for inclusion. We chose this restricted age group as we considered that they were more likely to show evidence of incident or progressive conjunctival scarring during the 4 years of the study. At the outset community meetings were held to introduce the study. Each household was then visited to meet the parents or legal guardians of children eligible for enrolment. A field worker explained the nature of the study in detail in either Swahili or Maasai language. There was an opportunity to discuss and ask questions. Finally, if the parent or guardian agreed to allow the child to be enrolled into the study this was documented on a consent form in Kiswahili, and witnessed by a third person.

Clinical Assessments and Sample Collection

The left eye of each child was examined by an ophthalmic nurse experienced in grading trachoma. The examinations were all performed under standardized conditions, using x2.5 loupes and a bright touch. The conjunctiva was anesthetized with a drop of preservative-free proxymetacaine hydrochloride 0.5%w/v (Minims®, Chauvin Pharmaceuticals Ltd, Surrey, UK). The eyelid was everted, examined and photographed (Nikon D90 with 105 mm Macro lens). Two conjunctival swab samples (Dacron polyester, Puritan Medical Products Company, Maine, USA) were collected for *C. trachomatis* detection and gene expression analysis. The swabs were passed across the upper tarsal conjunctiva four times, with a quarter turn between each pass. The first swab was placed directly into a tube containing RNAlater (Thermo Fisher Scientific, Massachusetts, USA) and the second into a dry tube. The samples were placed into a cool box. Later the same day the dry swab samples were stored directly at −80°C and the RNAlater samples kept at 4–8°C overnight and then stored at −80°C. Air control swabs were collected after every 50 samples by passing a swab 10 cm from a participant's everted eye, these were labeled and processed identically to participant samples.

Clinical signs were graded using the 1981 WHO Detailed Trachoma Grading System (FPC) (Dawson et al., 1981). This sub-divides the features into several four-point severity scales: follicles (F), papillary inflammation (P), and conjunctival scarring (C). This system corresponds to the WHO Simplified Trachoma Grading System in the following way: *Trachomatous inflammation-Follicular* (TF) is equivalent to F2/F3 and *Trachomatous inflammation-Intense* (TI) is equivalent to P3 (Thylefors et al., 1987). For the purpose of this study, we consider that both P2 and P3 represent clinically significant papillary inflammation, and refer to this as "TP" (Burton et al., 2015). We followed the widely used definition of "Clinically Active Trachoma": TF and/or TI. The digital photographs were graded by an ophthalmologist experienced in trachoma assessment, using the FPC system, supplemented by a previously described system for fine grading of conjunctival scarring, that quantifies the extent of the conjunctiva involved (Hu et al., 2011). The ophthalmologist was masked to the infection status.

Extensive public health education about trachoma was provided to the community through village level meetings and during the house-to-house visits, including the importance of face washing for children and environmental improvements. Trichiasis surgery was provided free of charge within the community. Subsequently, all residents of the three villages have been offered three rounds of annual mass antibiotic treatment with oral azithromycin (and topical tetracycline ointment for infants under 6 months and pregnant women), during the course of the longitudinal study.

C. trachomatis Detection

To detect *C. trachomatis,* we extracted DNA from swab samples stored in dry tubes using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, California, USA), according to the manufacturer's instructions. The cells attached to the swabs were initially disrupted by bead beating to release their contents. *C. trachomatis* DNA was detected using a previously described droplet digital PCR assay (Roberts et al., 2013). All samples were tested for the *C. trachomatis* plasmid and the human gene *RPP30*. Samples in which *C. trachomatis* plasmid was detected were retested for *C. trachomatis omcB* (a single-copy gene on the chlamydial chromosome). Five microliters of template DNA was added per reaction. PCR reaction conditions were as follows: 95°C for 10 min, then 40 cycles of 95°C for 10 s and 60°C for 30 s and finally 98°C for 12 min. Droplets were then examined for fluorescence on a QX100 Droplet Reader (Bio-Rad, UK), providing a quantitative result.

Human Gene Expression Analysis

Total RNA was extracted from the swab stored in RNALater using a DNA/RNA Purification Kit (Norgen Biotek Corp, Canada), following the manufacturer's instructions. RNA was reverse transcribed using the SuperScript® VILO® cDNA Synthesis Kit (Life Technologies). Quantitative real-time PCR was performed to measure the relative abundance of a panel of human transcripts, using the TaqMan® Microfluidic 384-well Low-Density Array (TLDA) (Life Technologies) on a ViiA7 real-time PCR instrument (Thermo Fisher Scientific, Massachusetts, USA). These cards have separate channels for eight samples, each containing 48 wells, pre-printed with the assay primers and probes. We used two different TLDA designs and measured the expression of 91 different genes of interest for each sample. The selected genes are listed in **Table 5**. We measured expression of three different reference genes: *HPRT1*, *GAPDH,* and *RPLP0. HPRT1* was selected as the most suitable for normalization as it was expressed at a relatively similar level to the majority of other transcripts of interest, whereas *GAPDH* and *RPLP0* were very highly expressed. The choice of transcripts tested was informed by previous gene expression studies including transcriptome analysis experiments on samples from The Gambia, Ethiopia

and Tanzania and by a genome-wide association study from The Gambia (Holland et al., 2010; Natividad et al., 2010; Burton et al., 2011b,c, 2015; Roberts et al., 2015).

Statistical Analysis

Data were managed in Access and transferred to STATA v14 for analysis. The ophthalmologist's grading of the digital photographs was used for analysis. The ddPCR results were initially exported to R for analysis; the critical cut-off value for designating a positive result for each target was 0.2 plasmid copies/μl of eluted DNA, 0.2 *omcB* copies/μl and >0.3 *RPP30* copies/μl. Samples were designated *C. trachomatis* positive if plasmid and human *RPP30* were detected.

The associations between each of the clinical signs (follicular inflammation, papillary inflammation and scarring) and *C. trachomatis* infection were assessed using univariable logistic regression with infection as the outcome variable. The associations between scarring and each of the other clinical signs, infection, sex and age were investigated with logistic regression, using presence of scarring as the outcome variable. Initially, univariable logistic regression was performed using each exposure in turn, and then a multivariable analysis was performed in order to provide an unbiased estimate of the association between exposure and outcome adjusting for all clinical features.

The gene expression data were normalized relative to the expression of *HPRT1* in the same sample, to adjust for variable concentrations of cDNA. This was done by the ΔC_T method. Distributions of ΔC_T values were plotted to assess them for normality. Fold change differences in gene expression between different phenotypic groups (Infection, follicular inflammation, papillary inflammation and scarring), vs. individuals without those clinical signs or infection, were calculated using the $\Delta\Delta C_T$ method. Linear regression was used to compare the two groups in each comparison, with ΔC_T values as the dependent variable and each phenotype group in turn as the independent variable, adjusting for age and sex. To take account of multiple comparisons we used a false discovery rate (FDR) of 5% (Benjamini and Hochberg, 1995). The linear regression analyses were repeated, but rather than taking the four phenotypic groups individually, they were all included as exposures in a multivariable model with age and sex, providing estimates of the fold-changes associated with each phenotype.

A heatmap of ΔC_T values for each gene of each participant was produced, to indicate visually whether the expression of individual genes was consistently of higher or lower expression in groups of individuals with clinically active Trachoma (TF and/or TI) and/or infection. A principal component analysis (PCA) was performed on the gene expression data and clinical disease and infection status were overlaid on plots in order to visually indicate any associations between the first two components and infection, active trachoma and scarring. These associations were tested formally using a logistic regression analysis, with the principal components as the exposure variables. For the heatmap and PCA, only individuals with complete expression data could be included. Therefore, so as not to lose too many individuals, any target with >5% of observations missing was excluded from these analyses, as well as the lasso regression and co-expression analyses described below. Any individuals with missing observations on the remaining targets were excluded from these analyses also.

For each of infection, active trachoma and scarring, a multivariable logistic regression was performed using this subset of gene expression levels as exposure variables, with the aim of retaining those expressions most strongly associated with the outcome of interest, adjusting for all other expression levels. Due to the large number of exposure variables, a penalized logistic regression was performed using the lasso technique (Tibshirani, 1997). The list of genes most strongly associated with each outcome was entered into ConsensusPathDB (http://cpdb.molgen.mpg.de/) gene set over-representation analysis, with all genes tested entered as background. Enriched pathway-based sets with a minimum overlap between pathway and input gene list of two and a $p < 0.05$ were identified.

A network graph based on the specimen-to-specimen Pearson correlation was generated using miru (https://kajeka.com/miru). The overall expression correlation matrix and graph were constructed from the raw cycle threshold expression values. The filtered dataset including only individuals and genes with complete expression data was used. Pearson correlation coefficients (r) ≥0.85 were retained and used as cut-offs in network construction. Nodes in the graph are individual mRNA transcripts linked by an edge if r was ≥0.85. The graph was then clustered using a Markov Clustering algorithm with an inflation value of 2.2. The partitioned clusters of expression contain sets of transcripts that exhibit a very high degree of co-expression across the sample set. The co-expression clusters or modules were then investigated for enrichment at the pathway level using ConsensusPathDB as described above, using all genes tested as background. A single value for each cluster of each individual was defined using the first principal component and this value was used to investigate differential expression associated with disease and infection phenotypes.

RESULTS

Study Participants

A total of 506 children between the ages of 6 and 10 years were recruited and examined. Their demographic and clinical characteristics are described in **Table 1**. There were almost equal numbers of male and female children, with a mean age of 7.5 years. Most (97.4%) of the children were of the Maasai ethnic group.

Active Trachoma

Trachomatous inflammation-Follicular (TF, F2/F3) was present in 170 (33.6%) children (**Table 1**). However, a further 135 (26.7%) had evidence of mild follicular conjunctivitis (F1). Significant conjunctival papillary inflammation (TP, P2/P3) was observed in 160 (31.7%) children, of which 64 (12.7%) had intense papillary inflammation (TI, P3).

TABLE 1 | Demographic characteristics, clinical signs and *C. trachomatis* infection status.

Category	*n*/506	(%)
Sex (Male)	251	(49.6%)
ETHNIC GROUP		
Maasai	493	(97.4%)
Chaga	7	(1.4%)
Sonjo	4	(0.8%)
Pare	2	(0.4%)
Age in years (mean and range)	7.5	(6–10)
SIMPLIFIED WHO GRADING		
TF	170	(33.6%)
TI	64	(12.7%)
TS	144	(28.5%)
DETAILED "FPC" WHO GRADING		
Follicles		
F0	201	(39.7%)
F1	135	(26.7%)
F2	81	(16.0%)
F3	89	(17.6%)
Papillae		
P0	180	(35.6%)
P1	166	(32.8%)
P2	96	(19.0%)
P3	64	(12.7%)
Scarring		
C0	362	(71.5%)
C1	120	(23.7%)
C2	21	(4.2%)
C3	3	(0.6%)
DETAILED TARSAL CONJUNCTIVA SCARRING GRADING SYSTEM		
S0	362	(71.5%)
S1a	71	(14.0%)
S1b	28	(5.5%)
S1c	26	(5.1%)
S2	19	(3.8%)
S3	0	–
***C. trachomatis* PLASMID DETECTED**		
No	428	(84.6%)
Yes	78	(15.4%)

The clinical signs were determined by grading of photographs.

C. trachomatis Infection and Active Trachoma

C. trachomatis plasmid was detected by ddPCR in 78 (15.4%) individuals. There was a strong association between the presence of TF and the detection of *C. trachomatis* plasmid; 62/78 (79.5%) of *C. trachomatis* positive individuals had TF (**Table 2**). Of the 16 infected children who did not have TF, 11 (68.8%) had signs of mild follicular conjunctivitis (F1). However, only 62/170 (36.5%) individuals with TF had detectable infection. There was a similar strong association between the presence of TP and chlamydial infection (**Table 2**).

Infection load and the concentration of *RPP30* endogenous control DNA were quantified. The concentration of plasmid DNA ranged from 0.22 to 3023 copies/μl, *omcB* ranged from 0.2 to 742 copies/μl and *RPP30* ranged from 0.32 to 1,081 copies/μl of eluted DNA. All samples had detectable *RPP30*. There was a marked positive trend in both the proportion infected and the load of infection with increasing *F*-Score and increasing *P*-Score (**Table 2**). There was a consistent ratio of plasmid to *omcB* copies/μl: geometric mean ratio of 4.57 to 1 (95% CI 3.91–5.34) and a median of 4.79 to 1 (95%CI 4.01–5.21). The correlation between plasmid and *omcB* copies/μl is illustrated in **Figure 1**. The ratio did not vary with either the follicular or papillary clinical severity scores (data not shown).

Conjunctival Scarring

Conjunctival scarring was relatively frequent, being found in 144 (28.5%) individuals; this was mostly mild scarring (**Table 1**). There was a significant association between the detection of *C. trachomatis* and scarring [$p = 0.002$, $OR = 2.23$ (95%CI 1.4–3.7)]. The load of infection also increased with scarring severity (**Table 2**). There were univariate associations between conjunctival scarring and TF, TP (P2/P3), *C. trachomatis* detection and female sex (**Table 3**). However, in a multivariable model only TP, female sex and age were independently associated with scarring.

There was strong evidence ($p = 0.0047$) that even at this young age females are proportionately more likely to have signs of established scarring than males (**Table 4**). Similarly, there was some evidence ($p = 0.048$) that females had slightly increased odds of TP. No evidence was found of an association between sex and the odds of TF or chlamydial infection ($p = 0.12$ and 0.25, respectively) (**Table 4**).

Conjunctival Gene Expression

The expression of 91 target genes was measured relative to that of *HPRT1*. Out of the 506 participants, samples from 12 individuals failed the qPCR for all target genes, leaving 494 individuals for gene expression analysis. The number of participant samples with detectable expression for each target is shown in Supplementary Table 1 (N). Multivariable linear regression analysis was performed using data for all 91 genes from 494 individuals.

Four sets of comparisons were performed: (1) infected v non-infected, (2) TF v no TF, (3) TP v no TP, and (4) TS v no TS. The relative Fold Change (FC) between these paired groups, calculated by the $\Delta\Delta C_T$ method, are presented in Supplementary Table 1 along with the *p*-values for the linear regression (adjusted for age and sex) for each comparison.

As clinical signs and infection may be highly correlated in their association with the expression of specific targets we tested for independent associations with each target's expression using multivariable linear regression models, **Table 5**. More genes were significantly differentially expressed in individuals with *C. trachomatis* infection (55/91) relative to individuals with TP (33/91), TF (11/91), and TS (17/91) in these multivariable models ($P < 0.05$). *C. trachomatis* Infection was associated with increased expression of multiple cytokines and chemokines (*CCL2, CXCL13, CCL18, CSF2, FOXP3, IFNG, IDO1, IL1B, IL6, IL8, IL10, IL12B, IL17A, IL19, IL21, IL22, IL23A*), cell

TABLE 2 | *C. trachomatis* plasmid detection and clinical signs; both the simplified and detailed WHO "FPC" grading system.

	C. trachomatis plasmid				OR	(95%CI)	*p*-value	Geometric mean	
	No	(%)	Yes	(%)				Plasmid	*omcB*
FOLLICULAR INFLAMMATION									
No TF	320	(95.2%)	16	(4.8%)	1	–	–	2.99	0.86
TF	108	(63.5)	62	(36.5%)	11.48	(6.4–20.7)	5.95E-16	16.27	4.26
FPC FOLLICLE SCORE									
F0	196	(97.5%)	5	(2.5%)	1	–	–	3.41	1.04
F1	124	(91.9%)	11	(8.2%)	3.48	(1.2–10.2)	0.024	2.82	0.81
F2	65	(80.3%)	16	(19.8%)	9.65	(3.4–27.4)	2.03E-05	20.05	4.17
F3	43	(48.3%)	46	(51.7%)	41.93	(15.7–111)	7.97E-14	15.13	4.29
PAPILLARY INFLAMMATION									
No TP	323	(93.4%)	23	(6.7%)	1	–	–	4.11	1.23
TP (P2/P3)	105	(65.6%)	55	(34.4%)	7.36	(4.3–12.5)	2.45E-13	17.67	4.64
FPC PAPILLARY SCORE									
P0	173	(96.1%)	7	(3.9%)	1	–	–	2.53	0.72
P1	150	(90.4%)	16	(9.6%)	2.63	(1.1–6.6)	0.038	5.09	1.41
P2	73	(76%)	23	(23.9%)	7.79	(3.2–18.9)	6.07E-06	13.47	2.65
P3	32	(50%)	32	(50.0%)	24.71	(10.0–60.8)	2.95E-12	21.47	6.99
SCARRING									
No	318	(87.9%)	44	(12.2%)	1	–	–	9.67	2.59
Yes	110	(76.4)	34	(23.6%)	2.23	(1.4–3.7)	0.002	14.38	4.35
FPC SCARRING SCORE									
C0	318	(87.9%)	44	(12.2%)	1	–	–	9.67	2.59
C1	89	(74.2%)	31	(25.8%)	2.52	(1.5–4.2)	0.0005	12.33	4.08
C2	18	(85.7%)	3	(14.3%)	1.20	(0.3–4.3)	0.77	70.24	7.85
C3	3	(100%)	0	–				–	–
DETAILED SCARRING SCORE									
S0	318	(87.9%)	44	(12.2%)	1	–	–	9.67	2.59
S1a	56	(78.9%)	15	(21.1%)	1.94	(1.0–3.7)	0.047	7.98	3.44
S1b	21	(75.0%)	7	(25.0%)	2.41	(1.0–6.0)	0.059	15.24	4.26
S1c	17	(65.4%)	9	(34.6%)	3.83	(1.6–9.1)	0.0024	21.61	5.32
S2	16	(84.2%)	3	(15.8%)	1.36	(0.4–4.8)	0.64	70.24	7.85
S3	0	–	0	–				–	–

P-values were calculated by Pearson χ2. TP is equivalent to P2 or P3 of the FPC papillary score.

cycle components (*CDC25C, TTK, TYMS*), matrix modifiers (MMP7, *MMP9, MMP12, TGFB1*), NK cell markers (*CD247, NCR1, NCAM1*) and intracellular signaling molecules/regulators (*CD274, IKZF1, RHOH, SAMSN1, SERPINB3, SERPINB4, STAT1, STAT4, SOCS1, TBX21*), **Table 5**. Infection was associated with particularly marked increases in expression of *IFNG* (FC: 7.12, *p*-value: 1.32E-42) and *IL22* (FC: 6.49, *p*-value: 1.6E-23). There was reduced expression of several factors including mucins (*MUC1, MUC4, MUC5AC, MUC7*) and *SPARCL1*.

Clinical signs of active trachoma were associated with increased expression of multiple cytokines, chemokines, antimicrobial peptides, matrix modifiers, following a similar pattern to infection (**Table 5**). TP was associated with more substantial increases in a wider range of factors than TF (**Table 5**): antimicrobial peptides (*DEFB4A, S100A7*), cytokine/chemokines (*CXCL5, CXCL13, CCL18, CCL2, IL1B, IL8, IL10, IL17A, IL19, IL23A, PTGS2*), matrix modifiers (*MMP7, MMP9, MMP12*) and intracellular signaling molecules/regulators (*CD274, SAMSN1, SERPINB3, SERPINB4, SOCS3*). Several cytokines and chemokines (*CCL18, CXCL13, IL19*, and *S100A7*) had a FC >2 in children with TP. Conjunctival scarring was associated with modestly increased expression (>1.2 FC) and borderline significance of several chemokines and cytokines (*CCL2, CCL20, CXCL5, CSF3, IL1B, IL8, IL10, IL21, IL33, FGF2*) in the multivariable linear regression models (**Table 5**). *SPARCL1* was significantly downregulated in TF and TP but not TS.

Only 108 individuals had detectable gene expression for all 91 targets, so in order to retain a reasonable number of individuals in the PCA and lasso analyses, which require complete records for all individuals, targets which were missing >5% of observations were excluded. This resulted in the exclusion of eight transcripts (*USP6, IL13, CTNND2, FGF2, SERPINB4, IL22, PREX2v1, PREX2v2*) for which there was no detectable expression in >5% individuals. In individuals with detectable expression of these

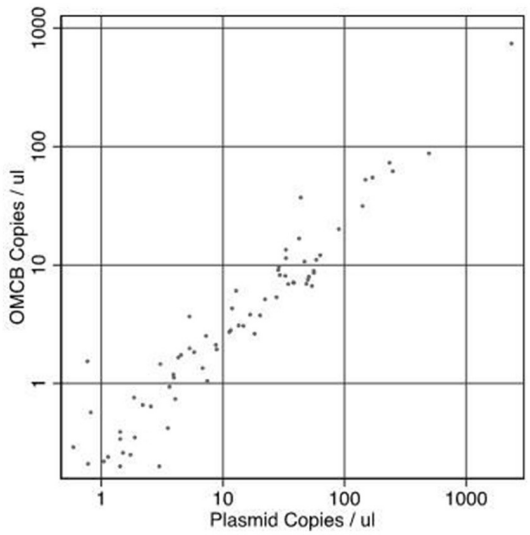

FIGURE 1 | The relationship between paired measures of copies of *Chlamydia trachomatis* plasmid/μl vs. *omcB*/μl in the same sample. Correlation *R*-squared = 0.989.

TABLE 3 | Univariable and multivariable associations between conjunctival scarring and other clinical features, *C. trachomatis* infection, sex and age.

Variable	OR	95% CI	*p*-value
UNIVARIABLE ANALYSIS			
TF	2.28	(1.53–3.40)	5.20E-05
TP (P2/P3)	2.98	(1.99–4.47)	1.22E-07
Plasmid	2.23	(1.36–3.67)	0.002
Sex (Female)	1.76	(1.19–2.61)	0.005
Age	1.06	(0.97–1.17)	0.217
MULTIVARIABLE LOGISTIC REGRESSION			
TF	1.39	(0.79–2.44)	0.251
TP (P2/P3)	2.60	(1.52–4.44)	4.83E-04
Plasmid	1.33	(0.75–2.37)	0.326
Sex (Female)	1.63	(1.08–2.45)	0.019
Age	1.16	(1.05–1.29)	0.005

eight targets, raw C_T values were generally very high (C_T > 33). These eight targets were excluded from the heatmap, PCA, lasso regression and co-expression network analyses, retaining a complete expression dataset for 83 genes. Among the 494 individuals, 36 were excluded as they had missing data among the 83 included targets, resulting in a final dataset of 458 individuals and 83 genes. Excluding further genes offered minimal gains in sample size. A heatmap of ΔC_T values separated by clinical and infection phenotype is presented in **Figure 2**. Infected individuals with or without active trachoma had a visibly distinct pattern of gene expression, whereas the ΔC_T values of normal healthy controls and individuals with active trachoma in the absence of infection appeared more similar overall.

PCA analysis was performed for 458 individuals that had complete gene expression data for 83 targets and active trachoma/infection and scarring status were overlaid on plots, Supplementary Figures 1A,B. Principal component 1 (PC1) was strongly associated with infection and active trachoma; for a one unit increase in PC1 there was an estimated 35% decrease in the odds of infection (95% CI 27–41%, $p < 0.001$) and an estimated 23% decrease in the odds of active trachoma (95% CI 9–28%, $p < 0.001$). PC1 loadings showed that as genes in the chemokines/cytokines and regulator/signaling pathways groups were up-regulated, PC1 became smaller, suggesting that the odds of infection/active trachoma was greater among individuals where these genes are up-regulated. The expression of mucins, *CDH1*, *SPARCL1,* and *S100A4* showed the reverse relationship, as when these genes were down-regulated the PC1 was smaller. Principal component 2 (PC2) was associated with increased odds of active trachoma alone, with a unit increase in PC2 being associated with an estimated 15% increase in the odds (95% CI 7–24%, $p < 0.001$), however there was no evidence of an association between PC2 and infection. There was evidence of a weaker association between PC1 and scarring, with an estimated 10% decrease in the odds of scarring (95% CI 6–14%, $p < 0.001$) per unit increase in PC1, Supplementary Figure 1B. There was no evidence of an association between scarring and PC2.

Three lasso logistic regressions were performed, first using infection (with or without active disease) as the outcome and the 83 targets, age and sex of 458 individuals as exposures. Then, excluding all infected individuals, active trachoma (uninfected only) was used as the outcome against uninfected individuals without active trachoma and the same 83 targets, age and sex as the exposures of interest. Finally, the regression was performed using scarring as the outcome and the same set of exposures in all 458 individuals. The transcripts most strongly associated with each of the three outcomes, and biological pathways with members that were over-represented in each relative to the background (83 targets) are listed in **Table 6**. Genes most strongly associated with infection were associated with Th1 cell development and NK cell pathways, whilst genes most associated with active trachoma in the absence of *C. trachomatis* infection were enriched for members of leukocyte transendothelial migration, TNF receptor, MMP activation, collagen formation and collagen fibrils assembly pathways. Genes most strongly associated with the TS phenotype were enriched for members of immunoregulatory interactions between lymphoid and non-lymphoid cells.

Networks of co-expression, independent of differential expression, were explored in the complete expression data of 83 transcripts and 458 individuals using Miru. The undirected graph contained 51 nodes connected by 121 edges. Markov clustering partitioned the network into 5 separate clusters of co-expressed genes that accounted for 38/51 (74.5%) of the transcripts in the original network. Cluster 1 contained 11 co-expressed transcripts, cluster 2 contained 10 transcripts, there were 6 transcripts in each of cluster 3 and 4, and 5 transcripts in cluster 5 (Supplementary Table 2A), the remaining nodes were not connected in the graph. The most connected gene transcript or hub in each module was MyD88/REL (cluster 1), IKZF1/VIM (cluster 2), STAT1 (cluster 3), SRGN (cluster 4), and IL10/IL17A/IL21 (cluster 5).

TABLE 4 | The relationship between sex and (i) clinical signs, (ii) *Chlamydia trachomatis* infection and (iii) infection load.

	Male	(%)	Female	(%)	OR	(95% CI)	*p*-value
FOLLICULAR INFLAMMATION							
No TF	175	(69.7%)	161	(63.1%)	1	–	–
TF	76	(30.3%)	94	(36.9%)	1.34	(0.9–1.9)	0.12
PAPILLARY INFLAMMATION							
No TP	182	(72.5%)	164	(64.3%)	1	–	–
TP (P2/P3)	69	(27.5%)	91	(35.7%)	1.46	(1.0–2.1)	0.0479
SCARRING							
No TS	194	(77.3%)	168	(65.9%)	1	–	–
TS	57	(22.7%)	87	(34.1%)	1.76	(1.2–2.6)	0.0047
INFECTION							
No	217	(86.5%)	211	(82.6%)	1	–	–
Plasmid	34	(13.6%)	44	(17.2%)	1.33	(0.8–2.2)	0.25
GEOMETRIC MEAN AMONG THOSE WITH INFECTION							
Plasmid	9.45	(4.6–19.3)	13.37	(7.5–23.7)			0.44[α]
omcB	2.93	(1.5–5.8)	3.50	(2.1–5.9)			0.67[α]

[α] *Differences between chlamydial load geometric means in males and females were calculated using a t-test.*

Pathway enrichment analysis was performed for each cluster relative to the background of 83 transcripts (Supplementary Table 2B). ΔC_T values for the transcripts in each cluster were collapsed into a single value for each of the 458 individuals, represented by the first principal component. Multivariable linear regression was then performed for each cluster, using the first principal component as the outcome variable and TF, TP, TS, infection, age, and sex as independent variables (Supplementary Table 2C). Cluster 1 was enriched for cell cycle and apoptosis pathways, however the combined expression value was not differentially expressed in any condition. Cluster 2 was significantly associated with infection and TF and was marginally enriched for the retinoblastoma pathway. Cluster 3 was highly enriched for Th1/2 cell differentiation, IFNγ, IL-12, CD8, and NK cell signaling pathways and was strongly associated with infection and age and marginally associated with sex, but interestingly not with TP or TF. Conversely, cluster 4 was enriched for TNF, inflammasome, TLR and NFkB signaling pathways and was associated with TP, TS, and age but not infection. Cluster 5 was enriched for the allograft rejection pathway and was associated with infection, TP, TS and age.

DISCUSSION

Clinical Disease and *C. trachomatis* Infection

In this study, conducted in communities prior to azithromycin MDA, there was a relatively high TF (34%) and moderate *C. trachomatis* (15.4%) prevalence in 6–10 year olds. There was a strong relationship between infection and clinical signs. The large majority (79.5%) of individuals with *C. trachomatis* detected by ddPCR had TF (F2/F3). Moreover, most individuals (68.8%) with detectable chlamydial infection but without TF had a mild follicular conjunctivitis (F1). There were few cases of infection in the absence of clinical signs. This is consistent with earlier studies which found a stronger correlation as the underlying prevalence of infection increased (Ramadhani et al., 2016b). However, in common with many other studies reporting the relationship between disease and infection, a minority (36%) of individuals with TF had detectable infection, probably due to the shorter duration of infection episodes relative to disease (Grassly et al., 2008; Burton et al., 2011a,c; Lee et al., 2014). With increasing severity of both TF and TP scores, there was an increasing proportion with infection and increasing loads of infection. Consistent with earlier studies, increasing load appeared to be more closely related to increasing TP rather than TF (Burton et al., 2003; Solomon et al., 2003; Michel et al., 2011; Derrick et al., 2016a).

In the present cross-sectional study conjunctival scarring was associated with TP, female sex and increasing age but not *C. trachomatis* infection, which is consistent with several earlier cohort studies (Dawson et al., 1990; West et al., 2001; Wolle et al., 2009b; Burton et al., 2015; Hu et al., 2016). However, longitudinal data supporting an association between TF or *C. trachomatis* infection and progressive scarring are limited (Ramadhani et al., 2016a). We found females had more TP and TS than males, whereas *C. trachomatis* infection and TF prevalence were not significantly different between the sexes (**Table 4**). This might suggest that there are additional determinants of TP and TS development beyond the initial *C. trachomatis* infection. It is generally believed that the difference in the proportion of males and females developing scarring sequelae of trachoma is attributable to a greater life-time exposure to repeated *C. trachomatis* infection among females (Taylor et al., 2014). While that may well be the case, the data from this study also suggest that even at a relatively young age, females appear more susceptible to developing TP and TS than males, despite comparable levels of *C. trachomatis* infection. This finding is consistent with the observation that in general, females generate stronger immune responses than males, making them

TABLE 5 | Multivariable linear regression models for conjunctival gene expression in the presence of clinical signs, *C. trachomatis* plasmid, female sex and age.

Target	TF		TP		TS		Plasmid		Sex (female)$^{\alpha}$		Age$^{\beta}$	
	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value
ANTIMICROBIAL PEPTIDES												
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	0.77	0.0919	**2.08**	**2.37E-06**	1.12	0.3844	1.28	0.1459	**1.37**	**0.0044**	**0.92**	**0.0022**
Psoriasin-1 (*S100A7*)	1.40	0.1605	**2.86**	**8.94E-06**	1.25	0.2520	1.77	0.0279	1.36	0.0672	**0.88**	**0.0039**
CELL CYCLE												
CD53 molecule (*CD53*)	0.98	0.7932	**1.19**	**0.0033**	1.08	0.1263	1.15	0.0340	1.03	0.4334	0.98	0.1419
M-phase inducer phosphatase 3 (*CDC25C*)	1.17	0.0490	1.08	0.3434	1.04	0.5710	**1.36**	**0.0005**	1.02	0.7103	0.99	0.4182
Cyclin-dependent kinase 13 (*CDK13*)	1.00	0.9318	**0.88**	**0.0037**	1.02	0.6393	0.92	0.0908	1.00	0.8874	1.01	0.1454
Catenin (cadherin-associated protein), delta 2 (*CTNND2*)	1.08	0.7376	0.81	0.3716	1.02	0.9196	0.79	0.3411	0.90	0.5413	1.06	0.1744
Sun Domain Family, Member 6 (*NSUN6*)	0.94	0.0874	0.98	0.5339	1.04	0.2220	1.01	0.8099	0.95	0.0359	1.01	0.1160
Phytanoyl-coa 2-hydroxylase (*PHYH*)	0.92	0.0678	0.95	0.2639	1.01	0.8297	**0.85**	**0.0010**	0.97	0.4291	1.02	0.0196
Tumor protein p53 (*TP53*)	1.01	0.7419	**0.85**	**0.0001**	1.03	0.3965	1.01	0.8485	0.97	0.3148	1.01	0.0460
TTK protein kinase (*TTK*)	1.16	0.0209	1.11	0.0933	1.10	0.0719	**1.31**	**0.0002**	0.98	0.7333	0.99	0.4067
Thymidylate synthetase (*TYMS*)	1.11	0.0590	**1.16**	**0.0050**	1.00	0.9654	**1.62**	**6.38E-15**	1.05	0.2448	0.99	0.1936
CYTOKINES/CHEMOKINES												
Chemokine ligand 18 (*CCL18*)	1.00	0.9903	**3.03**	**6.14E-10**	0.94	0.6534	**1.99**	**0.0004**	0.97	0.8422	**0.89**	**0.0006**
Chemokine ligand 2 (*CCL2*)	0.95	0.7092	**1.63**	**0.0008**	**1.39**	**0.0060**	**3.36**	**1.45E-13**	1.13	0.2284	**0.87**	**1.06E-07**
Chemokine ligand 20 (*CCL20*)	**1.45**	**0.0005**	1.04	0.7406	**1.33**	**0.0011**	1.23	0.0735	0.97	0.7140	**0.95**	**0.0035**
Chemokine receptor 6 (*CCR6*)	**1.53**	**0.0011**	0.83	0.1466	1.11	0.3398	1.10	0.4912	1.12	0.2129	0.96	0.1173
Colony stimulating factor 2 (*CSF2*)	1.19	0.0798	1.05	0.6564	1.13	0.1416	**2.45**	**2.83E-15**	1.14	0.0690	0.99	0.5702
Colony stimulating factor 3 (*CSF3*)	0.97	0.8494	1.40	0.0206	**1.52**	**0.0005**	0.85	0.3008	0.99	0.8901	**0.91**	**0.0008**
Chemokine ligand 13 (*CXCL13*)	1.41	0.0321	**2.01**	**1.22E-05**	1.14	0.3140	**2.11**	**2.29E-05**	1.23	0.0733	**0.87**	**3.46E-06**
Chemokine ligand 5 (*CXCL5*)	1.11	0.4860	**1.82**	**0.0001**	**1.60**	**0.0001**	0.82	0.2278	0.88	0.2371	**0.88**	**6.49E-06**
Forkhead box P3 (*FOXP3*)	1.02	0.7750	1.02	0.7818	0.91	0.0840	**1.25**	**0.0014**	0.95	0.2135	1.00	0.9477
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	1.17	0.1491	1.28	0.0187	**1.24**	**0.0139**	**1.73**	**3.53E-06**	**1.34**	**0.0001**	**0.89**	**1.05E-08**
Interferon gamma(*IFNG*)	**1.36**	**0.0109**	1.09	0.4791	1.12	0.2396	**7.12**	**1.32E-42**	**1.29**	**0.0025**	**0.91**	**2.97E-05**
Interleukin 10 (*IL10*)	1.13	0.2184	**1.49**	**3.48E-05**	**1.22**	**0.0141**	**1.78**	**7.86E-08**	1.09	0.2106	**0.94**	**0.0004**
Interleukin 12 beta (*IL12B*)	1.15	0.2315	1.19	0.1340	1.07	0.4853	**3.42**	**3.90E-21**	**1.23**	**0.0106**	0.97	0.2047
Interleukin 13 (*IL13*)	1.63	0.1091	0.66	0.1804	0.80	0.2701	1.38	0.2585	1.03	0.8545	1.07	0.1290
Interleukin 17A (*IL17A*)	1.16	0.2561	**1.65**	**0.0001**	1.19	0.0928	**2.34**	**1.85E-09**	1.18	0.0688	**0.90**	**3.56E-06**
Interleukin 19 (*IL19*)	0.96	0.7566	**2.63**	**5.69E-11**	1.16	0.2150	**2.11**	**3.75E-06**	**1.43**	**0.0006**	**0.88**	**6.69E-07**
Interleukin 1 beta(*IL1B*)	0.97	0.8261	**1.72**	**5.46E-06**	**1.36**	**0.0021**	**1.55**	**0.0009**	1.04	0.6251	**0.92**	**0.0002**
Interleukin 21 (*IL21*)	**1.68**	**0.0003**	1.36	0.0290	**1.34**	**0.0132**	**3.10**	**1.69E-12**	1.16	0.1432	**0.90**	**0.0001**
Interleukin 22 (*IL22*)	1.14	0.4452	1.26	0.1548	1.31	0.0511	**6.49**	**1.60E-23**	1.22	0.0973	0.93	0.0302
Interleukin 23A (*IL23A*)	0.98	0.8604	**1.70**	**3.81E-08**	1.10	0.2287	**1.87**	**3.62E-09**	0.99	0.8875	**0.93**	**3.13E-05**
Interleukin 33 (*IL33*)	0.96	0.6351	0.92	0.3465	**1.28**	**0.0009**	1.27	0.0164	0.96	0.5468	1.03	0.0472
Interleukin 6 (*IL6*)	0.92	0.5490	1.27	0.0721	1.27	0.0303	**1.80**	**0.0001**	0.92	0.3965	**0.94**	**0.0057**
Interleukin 8 (*IL8*)	0.87	0.1443	**1.43**	**0.0001**	**1.24**	**0.0041**	**1.32**	**0.0063**	1.00	0.9975	0.97	0.0751
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	0.90	0.3527	**1.45**	**0.0009**	**1.26**	**0.0116**	1.18	0.1781	0.98	0.7886	0.97	0.0978
Tumor necrosis factor (*TNF*)	**1.39**	**0.0040**	0.93	0.4934	1.16	0.1187	1.30	0.0383	1.15	0.0965	1.00	0.8808
EMT MARKERS												
Alpha smooth muscle actin (*ACTA2*)	1.10	0.1559	**0.83**	**0.0039**	1.14	0.0144	**1.29**	**0.0004**	1.04	0.3549	0.99	0.4144
Epithelial cadherin (*CDH1*)	0.94	0.2482	0.93	0.1746	1.05	0.2799	**0.78**	**4.22E-05**	0.99	0.7167	1.00	0.9175
Cadherin 1, type 1, E-cadherin (epithelial) (CDH1)	0.95	0.3727	0.92	0.1964	1.04	0.4101	**0.73**	**3.06E-06**	0.99	0.8120	1.01	0.5096

(Continued)

TABLE 5 | Continued

Target	TF		TP		TS		Plasmid		Sex (female)[α]		Age[β]	
	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value
Neuronal cadherin (*CDH2*)	0.98	0.8576	**0.71**	**0.0066**	1.18	0.1088	1.11	0.4339	1.00	0.9960	1.04	0.0558
S100 calcium binding protein A4 (*S100A4*)	0.88	0.0691	**0.71**	**1.83E-06**	1.15	0.0213	**0.47**	**1.99E-20**	0.90	0.0393	**1.03**	**0.0093**
Vimentin (*VIM*)	1.02	0.6112	1.04	0.4486	1.04	0.2589	**1.51**	**8.85E-15**	1.01	0.7266	1.01	0.3843
MATRIX MODIFIERS												
Connective tissue growth factor (*CTGF-1*)	0.98	0.8349	0.81	0.0191	0.98	0.7534	1.20	0.0721	**0.79**	**0.0003**	1.02	0.2769
Connective tissue growth factor (*CTGF-2*)	0.89	0.2310	0.85	0.0721	0.91	0.2245	1.21	0.0675	**0.82**	**0.0036**	1.03	0.0828
Fibroblast growth factor 2 (basic) (*FGF2*)	0.86	0.3151	1.11	0.4830	**1.43**	**0.0032**	**1.48**	**0.0124**	0.87	0.1988	1.01	0.7147
Matrix metallopeptidase 12 (*MMP12*)	1.31	0.0246	**1.48**	**0.0010**	1.26	0.0188	**2.00**	**1.84E-07**	1.11	0.2136	**0.92**	**0.0002**
Matrix metallopeptidase 7 (*MMP7*)	0.94	0.5657	**1.41**	**0.0024**	1.04	0.7042	**0.38**	**1.63E-14**	0.93	0.3375	0.96	0.0488
Matrix metallopeptidase 9 (*MMP9*)	**1.37**	**0.0048**	**1.43**	**0.0012**	1.20	0.0519	**1.77**	**3.45E-06**	0.98	0.8380	0.96	0.0371
Platelet-derived growth factor beta polypeptide (*PDGFB*)	1.00	0.9393	1.08	0.1904	**1.16**	**0.0043**	**1.43**	**1.55E-07**	1.06	0.1638	**0.96**	**0.0002**
SPARC-like 1 (hevin) (*SPARCL1*)	**0.53**	**0.0048**	**0.39**	**3.20E-05**	0.85	0.3842	**0.46**	**0.0020**	0.75	0.0794	**1.20**	**1.29E-05**
Transforming growth factor, beta 1 (*TGFβ1*)	1.02	0.6999	0.99	0.7693	1.08	0.0491	**1.23**	**0.0001**	0.99	0.6965	0.98	0.0305
Transforming growth factor, beta 2 (*TGFβ2*)	0.97	0.7251	0.96	0.6469	1.08	0.3172	0.91	0.3438	0.92	0.2258	1.01	0.6926
RESPONSE TO MICROBIOTA												
Arachidonate 5-lipoxygenase (*ALOX5*)	**0.88**	**0.0111**	0.97	0.4844	1.05	0.2353	**0.67**	**1.78E-13**	0.97	0.4387	1.01	0.2310
B-cell CLL/lymphoma 2 (*BCL2*)	1.00	0.9769	0.94	0.1509	1.09	0.0239	1.13	0.0212	0.97	0.4448	1.00	0.8130
CD40 molecule, TNF receptor superfamily member 5 (*CD40*)	0.98	0.6989	0.98	0.7901	1.06	0.2693	**1.25**	**0.0009**	1.06	0.1592	1.00	0.8334
Dual oxidase 2 (*DUOX2*)	0.92	0.3364	**1.38**	**0.0004**	1.09	0.2734	1.19	0.0852	**1.20**	**0.0047**	**0.94**	**0.0001**
V-rel avian reticuloendotheliosis viral oncogene homolog (*REL*)	0.96	0.3595	1.08	0.0911	1.05	0.1697	1.03	0.5774	1.05	0.1108	1.00	0.6653
Tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*)	0.87	0.1556	0.99	0.9463	1.07	0.3766	0.81	0.0448	0.96	0.5130	1.02	0.3991
Tumor necrosis factor receptor superfamily, member 1B (*TNFRSF1B*)	0.89	0.1026	1.18	0.0164	1.10	0.0956	1.02	0.8439	0.96	0.4386	1.00	0.7988
MUCINS												
Mucin 1, cell surface associated (*MUC1*)	0.92	0.1555	1.04	0.4586	1.03	0.4947	**0.82**	**0.0013**	1.00	0.9901	1.00	0.8866
Mucin 4, cell surface associated (*MUC4*)	0.93	0.3097	1.15	0.0472	1.06	0.3140	**0.70**	**1.02E-05**	0.96	0.4576	0.98	0.0865
Mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*)	0.77	0.0561	**0.61**	**0.0002**	1.25	0.0464	**0.56**	**0.0001**	0.97	0.7651	1.04	0.0811
Mucin 7, secreted (*MUC7*)	0.86	0.3401	0.69	0.0217	0.94	0.6225	**0.42**	**1.48E-06**	**0.63**	**0.0001**	**1.10**	**0.0012**
NK CELL MARKERS												
CD247 molecule (*CD247*)	1.12	0.0847	1.02	0.7505	1.00	0.9863	**1.80**	**1.78E-15**	1.06	0.2277	0.98	0.0376
Neural cell adhesion molecule 1 (*NCAM1*)	0.85	0.0590	**0.76**	**0.0007**	1.14	0.0524	**1.79**	**3.62E-10**	0.92	0.1385	1.00	0.9652
Natural cytotoxicity triggering receptor 1 (*NCR1*)	1.11	0.1621	1.00	0.9473	1.13	0.0531	**2.31**	**1.68E-22**	1.07	0.2349	**0.95**	**0.0002**
PATTERN RECOGNITION RECEPTORS												
Nucleotide-binding oligomerization domain containing 2 (*NOD2*)	0.88	0.1748	1.21	0.0348	1.13	0.1221	1.08	0.4293	0.90	0.0932	0.99	0.4252
Toll-like receptor 2 (*TLR2*)	1.01	0.8677	1.01	0.9108	**1.26**	**0.0013**	0.84	0.0615	1.02	0.7880	0.98	0.1690
Toll-like receptor 4 (*TLR4*)	0.99	0.9140	1.20	0.0184	**1.20**	**0.0039**	1.02	0.8485	0.94	0.2395	0.97	0.0550

(Continued)

TABLE 5 | Continued

Target	TF		TP		TS		Plasmid		Sex (female)[α]		Age[β]	
	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value	FC	*p*-value
REGULATORS/SIGNALING PATHWAYS												
CD274 molecule (*CD274*)	1.01	0.8828	**1.44**	**0.0001**	1.16	0.0507	**2.47**	**1.18E-18**	**1.20**	**0.0046**	**0.95**	**0.0030**
Chromodomain helicase DNA binding protein 8 (*CHD8*)	0.93	0.0593	1.01	0.7501	1.04	0.2423	1.00	0.9029	0.98	0.4950	1.01	0.2923
COMM domain containing 6 (*COMMD6*)	0.99	0.9205	0.96	0.6492	1.00	0.9510	0.89	0.2836	0.99	0.8589	0.99	0.6788
Hematopoietically expressed homeobox (*HHEX*)	**1.34**	**8.54E-06**	0.92	0.2204	1.07	0.1923	1.11	0.1411	1.04	0.3702	1.00	0.7584
IKAROS family zinc finger 1 (Ikaros) (*IKZF1*)	1.11	0.0319	0.99	0.9105	1.04	0.3969	**1.37**	**1.44E-08**	1.00	0.9898	1.01	0.2868
Myeloid differentiation primary response 88 (*MYD88*)	0.93	0.0303	1.05	0.1445	1.07	0.0151	1.00	0.9773	1.01	0.6856	1.00	0.6936
Marginal zone B and B1 cell-specific protein (*MZB1*)	1.16	0.2162	1.24	0.0712	0.95	0.6338	**2.07**	**8.27E-08**	1.21	0.0279	0.98	0.3593
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*)	0.95	0.1251	1.06	0.1139	1.04	0.1457	**1.13**	**0.0022**	0.99	0.7545	1.00	0.5891
Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2 (*PREX2v1*)	1.06	0.5998	0.81	0.0744	0.90	0.2736	**1.38**	**0.0115**	0.95	0.5644	1.02	0.4858
Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2 (*PREX2v2*)	1.07	0.5708	1.08	0.5194	0.84	0.0985	1.05	0.6832	0.86	0.0769	1.03	0.1608
Ras homolog family member H (*RHOH*)	**1.20**	**0.0034**	1.01	0.8137	1.03	0.6026	**1.47**	**4.37E-08**	1.11	0.0183	0.97	0.0227
SAM domain, SH3 domain and nuclear localization signals 1 (*SAMSN1*)	1.03	0.6236	**1.32**	**4.02E-05**	1.04	0.5155	**1.36**	**0.0001**	1.05	0.2859	0.97	0.0229
Serpin peptidase inhibitor B3 (*SERPINB3*)	0.87	0.3173	**1.90**	**6.73E-06**	1.32	0.0175	**2.65**	**8.33E-10**	0.86	0.1311	0.94	0.0185
Serpin peptidase inhibitor clade B member 4, (*SERPINB4*)	0.75	0.2648	**2.85**	**0.0001**	1.25	0.3075	**4.38**	**1.95E-07**	0.97	0.8763	1.02	0.7506
Suppressor of cytokine signaling 1 (*SOCS1*)	1.15	0.0606	1.10	0.2035	1.09	0.1646	**2.06**	**1.08E-17**	1.05	0.3178	**0.94**	**6.95E-06**
Suppressor of cytokine signaling 3 (*SOCS3*)	1.01	0.9205	**1.50**	**4.53E-05**	**1.34**	**0.0004**	1.21	0.0777	1.02	0.7518	**0.93**	**0.0001**
Serglycin (*SRGN*)	0.91	0.2568	**1.28**	**0.0018**	1.12	0.0767	0.97	0.7165	0.98	0.7288	0.99	0.6097
Signal transducer and activator of transcription 1 (*STAT1*)	1.02	0.8024	1.12	0.0565	1.11	0.0391	**1.97**	**1.34E-22**	1.09	0.0392	**0.95**	**9.97E-07**
Signal transducer and activator of transcription 3 (*STAT3*)	0.93	0.1345	1.06	0.1683	**1.14**	**0.0007**	1.00	0.9584	0.96	0.2153	0.98	0.0423
Signal transducer and activator of transcription 4 (*STAT4*)	1.02	0.8085	1.00	0.9741	1.04	0.4556	**1.85**	**5.37E-18**	1.04	0.3692	0.98	0.0535
T-box 21 (*TBX21*)	**1.21**	**0.0044**	1.00	0.9917	1.07	0.2307	**2.18**	**6.56E-25**	1.11	0.0197	**0.97**	**0.0060**
Ubiquitin specific peptidase 6 (Tre-2 oncogene) (*USP6*)	0.70	0.1911	0.87	0.6216	0.72	0.1582	0.97	0.9124	0.65	0.0322	0.99	0.7936

[α] *FC>1 indicates expression was greater in females than males.*

[β] *FC calculated with an age unit increase of 1 year.*

FC = fold change. Using the Benjamini and Hochberg approach for adjusting for multiple comparisons, in order to control the false discovery rate <5% only tests with a p-value below 0.0142 (highlighted in bold) are considered statistically significant.

more susceptible to diseases resulting from immune-mediated pathology (Klein and Flanagan, 2016).

Among infected individuals, the *C. trachomatis* plasmid:*omcB* ratio (4.57:1) was very similar to the ratio reported from a range of ocular and genital *C. trachomatis* isolates (mean 4.0:1) and from a population-based trachoma study in Guinea Bissau (5.3:1) (Pickett et al., 2005; Last et al., 2014). In common with the study from Guinea Bissau, we did not find evidence of variation

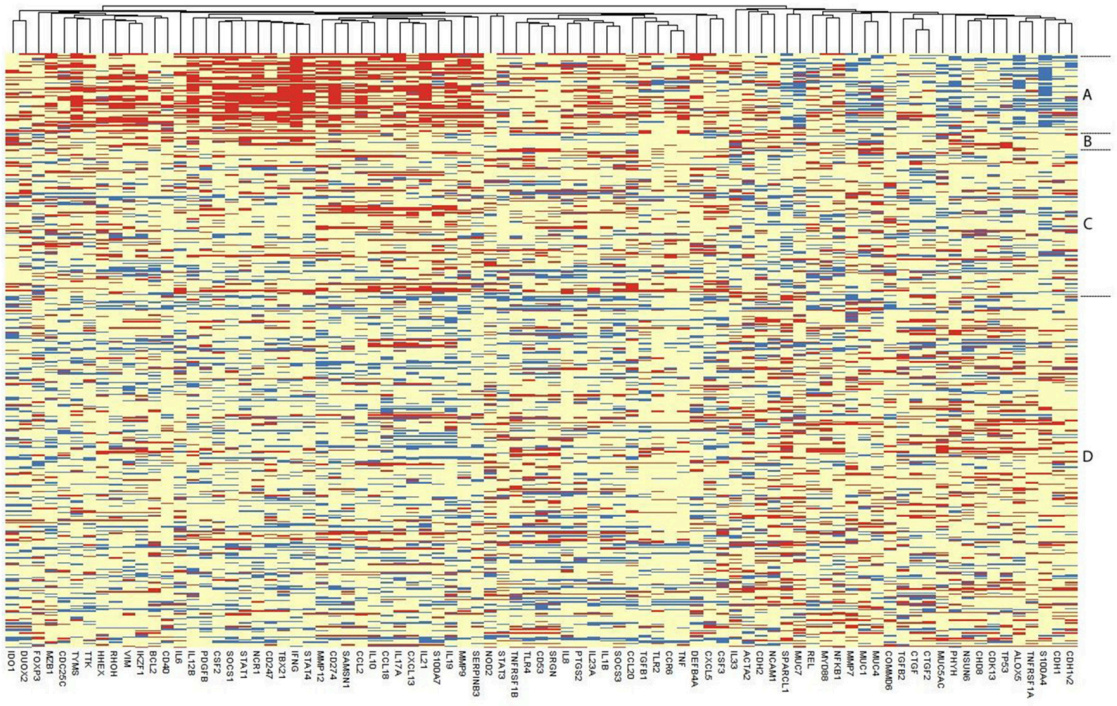

FIGURE 2 | Heatmap of the expression of each of the 83 retained genes in 458 individuals. Each row represents an individual, with individuals grouped as per their disease and infection status and the relevant group indicated on the right: Group **(A)** Infected, Active Trachoma; **(B)** Infected, no Active Trachoma; **(C)** Uninfected, Active Trachoma; **(D)** Uninfected, no Active Trachoma. ΔC_T values of genes are represented in the columns, with genes that tend to be expressed together located next to one another. Red means that an individual has a higher expression of that gene, blue, a lower expression, relative to that individual's expression of the endogenous control gene *HPRT1*.

in the plasmid copy number with increasing disease severity scores.

Host Gene Expression

IFNG was strongly upregulated in association with *C. trachomatis* infection, consistent with previous findings in trachoma endemic populations (Burton et al., 2004; Holland et al., 2006; Natividad et al., 2010). Several NK cell markers were also upregulated in association with *C. trachomatis* infection: *NCR1, CD56 (NCAM1),* and *CD247*. *IFNγ*, *NCAM1,* and *NCR1* were among the genes most strongly associated with increased odds of *C. trachomatis* infection, and Th1 and NK cell pathways were enriched in this gene set (**Table 6**). A strong *IFNG* response and transcriptional signatures associated with NK cell activation and cytotoxicity were closely associated with *C. trachomatis* infection and active trachoma in a West African population (Natividad et al., 2010), and infiltrates suggestive of NK cells were identified in scarred conjunctival biopsy tissue from individuals with trachomatous trichiasis from Tanzania (Hu et al., 2016). In peripheral blood from adults with trichiasis and controls that were stimulated *ex-vivo* with *C. trachomatis* antigens, NK cells and to a lesser extent CD8+ T cells were the major sources of IFNγ with <50% of the IFNγ produced by CD4+ T cells (Gall et al., 2011). In a non-human primate live-attenuated ocular chlamydial vaccine study, depletion of CD8+ cells abrogated protective immunity, however the contribution of CD4+ and NK cells was not investigated (Olivares-Zavaleta et al., 2014). IFNγ is a crucial component of the Th1 response to restrict *C. trachomatis* survival via tryptophan depletion, macrophage activation and promoting leukocyte adhesion, migration and activation (Beatty et al., 1994; Mosser and Edwards, 2008; Redgrove and McLaughlin, 2014). Interestingly, whilst *IFNG* was strongly upregulated in individuals with *C. trachomatis* infection, there was much less upregulation in individuals with disease but not infection, suggesting that the *IFNG* response is quickly down-regulated once *C. trachomatis* has been cleared. Supporting this observation, cluster 3, which was enriched for Th1, NK, IL-12, and IFNγ pathways, was strongly associated with infection but not with TF or TP. Cluster 3 expression was also marginally associated with female sex, and *IFNG*, *IDO1*, *IL12B,* and *IL19* were each upregulated in females relative to males (**Table 5**). Both the number of activated CD4+ cells and their relative expression of pro-inflammatory genes including *IFNG* have previously been shown to be higher in females than males (Hewagama et al., 2009; Sankaran-Walters et al., 2013). Our data suggest that increased expression of *IFNG* and related pathways in females may contribute to the observed differences in tissue responsiveness (TP and TS) between the sexes in the context of equal infection exposure. In support of this hypothesis, a longitudinal study conducted in The Gambia that visited

TABLE 6 | Lasso logistic regression of gene expression by infection, active disease (in only uninfected individuals) and scarring status.

C. trachomatis infection	Active trachoma (uninfected only)	Scarring
IFNG	HHEX	IL33
IL17A	CDH1v2	CXCL5
NCAM1	MMP9	IDO1
MZB1	MMP7	CCL2
CTGF	IDO1	MUC5AC
NCR1	IL21	IL21
TYMS	IFNG	MMP9
SERPINB3	CXCL5	TLR2
IKZF1	RHOH	PDGFB
MUC7	TNFRSF1B	CD40
CD53	S100A4	CDH2
IL21	SPARCL1	IL10
IL12B	VIM	SERPINB3
SOCS1	MYD88	NCR1
TBX21	TNFRSF1A	TTK
S100A7	NCAM1	BCL2
IL10	FOXP3	SOCS3
S100A4		FOXP3
MMP7		CCL18
CSF3		CTGF2
ALOX5		COMMD6
PDGFB		SAMSN1
ACTA2		CD247
CDC25C		
CDH2		
FOXP3		
STAT4		
IL6		
OVER-REPRESENTED PATHWAYS		
IL-12 and STAT4 dependent signaling pathway in Th1 development [15:3/3]*	Inflammatory Response Pathway [32:3/4]	Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell [211:3/4]
NO2-dependent IL-12 pathway in NK cells [9:3/3]	Activation of Matrix Metalloproteinases [31:2/2]	Signaling events mediated by PTP1B [53:3/4]
	Assembly of collagen fibrils and other multimeric structures [49:2/2]	
	Leukocyte transendothelial migration [116:2/2]	
	Collagen formation [94:2/2]	
	TNFs bind their physiological receptors [30:2/2]	

**[x:y/z] x = total number of genes in pathway, y = number of pathway genes present in input list, z = number of pathway genes present in background.*

For each outcome, the genes whose expression was most strongly associated are listed. Biological pathways with members that are over-represented ($P \leq 0.05$) in each gene list relative to the background (all genes tested) are shown for each phenotype, in descending order.

participants every 2 weeks found that the likelihood of developing infection or active disease and the duration of infection were independent of sex, however males had a reduced duration of active disease relative to females (Grassly et al., 2008). Whilst Th1 responses and *IFNG* are essential for controlling chlamydial infection, excessive responses are thought to cause collateral tissue damage and contribute to chlamydial pathology (Van Voorhis et al., 1997; Rank et al., 2000). It is therefore possible that a female predisposition to overproduce Th1 responses and *IFNG* may tip the balance from protection toward pathological tissue damage. However, further research is required to clarify this hypothesis and a direct causal relationship cannot be determined from this cross-sectional analysis.

The cytokine genes *IL1β*, *IL6*, *IL17, IL21, IL22*, *IL23,* and *TGFβ1* were strongly expressed in individuals with *C. trachomatis* infection. Th17 cells are generated in the presence of TGFβ1, IL-1β, IL-6, IL-21, and IL-23 and classically produce IL-17, IL-22, and IL-21 (Manel et al., 2008; Zielinski et al., 2012). *IL21* expression was also upregulated in individuals with TF and TS. Individuals with TP significantly upregulated *IL17* and *IL23* expression and *IL21* at marginal significance. The combined expression of cluster 5, containing *IL10, IL17A, IL21, CXCL13,* and *MMP12,* was significantly associated with *C. trachomatis* infection, TF, TP, and TS. Th17 cells have important roles in mucosal immunity against bacteria and fungi, however they are also associated with the inflammatory pathology of diseases such as Crohn's disease, psoriasis and rheumatoid arthritis (Tesmer et al., 2008). Epithelial cells stimulated by IL-17 and IL-22 upregulate chemokines, cytokines, antimicrobial peptides and growth factors such as IL-6, IL-8, S100A7, and GM-CSF (CSF2) (Wolk et al., 2006; Eyerich et al., 2010); these transcripts were upregulated in individuals with *C. trachomatis* infection (*S100A7* at marginal significance). *IL8* and *S100A7* were also upregulated in individuals with TP. IL-8 and S100A7, amongst other factors induced by Th17 cells, are chemotactic for neutrophils, an influx of which is associated with *C. trachomatis* infection and contributes to pathology in animal models of urogenital and ocular infection (Frazer et al., 2011; Lacy et al., 2011). IL-22, which was highly upregulated with infection, is also important in the maintenance of epithelial health and barrier function (Radaeva et al., 2004; Rutz et al., 2013). The relative contributions of *IL-17* and associated responses to protection from *C. trachomatis* infection and maintenance of barrier function vs. pathological tissue damage remain unclear. In murine models of genital *C. trachomatis* infection higher levels of IL-17/Th17 cells have been associated with greater pathology (Lu et al., 2012; Vicetti Miguel et al., 2016), whereas in IL-23 knock-out mice which had greatly reduced levels of IL-17 and IL-22 there was no difference in infection burden or oviduct pathology compared to wild type mice (Frazer et al., 2013). Longitudinal investigation of these factors is expected to further our understanding of the role of Th17 cell associated cytokines in human chlamydial pathogenesis.

STAT1 and *STAT4*, which promote Th1 cell development (Nishikomori et al., 2002; Mikhak et al., 2006; Zhu et al., 2010), were upregulated in response to *C. trachomatis* infection. *STAT1* and *STAT4* have previously been shown to be associated with active trachoma and ocular *C. trachomatis* infection, and *STAT1* was upregulated in cervical epithelial cells infected with *C. trachomatis in vitro* which was found to inhibit chlamydial

growth (Lad et al., 2005; Natividad et al., 2010). *STAT3*, which is required for Th17 development (Zhu et al., 2010; Yang et al., 2011), was not differentially regulated, contrasting the upregulation of Th17 cytokines. *SOCS1, IL10,* and *FOXP3* were upregulated in individuals with *C. trachomatis* infection and *SOCS3* and *IL10* were upregulated in individuals with TS and TP, indicative of inflammatory regulation. Upregulation of *IL10* expression has previously been reported in individuals with active trachoma and a genetic polymorphism in *IL10* was associated with trachomatous scarring (Mozzato-Chamay et al., 2000; Burton et al., 2004; Natividad et al., 2005; Faal et al., 2006; Skwor et al., 2008). However, the role of Tregs and IL-10 in trachoma remains unclear; dampening inflammation might result in less tissue damage, but conversely it might also prolong survival of *C. trachomatis* (Zhang et al., 2009).

MMP9, *MMP12, TGFβ1,* and *PDGF* were significantly upregulated in individuals with *C. trachomatis* infection. *MMP7*, *MMP9,* and *MMP12* were upregulated in individuals with TP, *MMP9 was* upregulated in TF and *FGF2* and *PDGF* were upregulated in individuals with TS. *MMP7* was downregulated in infected individuals. *MMP7* has consistently been found to be upregulated in scarring trachoma and trichiasis alongside *MMP9* and *MMP12* but there is less evidence for a role of *MMP7* in active trachoma (Holland et al., 2010; Burton et al., 2011b, 2015; Hu et al., 2012). Degradation of the extracellular matrix and basement membrane by MMP9 and MMP12, in part derived from macrophage and neutrophil degranulation (Shapiro et al., 1993; Kjeldsen et al., 1994), facilitates connective tissue remodeling and leukocyte migration into the epithelium (Ozdemir et al., 1999; Zeng et al., 1999; Misko et al., 2002). Whilst MMPs are an essential part of the wound healing process (Nwomeh et al., 1998; Wolf et al., 2017), MMP9 overexpression has been linked to aggressive contractile scarring in proliferative vitroretinopathy and failure of rat corneal cells to re-epithelialize following corneal injury (Fini et al., 1996; Kon et al., 1998).

SPARCL1 was down-regulated in individuals with *C. trachomatis* infection, TF and TP, consistent with previous studies (Hu et al., 2012; Burton et al., 2015). *SPARCL1* codes for a secreted glycoprotein which regulates extracellular matrix (ECM) and cell-matrix adhesion (Girard and Springer, 1996) SPARCL1 inhibits cell proliferation and migration and reduced *SPARCL1* expression has been linked to cancer metastases (Nelson et al., 1998; Sullivan and Sage, 2004). In a murine corneal injury model, knock-out of SPARCL1 led to excessive accumulation of collagen type IV from myofibroblasts and irregular fibrotic ECM formation (Chaurasia et al., 2013). Addition of exogenous SPARCL1 rescued the wild type phenotype with a cessation of collagen IV production, replacement with collagen I from keratinocytes and restoration of normal ECM architecture. Consistent with this, immunohistochemistry and histology studies using tissue from individuals with scarring trachoma have demonstrated a reduction in collagens I and III, an increase in collagens IV and V and progressive disorganization of collagen fibers in scarring trachoma relative to healthy conjunctival tissue (Abu El-Asrar et al., 1998; Hu et al., 2013b; Derrick et al., 2016b). Genes that were most strongly associated with active trachoma in the absence of *C. trachomatis* infection were enriched for pathways of leukocyte transendothelial migration, TNF receptors, MMP activation, collagen formation and assembly (**Table 6**). Active trachoma signs often persist after the clearance of *C. trachomatis* infection (Grassly et al., 2008; Burton et al., 2011a,c; Lee et al., 2014) and inflammation is linked to progressive scarring (Ramadhani et al., 2016a). We found that TP but not infection was associated with TS in the multivariable model (**Table 3**). These results suggest that the inflammatory phenotype following the clearance of *C. trachomatis* infection is characterized by collagen formation and assembly, MMP-mediated matrix remodeling and leukocyte influx, and that these pathways might be causative factors in the scarring process.

We found significantly reduced expression of *MUC1*, *MUC4, MUC5AC,* and *MUC7* in individuals with *C. trachomatis* infection, and *MUC5AC* expression was reduced in those with TP. MUC1 and MUC4 are primarily expressed by apical cells of the stratified epithelia, MUC7 by lacrimal gland epithelia and MUC5AC by goblet cells (Gipson and Argueso, 2003). *MUC7* expression was also significantly reduced in females relative to males. *MUC5AC* and *MUC7* down-regulation has previously been reported in individuals with trachomatous trichiasis, however expression of *MUC1* and *MUC4* was increased in this later stage of disease (Burton et al., 2011b). IL-22 stimulates the upregulation of MUC4 and MUC1, therefore, one might expect a corresponding increase in mucin expression in infected individuals (Turner et al., 2013). This inconsistency and the broad reduction in mucin expression during infection may reflect a loss of epithelial homeostasis and a potential reduction in barrier function. This in turn could result in increased access of bacteria to the epithelial cell surface, promoting inflammation. In patients with ulcerative colitis, reduction of goblet cells, mucin production and microbial diversity were identified as causative factors in disease etiology (Alipour et al., 2016). Microbial dysbiosis can itself be caused by inflammation, creating a positive feedback loop (Winter et al., 2013). Decreased diversity has previously been found in the conjunctival microbiome of individuals with scarring trachoma relative to healthy controls (Zhou et al., 2014). Furthermore, penetration of the mucin barrier and contact between epithelial cells and bacteria was demonstrated in ulcerative colitis patients with acute inflammation, whereas the mucin barrier of healthy controls was impenetrable (Johansson et al., 2014). It is tempting to speculate that reduced barrier function resulting from inflammation and loss of mucins causes microbial dysbiosis and contact between bacteria and epithelial cells, leading to persistent conjunctival inflammation, which in turn contributes to trachomatous scarring.

Consistent with our previous studies in various clinical stages of trachoma (Burton et al., 2011b,c, 2012, 2015; Hu et al., 2012), the antimicrobial peptide S100A7 was upregulated in individuals with TP and was marginally associated with *C. trachomatis* infection. S100A7 is secreted by epithelial cells in response to microbial challenge and expression can also be stimulated by IL-22 (Wolk et al., 2006). It is chemotactic for neutrophils and interferes with pathogen membrane permeability and enzyme function (Ganz, 2003; Guilhelmelli et al., 2013). The upregulation of S100A7 in various clinical stages of trachoma could support the

loss of epithelial barrier function hypothesis described above and could contribute to chronic inflammation.

There was differential expression of several cell cycle genes (*CD53, CDK13, CDC25C, PHYH, TYMS, TTK, TP53*) in individuals with *C. trachomatis* infection and/or TP (**Table 5**). These expression changes could reflect regulation of the cell cycle in response to infection or inflammation, such as the proliferation or contraction of infiltrating inflammatory cells and cells effecting the wound healing response. Studies have reported that progression of the cell cycle is slow in cells infected with *C. trachomatis* (Kun et al., 2013; Elwell et al., 2016) and *C. trachomatis* is known to interfere with host cell apoptosis as a survival mechanism (Fischer et al., 2004; Ying et al., 2007; Siegl et al., 2014). Cluster 1 was enriched for genes in apoptosis pathways, however the combined expression of this cluster was not associated with clinical phenotypes or infection.

CONCLUSIONS

Our results, illustrated in a graphical summary (**Figure 3**), suggest that *IFNG* and *IL22* were acutely upregulated in response to *C. trachomatis* infection and expression appeared to be reduced following the clearance of infection, despite the persistence of inflammation. This might suggest that the sources of these cytokines are important in the clearance of infection and that the inflammatory phenotype differs once infection has been cleared, shifting away from a Th1/NK cell dominated response. The upregulation of Th17 cell-associated cytokines in infection and TP phenotypes implies the involvement of Th17 cells, although it remains unclear whether they contribute more to protection or pathology. Epithelial cells appear to be highly active, with possible loss of epithelial homeostasis and barrier function resulting in loss of mucins and a pro-inflammatory anti-microbial phenotype, perhaps exacerbating inflammation. The upregulation of growth factors and MMPs, derived in part from infiltrating macrophages and neutrophils, and the down-regulation of SPARCL1 are likely to contribute to matrix remodeling, collagen deposition and conjunctival scarring in the post-infection inflammatory phase. Females were more susceptible to TP and TS and although this was not associated with a significant difference in infection prevalence, there was some evidence of increased *IFNG* related pathway activity in females. A caveat of this study is that gene expression may not correlate directly to protein expression and this will require further work. Despite this, we anticipate that

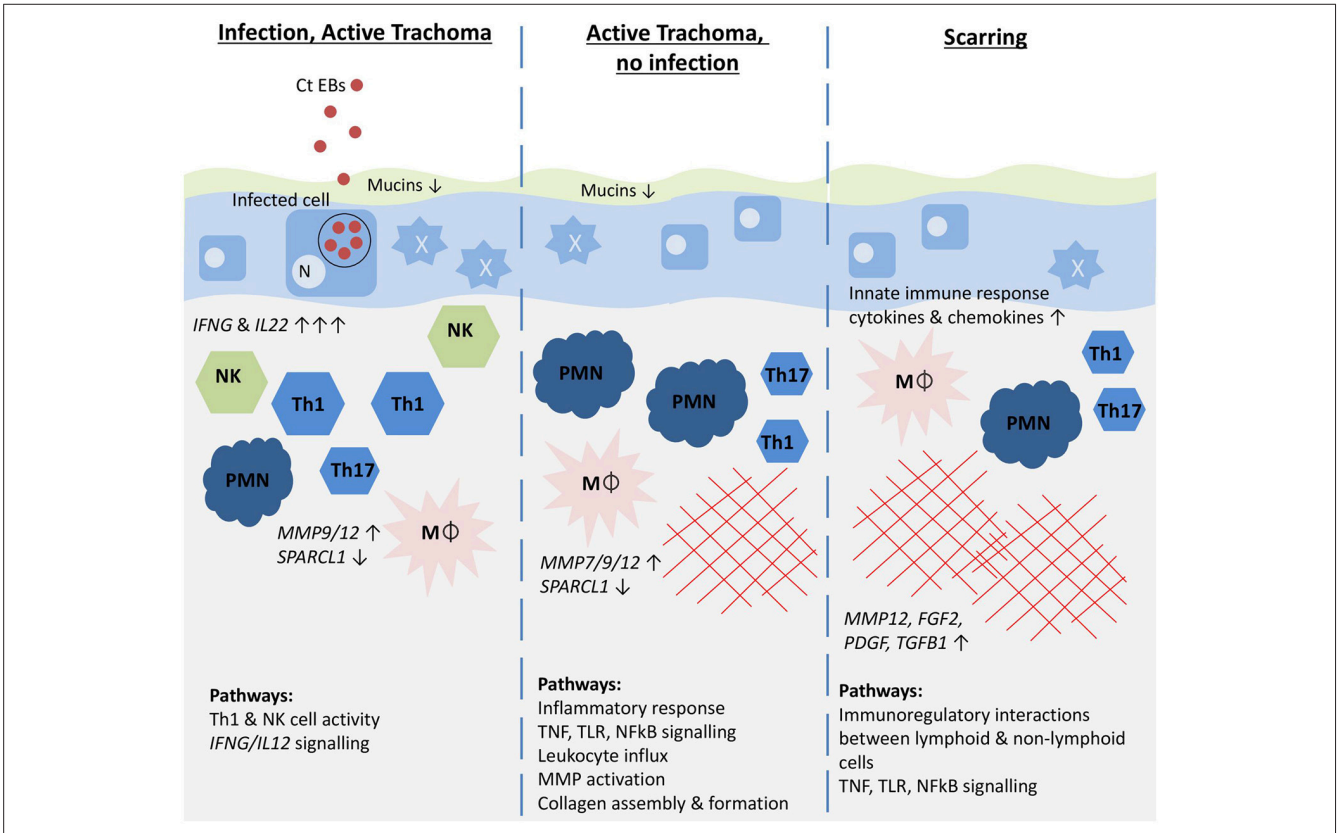

FIGURE 3 | Graphical summary illustrating the host immune pathways hypothesized to be associated with different trachoma phenotypes. Genes most strongly associated with each phenotype are shown in italics with the direction and strength of expression illustrated by arrows. Pathways that were most enriched for each phenotype are shown. Ct EBs, *C. trachomatis* elementary bodies; N, epithelial cell nucleus; NK, Natural killer cells; Th1, Th1 T cells; Th17, Th17 T cells; PMN, neutrophil; Mϕ, macrophage; red cross-hatching, fibrosis. The mucus layer is shown in green, the epithelial cell layer in blue, and the stroma in beige.

longitudinal investigation of these transcriptional signatures and relation to infection and ongoing clinical signs will enhance our understanding of their relative contributions to protection and pathology.

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception: MB, MH, DCM, and RB. Design of the work: MB, AR, MH, PM, TM and CR. Analysis: AR, MB, DM, MH, TD, and DJ. Interpretation of data for the work: AR, MB, MH, and TD. Drafting the work: AR and MB. Revising it critically for important intellectual content: AR, MB, MH, TD, DCM, RB, DM, CR, PM, TM, and DJ. Final approval of the version to be published: AR, MB, MH, TD, DCM, RB, DM, CR, PM, TM, and DJ. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: AR, MB, MH, TD, DCM, RB, DM, CR, PM, TM, and DJ.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00406/full#supplementary-material

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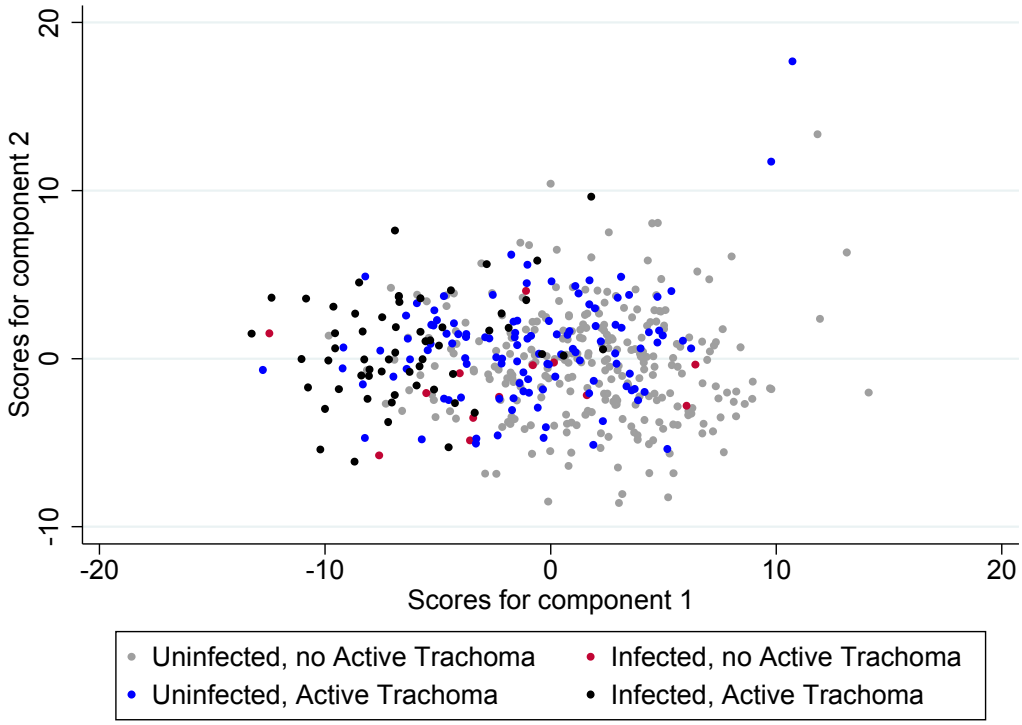
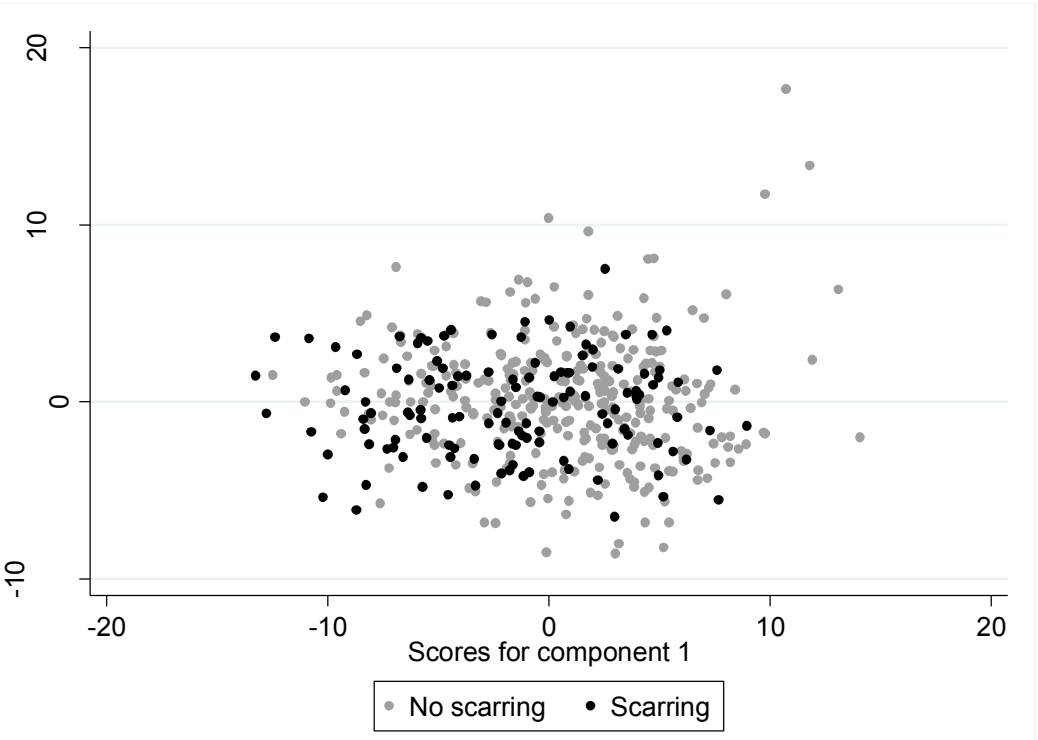
Supplementary figure 1: Principal component analysis. (a) Infection and Active Trachoma status. (b) Scarring status.

(a)



(b)



Supplementary Table 1: Comparison of conjunctival gene expression in the presence of *C. trachomatis* plasmid, follicular inflammation (TF), papillary inflammation (TP) and scarring trachoma, adjusted for age and sex. N – number of individuals with detectable expressions. FC – fold change. P-value. Using the Benjamini and Hochberg approach only tests with a p-value below 0.0350 have a False Discovery Rate of <5%.

		Infected vs not		TF vs no TF		TP vs no TP		TS vs no TS	
Target	**N**	**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**
Antimicrobial Peptides									
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	494	**1.57**	**0.0044**	**1.31**	**0.0330**	**1.96**	**4.70E-08**	**1.33**	**0.0258**
Psoriasin-1 (*S100A7*)	492	**3.43**	**5.81E-07**	**3.24**	**1.58E-09**	**4.30**	**3.88E-14**	**1.91**	**0.0012**
Cell Cycle									
Catenin (cadherin-associated protein), delta 2 (*CTNND2*)	327	0.75	0.1967	0.89	0.5377	0.79	0.2123	0.95	0.7887
CD53 molecule (*CD53*)	494	**1.24**	**0.0003**	**1.15**	**0.0022**	**1.24**	**2.78E-06**	**1.14**	**0.0049**
Cyclin-dependent kinase 13 (*CDK13*)	494	**0.87**	**0.0029**	**0.91**	**0.0080**	**0.86**	**4.29E-05**	0.97	0.4573
M-phase inducer phosphatase 3 (*CDC25C*)	485	**1.53**	**1.88E-07**	**1.36**	**2.03E-06**	**1.29**	**7.11E-05**	1.13	0.0548
Phytanoyl-coa 2-hydroxylase (*PHYH*)	494	**0.80**	**7.36E-07**	**0.85**	**6.84E-06**	**0.87**	**0.0001**	0.96	0.2410
Sun Domain Family, Member 6 (*NSUN6*)	494	0.98	0.4884	**0.94**	**0.0235**	0.96	0.1036	1.02	0.5273
Thymidylate synthetase (*TYMS*)	494	**1.82**	**8.78E-25**	**1.41**	**2.79E-13**	**1.41**	**5.38E-13**	**1.13**	**0.0144**
TTK protein kinase (*TTK*)	493	**1.51**	**6.25E-10**	**1.37**	**1.50E-09**	**1.34**	**3.26E-08**	**1.21**	**0.0005**
Tumor protein p53 (*TP53*)	494	0.95	0.2391	**0.93**	**0.0281**	**0.87**	**9.41E-06**	0.99	0.7425
Cytokines/Chemokines									
Chemokine ligand 2 (*CCL2*)	490	**4.29**	**3.77E-21**	**2.00**	**4.32E-08**	**2.39**	**3.53E-12**	**1.83**	**2.73E-06**
Chemokine ligand 5 (*CXCL5*)	493	1.22	0.1911	**1.63**	**6.09E-05**	**2.08**	**1.55E-09**	**1.88**	**2.64E-07**
Chemokine ligand 13 (*CXCL13*)	491	**3.48**	**2.10E-13**	**2.76**	**5.04E-14**	**3.11**	**2.55E-17**	**1.62**	**0.0006**
Chemokine ligand 18 (*CCL18*)	488	**3.16**	**9.96E-10**	**2.33**	**1.82E-08**	**3.59**	**3.75E-18**	1.37	0.0411
Chemokine ligand 20 (*CCL20*)	493	**1.59**	**1.86E-05**	**1.68**	**1.24E-09**	**1.46**	**8.91E-06**	**1.49**	**4.27E-06**
Chemokine receptor 6 (*CCR6*)	493	1.29	0.0493	**1.45**	**0.0003**	1.12	0.2586	1.17	0.1329
Colony stimulating factor 2 (*CSF2*)	489	**2.79**	**1.15E-22**	**1.68**	**1.93E-09**	**1.53**	**1.10E-06**	**1.32**	**0.0015**
Colony stimulating factor 3 (*CSF3*)	493	1.05	0.7614	1.22	0.0805	**1.47**	**0.0009**	**1.62**	**3.30E-05**
Forkhead box P3 (*FOXP3*)	491	**1.25**	**0.0004**	1.08	0.1071	1.07	0.1985	0.95	0.2866
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	494	**2.18**	**2.74E-12**	**1.69**	**3.79E-09**	**1.73**	**7.00E-10**	**1.47**	**1.99E-05**
Interferon gamma(*IFNγ*)	491	**8.81**	**2.54E-57**	**2.74**	**1.64E-17**	**2.28**	**6.80E-12**	**1.56**	**0.0003**
Interleukin 1 beta(*IL1β*)	493	**2.05**	**1.11E-08**	**1.64**	**6.93E-07**	**2.07**	**1.42E-13**	**1.65**	**7.84E-07**
Interleukin 6 (*IL6*)	493	**2.00**	**2.11E-07**	**1.35**	**0.0056**	**1.51**	**0.0001**	**1.43**	**0.0010**
Interleukin 8 (*IL8*)	493	**1.49**	**1.92E-05**	**1.23**	**0.0054**	**1.51**	**2.73E-08**	**1.38**	**2.27E-05**
Interleukin 10 (*IL10*)	492	**2.33**	**2.73E-16**	**1.78**	**2.63E-12**	**1.97**	**1.40E-16**	**1.50**	**1.91E-06**
Interleukin 12 beta (*IL12β*)	480	**3.99**	**2.91E-30**	**1.92**	**1.38E-10**	**1.84**	**2.20E-09**	**1.35**	**0.0045**
Interleukin 13 (*IL13*)	214	1.38	0.1496	1.37	0.1247	1.02	0.9098	0.83	0.3233
Interleukin 17A (*IL17A*)	492	**3.23**	**4.00E-18**	**2.11**	**6.23E-12**	**2.37**	**1.15E-15**	**1.57**	**5.34E-05**
Interleukin 19 (*IL19*)	493	**3.21**	**1.35E-13**	**2.20**	**3.83E-10**	**3.26**	**5.97E-22**	**1.63**	**0.0001**
Interleukin 21 (*IL21*)	480	**4.89**	**2.08E-24**	**3.09**	**2.34E-19**	**2.70**	**3.82E-15**	**1.87**	**1.99E-06**
Interleukin 22 (*IL22*)	427	**8.01**	**4.91E-33**	**2.66**	**1.26E-10**	**2.50**	**1.81E-09**	**1.81**	**0.0002**

Interleukin 23A (*IL23A*)	492	**2.38**	**3.16E-17**	**1.68**	**4.06E-10**	**2.04**	**1.15E-18**	**1.36**	**0.0002**
Interleukin 33 (*IL33*)	494	**1.26**	**0.0121**	1.04	0.6076	1.02	0.7717	**1.28**	**0.0007**
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	494	**1.37**	**0.0054**	**1.24**	**0.0175**	**1.52**	**3.22E-06**	**1.39**	**0.0003**
Tumor necrosis factor (*TNF*)	492	**1.53**	**0.0002**	**1.49**	**1.02E-05**	**1.25**	**0.0139**	**1.26**	**0.0123**
EMT Markers									
Alpha smooth muscle actin (*ACTA2*)	492	**1.28**	**0.0002**	1.10	0.0729	0.97	0.5791	**1.14**	**0.0118**
Epithelial cadherin (*CDH1*)	493	**0.73**	**4.34E-08**	**0.84**	**6.89E-05**	**0.84**	**0.0002**	0.98	0.7421
Cadherin 1, type 1, E-cadherin (epithelial) (CDH1)	494	**0.69**	**2.16E-09**	**0.82**	**9.65E-05**	**0.83**	**0.0002**	0.97	0.5543
Neuronal cadherin (*CDH2*)	482	0.98	0.8961	0.86	0.1328	**0.76**	**0.0049**	1.09	0.4063
S100 calcium binding protein A4 (*S100A4*)	493	**0.39**	**1.39E-32**	**0.58**	**8.58E-17**	**0.56**	**1.66E-19**	0.93	0.2657
Vimentin (*VIM*)	494	**1.57**	**3.65E-20**	**1.20**	**2.99E-06**	**1.19**	**1.55E-05**	**1.11**	**0.0073**
Matrix Modifiers									
Connective tissue growth factor (*CTGF-1*)	493	1.08	0.4138	0.91	0.2104	**0.83**	**0.0117**	0.94	0.3853
Connective tissue growth factor (*CTGF-2*)	494	1.04	0.6659	**0.84**	**0.0218**	**0.81**	**0.0052**	0.87	0.0603
Fibroblast growth factor 2 (basic) (*FGF2*)	399	**1.54**	**0.0021**	1.12	0.3424	1.25	0.0569	**1.51**	**0.0005**
Matrix metallopeptidase 7 (*MMP7*)	493	**0.42**	**1.38E-13**	0.84	0.0674	1.05	0.6140	0.99	0.9536
Matrix metallopeptidase 9 (*MMP9*)	493	**2.51**	**8.58E-15**	**2.11**	**1.91E-15**	**2.11**	**2.14E-15**	**1.51**	**2.08E-05**
Matrix metallopeptidase 12 (*MMP12*)	493	**2.85**	**3.14E-16**	**2.17**	**3.60E-14**	**2.23**	**3.53E-15**	**1.62**	**4.33E-06**
Platelet-derived growth factor beta polypeptide (*PDGFβ*)	492	**1.53**	**1.88E-11**	**1.22**	**9.88E-05**	**1.25**	**1.59E-05**	**1.24**	**3.14E-05**
SPARC-like 1 (hevin) (*SPARCL1*)	484	**0.22**	**1.74E-10**	**0.23**	**1.04E-14**	**0.21**	**1.54E-16**	**0.52**	**0.0009**
Transforming growth factor, beta 1 (*TGFβ1*)	493	**1.25**	**3.23E-06**	**1.10**	**0.0156**	1.08	0.0572	**1.11**	**0.0077**
Transforming growth factor, beta 2 (*TGFβ2*)	492	0.89	0.2044	0.93	0.3235	0.93	0.3581	1.05	0.5253
Microbiota Response									
Arachidonate 5-lipoxygenase (*ALOX5*)	494	**0.62**	**1.44E-20**	**0.77**	**1.62E-10**	**0.81**	**1.06E-06**	0.96	0.3789
B-cell CLL/lymphoma 2 (*BCL2*)	494	**1.11**	**0.0217**	1.02	0.6071	0.99	0.7735	**1.09**	**0.0241**
CD40 molecule, TNF receptor superfamily member 5 (*CD40*)	494	**1.24**	**0.0004**	1.05	0.2999	1.05	0.3513	1.08	0.1334
Dual oxidase 2 (*DUOX2*)	494	**1.32**	**0.0022**	**1.19**	**0.0180**	**1.40**	**2.70E-06**	**1.19**	**0.0202**
Tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*)	494	**0.76**	**0.0046**	**0.82**	**0.0105**	0.88	0.0995	1.01	0.8651
Tumor necrosis factor receptor superfamily, member 1B (*TNFRSF1B*)	494	1.05	0.5043	1.01	0.8897	**1.14**	**0.0190**	**1.13**	**0.0337**
V-rel avian reticuloendotheliosis viral oncogene homolog (*REL*)	494	1.05	0.2782	1.02	0.5318	1.08	0.0413	1.07	0.0657
Mucin									
Mucin 1, cell surface associated (*MUC1*)	494	**0.80**	**0.0001**	**0.89**	**0.0110**	0.95	0.2538	1.00	0.9778
Mucin 4, cell surface associated (*MUC4*)	494	**0.73**	**1.17E-05**	0.91	0.1154	1.02	0.7338	1.04	0.4983
Mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*)	494	**0.41**	**2.69E-10**	**0.50**	**6.86E-10**	**0.48**	**2.05E-11**	0.96	0.7406
Mucin 7, secreted (*MUC7*)	494	**0.33**	**1.76E-11**	**0.52**	**6.24E-07**	**0.49**	**8.02E-08**	**0.74**	**0.0248**
NK Cell Markers									
CD247 molecule (*CD247*)	493	**1.93**	**6.19E-22**	**1.37**	**1.65E-08**	**1.28**	**1.14E-05**	1.11	0.0776
Natural cytotoxicity triggering receptor 1 (*NCR1*)	492	**2.50**	**1.13E-30**	**1.50**	**1.27E-09**	**1.38**	**1.14E-06**	**1.28**	**0.0003**
Neural cell adhesion molecule 1 (*NCAM1*)	493	**1.50**	**2.08E-06**	0.90	0.1171	**0.83**	**0.0080**	1.10	0.1742
Pattern Recognition Receptors									
Nucleotide-binding oligomerization domain containing 2 (*NOD2*)	491	1.13	0.1847	1.04	0.6135	**1.19**	**0.0174**	1.17	0.0377

Toll-like receptor 2 (*TLR2*)	493	0.89	0.1577	1.01	0.8602	1.03	0.6513	**1.24**	**0.0018**
Toll-like receptor 4 (*TLR4*)	493	1.13	0.1092	**1.15**	**0.0218**	**1.26**	**0.0002**	**1.26**	**0.0002**
Regulators/Signalling Pathways									
CD274 molecule (*CD274*)	493	**2.99**	**5.54E-29**	**1.73**	**1.58E-11**	**1.92**	**3.44E-16**	**1.43**	**1.41E-05**
Chromodomain helicase DNA binding protein 8 (*CHD8*)	494	0.97	0.4299	0.95	0.0481	0.98	0.4673	1.02	0.4319
COMM domain containing 6 (*COMMD6*)	493	0.87	0.1506	0.93	0.3440	0.92	0.2886	0.97	0.6802
Hematopoietically expressed homeobox (*HHEX*)	493	**1.26**	**0.0004**	**1.35**	**1.19E-08**	**1.15**	**0.0083**	**1.14**	**0.0177**
IKAROS family zinc finger 1 (Ikaros) (*IKZF1*)	494	**1.45**	**2.24E-13**	**1.24**	**2.04E-07**	**1.16**	**0.0002**	**1.10**	**0.0232**
Marginal zone B and B1 cell-specific protein (*MZB1*)	493	**2.44**	**1.08E-12**	**1.65**	**5.78E-07**	**1.64**	**1.03E-06**	1.15	0.1880
Myeloid differentiation primary response 88 (*MYD88*)	494	1.00	0.8889	0.97	0.2443	1.02	0.3767	**1.07**	**0.0156**
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*)	494	**1.13**	**0.0005**	1.03	0.3739	**1.07**	**0.0168**	**1.06**	**0.0341**
Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2 (*PREX2v1*)	462	**1.28**	**0.0335**	1.02	0.7928	0.89	0.2381	0.90	0.2483
Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2 (*PREX2v2*)	445	1.09	0.4465	1.10	0.3299	1.09	0.3703	0.88	0.2025
Ras homolog family member H (*RHOH*)	494	**1.63**	**4.48E-14**	**1.38**	**4.78E-10**	**1.26**	**8.00E-06**	**1.13**	**0.0271**
SAM domain, SH3 domain and nuclear localization signals 1 (*SAMSN1*)	494	**1.57**	**2.47E-10**	**1.35**	**8.85E-08**	**1.48**	**2.48E-12**	**1.17**	**0.0067**
Serglycin (*SRGN*)	494	1.05	0.5256	1.07	0.2821	**1.24**	**0.0006**	**1.17**	**0.0122**
Serpin peptidase inhibitor B3 (*SERPINB3*)	493	**3.43**	**2.87E-16**	**1.83**	**7.66E-07**	**2.45**	**8.52E-14**	**1.72**	**1.21E-05**
Serpin peptidase inhibitor clade B member 4, (*SERPINB4*)	406	**5.93**	**1.95E-11**	**2.32**	**0.0002**	**3.80**	**8.81E-10**	**1.80**	**0.0089**
Signal transducer and activator of transcription 1 (*STAT1*)	493	**2.13**	**1.90E-31**	**1.38**	**2.63E-09**	**1.40**	**5.78E-10**	**1.25**	**6.72E-05**
Signal transducer and activator of transcription 3 (*STAT3*)	493	1.02	0.6831	1.00	0.9047	1.06	0.1165	**1.14**	**0.0003**
Signal transducer and activator of transcription 4 (*STAT4*)	493	**1.88**	**2.22E-22**	**1.25**	**3.32E-05**	**1.21**	**0.0005**	**1.12**	**0.0312**
Suppressor of cytokine signalling 1 (*SOCS1*)	493	**2.34**	**4.52E-27**	**1.56**	**6.81E-12**	**1.48**	**1.55E-09**	**1.26**	**0.0006**
Suppressor of cytokine signalling 3 (*SOCS3*)	493	**1.53**	**3.68E-05**	**1.44**	**6.37E-06**	**1.71**	**2.48E-11**	**1.53**	**2.02E-07**
T-box 21 (*TBX21*)	493	**2.42**	**7.94E-36**	**1.57**	**2.30E-14**	**1.40**	**1.79E-08**	**1.22**	**0.0011**
Ubiquitin specific peptidase 6 (Tre-2 oncogene) (*USP6*)	182	0.72	0.2233	**0.61**	**0.0298**	0.66	0.0766	0.66	0.0716

Supplementary Table 2a. Network co-expression analysis of the filtered dataset using Miru and Markov clustering revealed five clusters of co-expressed genes.

Cluster 1		Cluster 2		Cluster 3		Cluster 4		Cluster 5	
Genes	**Connections**	**Genes**	**Connections**	**Genes**	Connections	**Genes**	**Connections**	**Genes**	**Connections**
MYD88	13	IKZF1	11	STAT1	6	SRGN	6	IL10	3
REL	13	VIM	11	CD247	5	TLR4	4	IL17A	3
NFKB1	12	CD53	10	STAT4	5	TNFRSF1B	4	IL21	3
CHD8	11	SAMSN1	10	IFNG	2	IL1B	3	CXCL13	1
BCL2	10	RHOH	6	NCR1	2	PTGS2	3	MMP12	1
TP53	9	TBX21	6	PDGFB	1	SOCS3	3		
CDK13	8	TYMS	6						
NSUN6	8	CD274	4						
PHYH	6	HHEX	3						
CD40	5	TTK	1						
ALOX5	1								

Supplementary Table 2b. Pathway enrichment analysis of genes in each cluster against background, using ConsensusPathDB. The first six most enriched pathways (P < 0.05) are shown.

pathway name	set size	candidates	p-value	q-value	pathway source
Cluster 1					
Neurotrophin signaling pathway	121(3)	3 (100.0%)	0.00217	0.0286	KEGG
Apoptosis Modulation and Signaling	92(6)	4 (66.7%)	0.00296	0.0286	Wikipathways
DNA Damage Response (only ATM dependent)	110(4)	3 (75.0%)	0.00798	0.0286	Wikipathways
IL1	56(4)	3 (75.0%)	0.00798	0.0286	NetPath
Downstream signaling events of B Cell Receptor (BCR)	147(4)	3 (75.0%)	0.00798	0.0286	Reactome
Signaling by the B Cell Receptor (BCR)	242(4)	3 (75.0%)	0.00798	0.0286	Reactome
Viral carcinogenesis	203(4)	3 (75.0%)	0.00798	0.0286	KEGG
Prostate cancer	89(4)	3 (75.0%)	0.00798	0.0286	KEGG
Cluster 2					
Retinoblastoma (RB) in Cancer	89(3)	2 (66.7%)	0.0418	0.155	Wikipathways
Cluster 3					
Th1 and Th2 cell differentiation	92(7)	4 (57.1%)	0.000344	0.0235	KEGG
SHP2 signaling	59(4)	3 (75.0%)	0.00102	0.0235	PID
Downstream signaling in naïve CD8+ T cells	68(4)	3 (75.0%)	0.00102	0.0235	PID
IL12-mediated signaling events	64(10)	4 (40.0%)	0.00193	0.0333	PID
Natural killer cell mediated cytotoxicity	135(5)	3 (60.0%)	0.00247	0.0341	KEGG
IL12 signaling mediated by STAT4	32(6)	3 (50.0%)	0.00479	0.0431	PID
IFN gamma signaling pathway	6(2)	2 (100.0%)	0.005	0.0431	BioCarta
IFN gamma signaling	6(2)	2 (100.0%)	0.005	0.0431	INOH
Cluster 4					

TNF signaling pathway	110(14)	4 (28.6%)	0.00837	0.191	KEGG
Nanomaterial induced inflammasome activation	3(3)	2 (66.7%)	0.0145	0.191	Wikipathways
TLR ECSIT MEKK1 JNK	27(3)	2 (66.7%)	0.0145	0.191	INOH
NF-kappa B signaling pathway	95(10)	3 (30.0%)	0.0253	0.191	KEGG
Glucocorticoid Receptor Pathway	71(4)	2 (50.0%)	0.0279	0.191	Wikipathways
TLR NFkB	70(4)	2 (50.0%)	0.0279	0.191	INOH
Nuclear Receptors Meta-Pathway	316(12)	3 (25.0%)	0.0435	0.197	Wikipathways
Type II interferon signaling (IFNG)	37(5)	2 (40.0%)	0.0448	0.197	Wikipathways
Cluster 5					
Allograft Rejection	80(14)	4 (28.6%)	0.00313	0.0532	Wikipathways
Cytokine-cytokine receptor interaction	265(25)	4 (16.0%)	0.0343	0.272	KEGG

Supplementary Table 2c. Differential regulation of clusters 1-5 in individuals with *C. trachomatis* infection and clinical signs. A multivariable linear regression was performed using the first principle component as the combined cluster expression value for each individual. Using the Benjamini and Hochberg approach for adjusting for multiple comparisons, in order to control the false discovery rate <5% only tests with a p-value below 0.018 (highlighted in bold) are considered statistically significant.

	Cluster 1		**Cluster 2**		**Cluster 3**		**Cluster 4**		**Cluster 5**	
	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)
TS	0.063	0.63 0.38 - 1.03)	0.334	1.24 (0.80 - 1.93)	0.036	1.47 (1.03 - 2.10)	**0.014**	1.75 (1.12 - 2.74)	**0.010**	1.57 (1.12 - 2.22)
TF	0.174	1.51 (0.84 - 2.72)	**0.016**	1.91 (1.13 - 3.25)	0.351	1.23 (0.80 - 1.88)	0.370	0.78 (0.46 - 1.33)	0.028	1.59 (1.05 - 2.40)
TP	0.489	1.23 (0.69 - 2.20)	0.044	1.71 (1.02 - 2.88)	0.361	1.22 (0.80 - 1.86)	**3.94E-04**	2.60 (1.54 - 4.40)	**2.53E-05**	2.41 (1.61 - 3.62)
Infection	0.590	1.19 (0.63 - 2.27)	**1.55E-15**	11.29 (6.36 - 20.06)	**2.00E-16**	17.05 (10.71 - 27.14)	0.244	1.41 (0.79 - 2.52)	**1.23E-10**	4.51 (2.88 - 7.05)
Age	0.086	0.91 (0.82 - 1.01)	0.111	0.92 (0.84 - 1.02)	**2.08E-06**	0.82 (0.76 - 0.89)	**0.016**	0.89 (0.80 - 0.98)	**7.53E-08**	0.81 (0.75 - 0.87)
Sex (F)	0.584	1.13 (0.74 - 1.72)	0.081	1.40 (0.96 - 2.06)	0.028	1.41 (1.04 - 1.92)	0.724	0.93 (0.64 - 1.37)	0.078	1.31 (0.97 - 1.76

7. Ocular Immune Responses and Clinical Signs of Trachoma Before and After Azithromycin Mass Drug Administration in a Treatment Naïve Trachoma-Endemic Tanzanian Community

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Athumani M. Ramadhani
Principal Supervisor	Prof. Matthew J. Burton
Thesis Title	Studies on the Development of Scarring Trachoma in Tanzania

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	N/A		
When was the work published?	N/A		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	N/A		
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Frontiers in Cellular and Infection Microbiology
Please list the paper's authors in the intended authorship order:	Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Patrick Massae, Aiweda Malisa, Kelvin Mbuya, Tara Mtuy, William Makupa, Chrissy h Roberts, Robin L. Bailey, David C. W. Mabey, Martin J. Holland and Matthew J. Burton
Stage of publication	**Submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed laboratory work and analysed the data with some guidance from Matthew Burton, David Macleod and Tamsyn Derrick. I wrote a first draft of this paper and comments from co-authors were considered. I

	periodically attended field work, doing clinical data entry from the study participants and QA of sample collection process and grading. I was involved in mass drug administration.

Student Signature: [redacted] Date: 29.10.2018

Supervisor Signature: [redacted] Date: 27/10/2018

Ocular Immune Responses and Clinical Signs of Trachoma Before and After Azithromycin Mass Drug Administration in a Treatment Naïve Trachoma-Endemic Tanzanian Community

Authors:
Athumani M. Ramadhani[1,2], Tamsyn Derrick[1,2], David Macleod[3], Patrick Massae[2], Aiweda Malisa[2], Kelvin Mbuya[2], Tara Mtuy[1,2], William Makupa[2], Chrissy h Roberts[1], Robin L. Bailey[1], David C. W. Mabey[1], Martin J. Holland[1] and Matthew J. Burton[1]

Affiliations:
1. Clinical Research Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK
2. Kilimanjaro Christian Medical Centre, Moshi, Tanzania
3. Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK

Corresponding Author:
Athumani M. Ramadhani
International Centre for Eye Health
Clinical Research Department
Faculty of Infectious and Tropical Diseases
London School of Hygiene & Tropical Medicine
Keppel Street
London
WC1E 7HT
UK
Email: Athumani.Ramadhani@lshtm.ac.uk

Running Title: Azithromycin Effect on Gene Expression

ABSTRACT

Background; Trachoma, caused by *Chlamydia trachomatis*, remains the leading infectious cause of blindness worldwide. Persistence and progression of the resulting clinical disease appears to be an immunologically mediated process. Azithromycin, which is distributed at the community level for trachoma control, has immunomodulatory properties. We investigated the impact of one round of oral azithromycin on conjunctival immune responses three and six months post treatment.

Methodology; A cohort of children aged 6 to 10 years were recruited from a trachoma endemic region of northern Tanzania and were visited five times in a 12-months period. They were examined for clinical signs of trachoma and conjunctival swabs were collected for laboratory analysis. *C. trachomatis* infection was detected and the expression of 46 host genes was quantified using quantitative PCR. All community members were offered azithromycin treatment immediately after the six-month timepoint according to international guidelines.

Findings; The prevalence of *C. trachomatis* infection and inflammatory disease signs were significantly reduced three and six months post-mass drug administration (MDA). *C. trachomatis* infection was strongly associated with clinical signs at all five time-points. A profound anti-inflammatory effect on conjunctival gene expression was observed 3 months post-MDA, however, gene expression had largely returned to pre-treatment levels of variation by 6 months. This effect was less marked, but still observed, after adjusting for *C. trachomatis* infection and when the analysis was restricted to individuals who were free from both infection and clinical disease at all five time-points. Interestingly, a modest effect was also observed in individuals who did not receive treatment.

Conclusion; Conjunctival inflammation is the major clinical risk factor for progressive scarring trachoma, therefore, the reduction in inflammation associated with azithromycin treatment may be beneficial in limiting the development of potentially blinding disease sequelae. Future work should seek to determine whether this effect is mediated directly through inhibition of pro-inflammatory intracellular signalling molecules, through reductions in concurrent, sub-clinical infections, and/or through reduction of infection exposure.

INTRODUCTION

Trachoma remains the leading infectious cause of blindness worldwide, with the greatest burden in sub-Saharan Africa (WHO, 2016). Trachomatous disease is initiated by repeated conjunctival infection with C*hlamydia trachomatis,* which triggers prolonged inflammatory episodes that contribute to the development of conjunctival scarring (reviewed in Ramadhani et al., 2016a). Infection and clinical signs of active trachoma (follicular and papillary inflammation) are most frequently found in younger children (WHO, 2017). Conjunctival scarring gradually accumulates through childhood, adolescence and into adult life. Eventually this results in the in-turning of the eyelid (entropion) and eyelashes (trichiasis), abrasion of the eyelashes against the cornea, severe visual impairment and blindness in later life. According to recent World Health Organisation (WHO) estimates, around 165.1 million people live in trachoma-endemic areas (of whom 89% are from WHO's African region) (WHO, 2018) and 2.8 million have trichiasis (Flueckiger et al., 2018). The WHO advocates the use of the **SAFE** Strategy for trachoma control: **S**urgery to correct trichiasis, **A**ntibiotics to treat *C. trachomatis* infection, **F**acial cleanliness and **E**nvironmental improvements to suppress transmission (WHO, 1998).

Annual mass drug administration (MDA) with oral azithromycin for a minimum of 3 years is recommended for communities where the initial prevalence of the clinical sign *trachomatous inflammation-follicular* (TF) is ≥10% in children aged 1 to 9 years, with a recommended coverage of 80% of the whole community (WHO, 2004). In low-prevalence settings this usually leads to a sustained reduction in *C. trachomatis* infection prevalence over time (Pant et al., 2016, Solomon et al., 2008, Burton et al., 2010, Burton et al., 2005), however, in highly endemic areas infection can re-emerge shortly after MDA (Melese et al., 2004).

Inflammatory disease signs are reported to persist longer than infection at both the individual and population levels, resulting in the observation of clinical signs in the absence of infection (Grassly et al., 2008, Ramadhani et al., 2016b, Bailey et al., 1999, Faal et al., 2005). The correlation between clinical signs and *C. trachomatis* infection in communities prior to MDA is further reduced following treatment (Ramadhani et al., 2016b).

Previously we reported on the relationship between clinical signs, *C. trachomatis* infection and the expression of 91 immuno-fibrogenic and cell marker genes at the baseline time-point of a long-term cohort study of Tanzanian children (Ramadhani et al., 2017). We found an increase in transcripts related to Th1 and NK cell activity in individuals with *C. trachomatis* infection and an increase in matrix and fibrogenic factors in individuals with active disease in the absence of infection, supporting the findings of several earlier studies (Burton et al., 2015, Hu et al., 2012, Burton et al., 2012, Burton et al., 2004, Burton et al., 2011, Faal et al., 2006, Faal et al., 2005, Bobo et al., 1996). However, the stability of these transcriptional responses in an untreated population and the changes that might occur following MDA with azithromycin have not previously been investigated.

Azithromycin is a macrolide antibiotic which has anti-inflammatory and immunomodulatory properties via inhibition of the transcription factor Nuclear Factor Kappa-B (Stellari et al., 2014). Azithromycin has been reported *in vitro* to suppress T-cell proliferation and activation and to reduce the expression of mucins and pro-inflammatory cytokines (Ratzinger et al., 2014, Murphy et al., 2008a, Poachanukoon et al., 2014). As a result azithromycin is found to be beneficial in the treatment of diseases characterised by pathological inflammation (Giamarellos-Bourboulis, 2008). Azithromycin therefore has the potential to exert broad anti-inflammatory effects on conjunctival gene expression, independently of the clearance of *C. trachomatis*.

Here we investigate the stability of clinical signs of active trachoma, *C. trachomatis* infection and host immune responses in a cohort of Tanzanian children prior to azithromycin MDA and the changes induced following treatment.

METHODS

Ethics Statement

This study was reviewed and approved by the Ethics Committees of the Tanzania National Institute for Medical Research, Kilimanjaro Christian Medical University College and the London School of Hygiene & Tropical Medicine. The study adhered to the tenets of the Declaration of Helsinki. A field worker explained the nature of the study in detail in either Kiswahili or Maasai. Prior to enrolment of a child into this study, their parent or guardian provided written informed consent, on a consent form in Kiswahili, which was witnessed by a third person.

Study Design and Population

This study was conducted in three adjacent trachoma endemic communities in Kilimanjaro and Arusha regions, Northern Tanzania. In January 2012 we recruited a cohort of children aged 6-10 years from these communities to study the pathogenesis of trachomatous conjunctival scarring. The cohort has subsequently been followed-up every three months for four years. All children aged 6-10 years, who were normally resident in the three villages, were eligible for inclusion. We chose this restricted age group as we considered that they were more likely to show evidence of incident or progressive conjunctival scarring during the four years of the study.

The study population and participant recruitment process are described in more detail in the report of baseline (time-point 1) findings (Ramadhani et al., 2017). In brief, these villages are relatively remote, geographically neighbours and have similar patterns of life and traditions. This area is predominately inhabited by people of the Maasai ethnic group. Pastoral activities are the main occupation. The area is dry for much of the year, except for the rainy season (February to May). Water supply is therefore limited, and largely depends on a long-distance water pipe scheme from Mount Kilimanjaro. Family units are organised in *Boma*, with living huts arranged in a circle around a central animal enclosure, which is often characterised by a high density of flies.

Clinical assessments and sample collection

We visited the cohort of children every three months at their homes or schools. An experienced ophthalmic nurse examined their left eye for clinical signs of trachoma using x2.5 loupes and a bright torch. Signs were graded using the 1981 WHO 'FPC' detailed grading system (Dawson et al., 1981). This sub-divides the features into several four-point severity scales: follicles (F), papillary inflammation (P) and conjunctival scarring (C). This system corresponds to the WHO Simplified Trachoma Grading System in the following way: *Trachomatous inflammation-Follicular* (TF) is equivalent to F2/F3 and *Trachomatous inflammation-Intense* (TI) is equivalent to P3 (Thylefors et al., 1987). We followed the widely used definition of "Clinically Active Trachoma": TF and/or TI. However, for the purpose of this study, we also consider that both P2 and P3 represent clinically significant papillary inflammation, and refer to this as "TP" (Burton et al., 2015). High resolution photographs (Nikon D90 camera with 105mm Macro lens) were taken of the examined eye for independent grading.

The conjunctiva was anaesthetised with a drop of preservative-free proxymetacaine hydrochloride 0.5% w/v (Minims®, Chauvin Pharmaceuticals Ltd, Surrey, UK). Two conjunctival swab samples (Dacron polyester, Puritan Medical Products Company, Maine, USA) were collected for *C. trachomatis* detection and gene expression analysis. The swabs were passed across the upper tarsal conjunctiva four times, with a quarter turn between each pass. The first swab was placed directly into a tube containing RNAlater solution (Thermo Fisher Scientific, Massachusetts, USA) and the second into a dry tube. The samples were placed into a cool box. Later the same day the dry swab samples were stored directly at -80^{o}C and the RNAlater samples kept at 4-8^{o}C overnight and then stored at -80^{o}C.

Trachoma control measures

The SAFE Strategy is being implemented in this region of Tanzania. Community members who had trachomatous trichiasis were offered free surgery in the local health facility. Azithromycin MDA was distributed to the members of the three villages by our field team under the auspices of the Tanzanian National NTD Control Programme in the Ministry of Health and with supervision by district eye coordinators. Azithromycin, donated to the National NTD Control Programme by Pfizer through the International Trachoma Initiative, was offered to all community members over the age of 6 months. Single-dose azithromycin MDA (1g for adults and 20mg/kg for children) was distributed annually for three years. For infants under six months, tetracycline eye ointment was provided to their primary carer to be applied twice a day to both eyes for six weeks. The project team provided repeated health education messages around hygiene and sanitation. The first round of MDA was administered to all individuals in the three cohort villages immediately after time-point 3. No adverse effects were reported.

***C. trachomatis* detection**

Two protocols were used for both genomic DNA extraction and *C. trachomatis* detection. For time-point 1 samples, genomic DNA was extracted from dry swabs using the PowerSoil DNA isolation Kit (MO Bio Laboratories, California, USA) according to manufacturer's instructions. For time-points 2 to 5, genomic DNA was extracted from samples stored in RNAlater using the Norgen DNA/RNA Purification Kit (Norgen Biotek Corp, Canada) following the manufacturer's instructions.

C. trachomatis was detected in the time-point 1 samples using a droplet digital PCR assay (ddPCR) and at time-points 2 to 5 using multiplex quantitative real-time PCR (qPCR) previously evaluated against ddPCR (Ramadhani et al., 2017, Roberts et al., 2013, Butcher et al., 2017). Both assays detect chlamydial plasmid open reading frame 2 (*pORF2*), *C. trachomatis* outer membrane complex protein B (*omcB*) and human endogenous control gene ribonuclease P/MRP Subunit P30 (*RPP30*) (Butcher et al., 2017), using the same primer and probe sequences. The ddPCR reaction contained 5µl of DNA template and primers/probes at a final concentration of 0.3nM using Taqman mastermix. PCR reaction conditions were as follows: 95^{O}C for 10 minutes, then 40 cycles of 95^{O}C for 10 seconds and 60^{O}C for 30 seconds and finally 98^{O}C for 12 minutes. Droplets were then examined for fluorescence on a QX200TM Droplet Reader (Bio-Rad, UK), providing a quantitative result. The qPCR assay was performed on a ViiA7 thermal cycler (Thermo Fisher Scientific, Massachusetts, USA) using TaqMan Multiplex Master mix in a final volume of 20 µl, containing 4µl of DNA template and primers and probes each at a final concentration of 0.3nM. Cycling conditions were as follows: 95°C hold for 20 seconds followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Samples were tested in duplicate and were considered *C. trachomatis* positive if either replicate amplified *omcB* and/or *pORF2* with a cycle threshold (CT) value <40. In order to compare agreement between ddPCR and qPCR assays, Norgen-extracted DNA from time-point 2 samples (extracted from the first swab stored in RNAlater) were tested using both methods and the results are shown in Supplementary Table 1a.

Analysis of human gene expression

Total RNA was extracted from samples stored in RNAlater using the Norgen DNA/RNA Purification Kit (Norgen Biotek Corp, Canada) and reverse transcribed using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies) following the manufacturer's instructions. Relative abundance of host gene targets was quantified by real-time PCR using customized TaqMan® Microfluidic 384-well Array Cards (Thermo Fisher Scientific, Massachusetts, USA) on a ViiA7 real-time PCR machine (Thermo Fisher Scientific, Massachusetts, USA), as previously described (Ramadhani et al., 2017). A total of 46 genes of interest were selected based on our previously reported time-point 1 findings, in which we selected genes that were significantly associated with clinical signs and/or *C. trachomatis* infection status. *HPRT1* was included in each PCR run as an endogenous control gene.

Statistical analysis

Data were managed in Microsoft Access. The ΔCT method was used to adjust for the concentration of input RNA by subtracting the cycle threshold (CT) value of each gene from the CT value of *HPRT1* in the same sample (Livak and Schmittgen, 2001). The distribution of ΔCT values were plotted to assess normality. Host gene expression, *C. trachomatis* infection and clinical data were analysed in STATA v14.

For each time-point the prevalence of clinical signs and *C. trachomatis* infection was estimated and the association between infection and each of TF and TP was estimated using logistic regression. The effect of MDA on infection, TF, TP and AT (Active Trachoma) was estimated using a random effects logistic regression. Each of infection, TF, TP and AT were used as the outcome variable in four separate regressions, the observations from the first three time-points were compared with the observations from time-point 4 (the first observation after the MDA) and participant ID was included as a random effect to account for the fact that these were repeated observations within individuals. An identical analysis was repeated comparing the observations from time-point 5 to those from before MDA to assess whether the estimated effect persisted at six months post treatment.

The change in mean ΔCT value from time-point 1 was plotted for 46 genes at each of the four subsequent time-points and inspected to identify any clear differences between time-points. The change in mean ΔCT following MDA was formally tested by comparing the mean ΔCT in the first three time-points with that from the fourth time-point using a random effects linear regression, with the ΔCT value of each gene as the outcome variable, whether an observation was before or after MDA and whether individuals received treatment as the exposures of interest and participant ID was included as a random effect to again account for repeated observations of the same individuals. The relationship between pre-and post- MDA, and between treated and untreated was analysed separately. Then an interaction term was included between before/after MDA and treated/untreated variables to provide evidence of whether gene expression response after MDA was different in treated versus untreated groups. These analyses were also repeated comparing the fifth time-point with the three pre-MDA time-points to identify if the effect persisted. Gene expression was then compared at time-point 4 only between treated and untreated individuals. These analyses were initially performed unadjusted and then adjusted for infection status (clinical signs were not adjusted for as they were likely to be caused by both infection and gene expression, rather than the other way around so adjusting for these could bias our estimates).

Multivariable linear regression was used at each of the five time-points presented in this report, to test the association of each gene's expression with clinical signs and infection, adjusting for age and sex and assuming a false discovery rate (FDR) of 5% in multiple comparisons (Benjamini and Hochberg, 1995).

RESULTS

Study participants

We registered a total of 666 children aged between 6 and 10 years who were eligible for recruitment at the beginning of this study from three trachoma-endemic villages. Demography, clinical signs and infection status of the 506 participants seen at time-point 1 have previously reported in detail (Ramadhani et al., 2017). In general participants were predominantly from the Maasai ethnic group (652/666, 97.9%) with a similar number of males (332, [49.9%]) and females (334 [50.1%]) and a mean age of 7.01 years (SD 2.0) at the time of commencing the study. Following baseline recruitment new enrolment into the cohort was permitted at the second time-point. There were some few new enrolments after the second time-point, however these were not included in the analysis. At time-points 2, 3, 4, and 5 we assessed 537 (87.2%), 466 (75.6%), 467 (75.8%) and 477 (77.4m %) children, respectively. At each time-point some children were not examined due to being absent in the village, having moved away or declining to participate. MDA was offered immediately after time-point 3 to all members of the three cohort villages. The reported community-wide coverage was 68.7%. All study participants examined in time-point 3 (466) were treated except one who refused. At time-point 4, 392/466 (84.1%) of the individuals seen had been treated.

Clinical signs of trachoma

At time-point 1 the clinical signs previously reported were based on grading of conjunctival photographs, to enable subsequent comparison with the final time-point for determination of scarring incidence and progression (Ramadhani et al., 2017). However, for consistency within this analysis of the first five time-points, the field grading data was used. The agreement between field and photograph grading for time-point 1 is shown in Supplementary Table 1b. Kappa scores between field and photographs grading were 0.92 for TF and 0.68 for TP, with TP being slightly under-reported by field graders.

The prevalence of TF (F2/F3) in the first three time-points prior to MDA was fairly consistent (171/506 [33.8%], 163/537 [30.2%] and 104/467 [22.3%]), dropping to 52/467 (11.1%) and 61/479 (12.6%) post-MDA at time-points 4 and 5 (Figure 1). The prevalence of TP (P2/P3) was consistently lower than TF and also dropped substantially following MDA (Figure 1). There were no statistically significant differences between males and females in terms of the proportion showing signs of TF and/or TP at any time-point (Supplementary Table 2), with the exception of time-point 1 where there was possibly a weak association between TP and female sex.

C. trachomatis infection

The prevalence of infection was fairly consistent prior to MDA ranging from 11-16%, dropping to 1.3% three months after azithromycin MDA (time-point 4) and increasing slightly to 2.5% at time-point 5 (Figure 1). There was strong evidence for an association between *C. trachomatis* infection and clinical signs (TF, TP) at all five time-points (Table 1), with the exception of TP at time-point 5. There was a significant reduction in TF, TP and *C. trachomatis* detection in post-MDA time-points 4 and 5 relative to the combined odds at pre-MDA time-points 1-3 (Table 2). The inflammatory disease (TF and/or TI (active trachoma)) and infection status for each individual at each time-point is shown in Figure 2; participants were grouped by infection and disease status at baseline. There were no statistically significant differences between males and females in terms of the proportion testing positive for *C. trachomatis* at any time-point (Supplementary Table 2).

Conjunctival gene expression

Forty-six genes of interest were quantified in all individuals who were sampled at each of the five time-points. All amplified targets were included in the analyses. For each time-point, multivariate linear regression models were constructed for expression of each gene to investigate associations with TF, TP and *C. trachomatis* infection, adjusting for age and sex (Supplementary Table 3). The

associations between the expression of specific genes with clinical signs and *C. trachomatis* infection was similar at each time-point and was consistent with the baseline (time-point 1) report (Ramadhani et al., 2017). Briefly, in individuals with *C. trachomatis* infection, *IFNG*, *IL22*, *CCL2*, *IL12B*, *CD274*, *IL21*, *IL17A* and *SOCS1* genes were consistently the most upregulated and *S100A4*, *ALOX5*, *MMP7*, *MUC5AC*, *MUC7*, *MUC4*, *MUC1*, *CDH2* and *CDH1* genes were the most downregulated. In individuals with TF and TP, *S100A7*, *CCL18*, *MMP12*, *CXCL13*, *IL10*, *IL19*, *IL21* and *IL17A* were the most upregulated while *S100A4*, *SPARCL1*, *ALOX5* and *MUC5AC* were the most downregulated. Thus, suggesting that some of host immune responses are important in the clearance of infection, whereas others are associated with both clearance of infection and the clinical inflammation modulation.

For each target, the difference in mean gene expression (across all individuals) was calculated at each time-point relative to the mean expression at time-point 1 (Figure 3). There was only modest variability between these time-points, with the exception of time-point 4, three months after MDA, which showed marked differences compared to time-point 1 (and the other time-points). The largest increases in expression at time-point 4 relative to time-point 1 were found in *SPARCL1*, *MUC5AC*, *CDH2*, *CTGF*, *NCAM1*, *CDH1*, *MUC7*, *S100A4*, and *IL12B* (Figure 3). The largest decreases were in *S1007A*, *CCL18*, *CXCL5*, *DEFB4A*, *CXCL13*, *IL19*, *MMP12*, *IDO1*, *IL1B*, and *IL17A* (Figure 3). By time-point 5, six months after MDA, difference in mean gene expression had mostly returned to levels that were similar to those prior to treatment (Figure 3).

The change in mean ΔCT for the expression of each gene from the three time-points before MDA to the fourth time-point three months after MDA was estimated for all participants, and also separately for both individuals who received MDA and those who did not, adjusting for changes in *C. trachomatis* infection status (Table 3). This showed a significant change in mean ΔCT of most targets from before MDA compared with three months after MDA. Interestingly, this change was still observed in the untreated sub-group, albeit at a much reduced scale. The changes in mean ΔCT were larger when the analysis was not adjusted for *C. trachomatis* infection (Supplementary table 4).

To investigate the differences between the treated and untreated groups further, mean ΔCT were compared between the groups at time-point 4 only (Supplementary Table 5). This revealed only subtle differences in mean ΔCT between treated and untreated individuals. The anti-inflammatory effect of MDA on gene expression was observed even in individuals without any detectable episodes of chlamydial infection or clinical signs at any of these five time-points (Supplementary Table 6).

DISCUSSION

Infection

The prevalence of *C. trachomatis* was similar across the three time-points before MDA, suggesting that the infection prevalence was relatively stable in this antibiotic-naïve community at around 11% - 16%. There was a substantial reduction in infection prevalence at three months post-MDA, however, it rose slightly by six months post-MDA, suggesting some limited re-emergence of infection. This may be due to insufficient MDA coverage within the community, contact with individuals from surrounding untreated communities, or failure to complete the 6-week daily treatment course of tetracycline eye ointment for infants under 6 months. Members of these communities travel quite frequently to search for pastures and water for livestock, to visit markets and for social interactions with other communities. As a result, it was difficult to achieve high MDA coverage. Previous studies from Tanzania and The Gambia have also reported on the importance of contact between communities as a risk factor for reinfection following treatment (West et al., 2015, Burton et al., 2005, Burton et al., 2010).

Disease

Clinical signs prevalence was similar prior to treatment (first three time-points) suggesting stability within the community however, following MDA was strongly reduced. Clinical signs of inflammation were strongly correlated with *C. trachomatis* infection at all five time-points. In our previous systematic review and meta-analysis we found a strong correlation between TF and *C. trachomatis* infection and a moderate correlation between intense papillary inflammation (TI) and infection prior to initiation of MDA, however, after treatment the correlation was weaker for TF and no correlation was found for TI (Ramadhani et al., 2016b). Most of these earlier studies included multiple rounds of MDA and reported data several years after initiating treatment, therefore it might be too early to see this trend in our cohort. There was no consistent difference in the prevalence of clinical signs of inflammation between males and females, with the exception of the first timepoint which showed a non-significant trend of more TP in females.

Gene Expression

Our findings in this study of the associations between host gene expression, *C. trachomatis* infection and clinical signs of inflammation were consistent with previous reports from ourselves and others (Burton et al., 2015, bu El-Asrar et al., 2006, Bobo et al., 1996, Burton et al., 2004, Faal et al., 2005, Skwor et al., 2008, Burton et al., 2011). Targets that were consistently associated with clinical signs (TF/TP) at all five time-points included antimicrobial peptides (*S100A7),* pro-inflammatory cytokines and chemokines *(CCL18, CXCL13, IL10, IL19, IL21, IL17A),* matrix modifiers *(MMP12* and *SPARCL1),* epithelial-mesenchymal transition markers *(S100A4),* microbiota responses (*ALOX5)* and mucins (*MUC5AC).* Likewise, *C. trachomatis* infection was consistently associated with pro-inflammatory cytokines and chemokines *(IFNG, IL22, CCL2, IL12B, IL21, IL17A),* regulators/signalling pathways *(SOCS1, CD274), S100A4, ALOX5,* matrix modifiers *(MMP7, SPARCL1)* and mucins (*MUC7)*. We discussed the functions of these genes and their potential roles in the clearance of *C. trachomatis* infection and immunopathology in detail in our baseline paper (Ramadhani et al., 2017). The results at each of the subsequent time-points support the data from baseline, suggesting that strong *IFNG*/*IL12* responses are important in the clearance of infection, whilst Th17 cell associated cytokines and matrix factors are associated with both infection and the clinical inflammation which persists after infection has been cleared.

Large changes in gene expression were detected at time-point 4, three months after MDA with azithromycin, relative to the three time-points prior to MDA. This variation in gene expression largely returned to pre-MDA levels by time-point 5, six months post-MDA. Azithromycin appeared to have an anti-inflammatory effect on gene expression, reversing the direction of gene expression change usually associated with clinical signs and *C. trachomatis* infection. Genes normally downregulated

in individuals with *C. trachomatis* infection and/or inflammation were upregulated post-MDA (*SPARCL1*, *MUC5AC*, *CDH2*, *CTGF*, *NCAM1*, *CDH1*, *S100A4*, *MUC7*, and *FGF2*), whilst genes normally upregulated (*S100A7*, *CCL18*, *CXCL5*, *CXCL13*, *IL19*, *IDO1*, *MMP12*, *IL17A*, *IL1B* and *IL21*) were strongly downregulated post-MDA. The effect was greatest when *C. trachomatis* infection was not adjusted for, however, the effect was still large after adjustment for infection, suggesting that azithromycin has an immunomodulatory effect on gene expression that is independent of the concurrent reduction in infection. This effect was also seen in individuals without any episodes of *C. trachomatis* infection and/or clinical signs of inflammation across all 5 time-points, supporting this hypothesis. However, we cannot exclude the possibility that azithromycin treatment reduced ocular infections with other sub-clinical or mild inflammation-causing organisms in these individuals. Interestingly, a change in mean gene expression post-MDA was also observed in individuals who did not receive treatment. This could be due to a reduction in transmission and therefore exposure to *C. trachomatis* and other infectious organisms within the community as a whole.

Azithromycin has previously been reported to have anti-inflammatory effects in humans, animal and *in vitro* models, leading to improved clinical outcomes through a combined approach of clearing infection and reducing pathological host inflammatory responses (Amsden, 2005). One pre-surgical dose of azithromycin reduced the level of pro-inflammatory cytokines and chemokines detected in oral fluid 6 days following dental implant surgery relative to amoxicillin (Escalante et al., 2015). Relative to other non-macrolide antibiotics, azithromycin reduced levels of IL-6, IL-8, TNF-α and GM-CSF proteins in individuals with pneumonia and rhinovirus infections (Lorenzo et al., 2015, Gielen et al., 2010, Murphy et al., 2008b). MMP9 expression was reduced in the airways of lung transplanted individuals treated with azithromycin between 3 and 6 months (Verleden et al., 2011), and in an experimental laminectomy model in rats, azithromycin was associated with reduction of fibrosis and inflammatory cell density six weeks after administration (Emmez et al., 2011). Immunomodulatory effects of azithromycin are thought to be enhanced by its long half-life in tissue, lasting for several weeks (Langtry and Balfour, 1998, Leach et al., 1997). In addition to localised anti-inflammatory effects, one round of azithromycin, administered for trachoma control, was associated with a large reduction in infectious and all-cause childhood mortality (Keenan et al., 2011); a finding which was reinforced by a large multi-country placebo-controlled clinical trial (Keenan et al., 2018). Our findings of an immunomodulatory effect of azithromycin are therefore consistent with published evidence and suggest that MDA for trachoma control may have an additional protective effect through a systemic reduction in inflammation.

This study has several limitations. It was only feasible to sample one eye from study participants, thus only the left eye was examined and sampled throughout the longitudinal study. The age range of study participants was limited due to the study design of the overall longitudinal study, which this investigation was nested within. The method of *C. trachomatis* detection was changed after the first time-point, which could introduce inconsistencies between the infection results of the first relative to later time-points. Agreement between the two methods used was however, deemed acceptable (Supplementary table 1a). The infection loads of discrepant results were very low and at around the limit of detection. Given the large sample size and the use of three pre-MDA time-points, this variation is not expected to significantly alter the results or their interpretation.

Conclusions

We present evidence that one round of oral azithromycin treatment exerted a strong anti-inflammatory effect on conjunctival gene expression, detectable three months following treatment but mostly returning to background levels by six months. This effect was also observed in individuals without *C. trachomatis* infection or active trachoma across all five time-points, indicating that the immunomodulatory effect was at least in part independent of the reduction of *C. trachomatis* infection. Interestingly, a reduced effect was also seen in individuals who did not receive treatment, which could reflect a community reduction in infection transmission and exposure. A limitation of

this study is that we cannot determine whether this effect is mediated directly through inhibition of pro-inflammatory intracellular signalling molecules, through reductions in concurrent, sub-clinical infections, and/or through reduction of infection exposure, and future work should seek to understand these mechanisms. Conjunctival papillary inflammation is a significant risk factor for scarring progression (Ramadhani et al., 2016a), therefore the anti-inflammatory effect of azithromycin might have therapeutic potential in limiting the development of disease sequelae, that goes beyond its effect on the prevalence of ocular *C. trachomatis* infection.

Figure 1. **The prevalence of *C. trachomatis* infection and clinical signs before and after MDA.**

Figure 2. **The clinical disease (Active Trachoma, TF and/or TI) and infection status of individuals at each time-point, ordered by status at baseline.** Each row represents an individual and each column represents a time-point.

Figure 3. Stability of conjunctival gene expression. The difference in mean ΔCT value (across all individuals) for each gene at time-points 2, 3, 4 and 5 is shown relative to time-point 1. Values are adjusted for *C. trachomatis* infection and clinical phenotypes and are ordered by difference in mean gene expression at Time-point 4.

Table 1. The association between *C. trachomatis* detection and clinical signs by time-point. Clinical signs are based on field grading from all five time-points.

Time-point		Follicular Inflammation (TF)		Papillary inflammation (TP)	
		No TF (%)	**TF (%)**	**No TP (%)**	**TP (%)**
1 (n=506) Jan/Feb	Ct infected (%)	18/335 (5.4)	60/171 (35.1)	38/407 (9.3)	40/99 (40.4)
	OR (95%CI)	-	9.52 (5.4-16.8)	-	6.58 (3.9-11.1)
	p-value	-	<0.0001	-	<0.0001
2 (n=536) Apr/May	Ct infected (%)	29/374 (7.8)	53/162 (32.7)	38/429 (8.9)	44/107 (41.1)
	OR (95%CI)	-	5.78 (3.5-9.5)	-	7.19 (4.3-12.0)
	p-value	-	<0.0001	-	<0.0001
3 (n=466) Aug/Sept	Ct infected (%)	19/362 (5.3)	35/104 (33.7)	30/398 (7.5)	24/68 (35.3)
	OR (95%CI)	-	9.16 (4.9-16.9)	-	6.69 (3.6-12.5)
	p-value	-	<0.0001	-	<0.0001
Azithromycin MDA					
4 (n=467) November	Ct infected (%)	3/415 (0.7)	3/52 (5.8)	4/457 (0.9)	2/10 (20.0)
	OR (95%CI)	-	8.41 (1.7-42.8)	-	28.31 (4.5-177.5)
	p-value	-	0.010	-	<0.0001
5 (n=477) Jan/Feb	Ct infected (%)	4/417 (1.0)	8/60 (13.1)	10/442 (2.3)	2/35 (5.7)
	OR (95%CI)	-	15.88 (4.6-54.5)	-	2.62 (0.6-12.4)
	p-value	-	<0.0001	-	0.226

Table 2. Comparison of *C. trachomatis* infection and disease signs between the pre-treatment (odds of Combined time-points 1, 2 and 3) to the post-treatment time-points (4 and 5 analysed separately). Clinical signs are based on field grading from all five time-points.

		Infection	***TF***	***TP***	***Active Trachoma***
Odds of combined pre-MDA time-points vs. time-point 4 (3 months post-MDA)	OR	0.01	0.10	0.03	0.08
	(95%CI)	(0.004-0.04)	(0.06-0.17)	(0.01-0.07)	(0.05-0.13)
	p-value	<0.0001	<0.0001	<0.0001	<0.0001
Odds of combined pre-MDA time-points vs. time-point 5 (6 months post-MDA)	OR	0.04	0.15	0.18	0.14
	(95%CI)	(0.02-0.10)	(0.10-0.23)	(0.11-0.30)	(0.10-0.22)
	p-value	<0.0001	<0.0001	<0.0001	<0.0001

Table 3. Estimated Fold Changes (FC) with their respective p-values comparing the expression of each gene between the combined first three time-points (1, 2 and 3) before MDA and time-point 4 (three months after MDA), adjusted for *C. trachomatis* infection. A FC of >1 indicates increased expression of the gene at time-point 4. Random effects multivariable linear regression of all individuals (first panel), untreated only (second panel) and treated only (third panel). The final column (p-value for interaction) provides evidence as to whether the fold change from before to after MDA is different in the treated and untreated groups. Results are ordered by FC of ''**All**'' individuals. The Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control the false discovery rate <5%, only tests with a p-value <0.034 are considered statistically significant.

Target	All		Untreated Only		Treated Only		p-value for interaction
	FC	p-value	FC	p-value	FC	p-value	
SPARCL1	11.89	7.76 x10^-186	6.81	9.04 x10^-19	13.13	2.54 x10^-173	0.005
MUC5AC	4.02	3.95 x10^-145	2.89	1.20 x10^-14	4.26	5.35 x10^-136	0.009
CDH2	3.42	2.97 x10^-141	2.67	9.16 x10^-16	3.58	3.30 x10^-130	0.026
NCAM1	2.23	9.10 x10^-89	1.83	1.95 x10^-9	2.31	8.32 x10^-83	0.031
CTGF	2.20	3.57 x10^-96	2.25	1.27 x10^-17	2.20	4.10 x10^-81	0.818
MUC7	1.89	7.10 x10^-29	2.17	1.01 x10^-7	1.84	2.24 x10^-23	0.291
S100A4	1.82	7.83 x10^-79	1.75	1.56 x10^-12	1.83	5.50 x10^-69	0.612
CDH1	1.72	1.23 x10^-80	1.69	9.80 x10^-14	1.72	2.55 x10^-69	0.808
FGF2	1.50	2.86 x10^-9	1.48	0.019	1.50	4.19 x10^-8	0.934
SERPINB4	1.43	1.91 x10^-3	1.65	0.067	1.39	0.009	0.570
IL12B	1.42	5.47 x10^-15	1.49	4.10 x10^-4	1.41	1.97 x10^-12	0.657
TGFB1	1.34	2.53 x10^-45	1.43	4.80 x10^-12	1.33	6.55 x10^-36	0.178
ALOX5	1.29	7.49 x10^-39	1.37	7.71 x10^-11	1.27	1.24 x10^-30	0.133
MUC1	1.24	8.04 x10^-17	1.35	4.05 x10^-6	1.22	9.95 x10^-13	0.161
NCR1	1.23	2.51 x10^-10	1.25	0.007	1.23	7.76 x10^-9	0.835
PDGFB	1.22	7.96 x10^-14	1.35	1.26 x10^-5	1.20	2.59 x10^-10	0.130
GAPDH	1.20	5.05 x10^-11	1.49	1.68 x10^-8	1.16	1.33 x10^-6	0.001
SOCS1	1.20	2.64 x10^-8	1.49	5.69 x10^-7	1.15	6.66 x10^-5	0.003
MUC4	1.16	1.80 x10^-7	1.34	6.1 x10^-5	1.13	6.01 x10^-5	0.034
VIM	1.15	9.83 x10^-10	1.22	4.08 x10^-4	1.14	2.11 x10^-7	0.242
CD247	1.01	0.757	1.10	0.196	0.99	0.856	0.205
MMP9	0.95	0.242	0.98	0883	0.95	.0224	0.731
IFNG	0.95	0.219	0.93	0.521	0.95	0.293	0.860
MMP7	0.93	0.033	1.08	0.441	0.90	0.009	0.086
IL23A	0.88	5.88 x10^-4	1.00	0.989	0.86	1.73 x10^-4	0.127
IL22	0.83	0.020	1.26	0.232	0.76	0.002	0.016
IL6	0.83	5.24 x10^-4	0.83	0.150	0.83	0.001	0.959
CCL20	0.82	3.80 x10^-6	0.92	0.447	0.80	2.29 x10^-6	0.240
IL8	0.72	1.42 x10^-18	0.72	4.81 x10^-4	0.72	4.02 x10^-16	0.980
PTGS2	0.70	1.97 x10^-17	0.79	0.02.52	0.69	8.27 x10^-17	0.210
DUOX2	0.69	7.01 x10^-25	0.87	0.134	0.67	1.81 x10^-26	0.006
IL10	0.67	7.68 x10^-24	0.80	0.026	0.64	6.84 x10^-24	0.047
MZB1	0.65	3.20 x10^-17	0.71	0.008	0.64	6.23 x10^-16	0.466
CD274	0.63	6.37 x10^-37	0.77	0.004	0.61	1.28 x10^-36	0.018
CCL2	0.60	2.63 x10^-22	0.63	5.52 x10^-4	0.59	5.94 x10^-20	0.617
SOCS3	0.59	1.13 x10^-38	0.76	0.006	0.57	5.67 x10^-39	0.007
IL1B	0.52	1.78 x10^-43	0.61	2.81 x10^-5	0.50	1.55 x10^-40	0.140
IL21	0.51	3.18 x10^-24	0.72	0.044	0.48	1.03 x10^-24	0.022
IL17A	0.48	6.01 x10^-44	0.64	7.36 x10^-4	0.46	2.57 x10^-43	0.017
IDO1	0.44	6.25 x10^-90	0.68	1.69 x10^-4	0.41	4.74 x10^-93	9.55 x10^-6
MMP12	0.43	9.98 x10^-70	0.56	1.87 x10^-6	0.41	6.82 x10^-67	0.018
IL19	0.40	5.00 x10^-63	0.55	1.42 x10^-5	0.37	4.32 x10^-61	0.012
CXCL5	0.39	2.79 x10^-65	0.61	3.88 x10^-4	0.36	6.18 x10^-66	7.64 x10^-4
DEFB4A	0.38	1.32 x10^-64	0.52	1.16 x10^-5	0.36	1.94 x10^-62	0.015
CXCL13	0.38	1.19 x10^-47	0.50	3.58 x10^-5	0.36	3.28 x10^-45	0.067
CCL18	0.33	8.10 x10^-49	0.47	5.75 x10^-5	0.31	7.95 x10^-47	0.032
S100A7	0.28	4.90 x10^-55	0.53	0.002	0.25	3.61 x10^-56	7.03 x10^-4

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception: MB, MH, DCM, and RB.
Design of the work: AR, TD, DM, PM, AM, KM, TM, WM, CR, MH and MB
Analysis: AR, TD, DM and MB
Interpretation of data for the work: AR, TD, DM, MH, and MB
Drafting the work: AR, TD, DM and MB.
Revising it critically for important intellectual content: AR, TD, DM, PM, AM, KM, TM, WM, CR, RB, DCM, MH and MB
Final approval of the version to be published: AR, TD, DM, PM, AM, KM, TM, WM, CR, RB, DCM, MH and MB
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: AR, TD, DM, PM, AM, KM, TM, WM, CR, RB, DCM, MH and MB

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CONFLICT OF INTEREST

Authors declared there was no conflict of interest.

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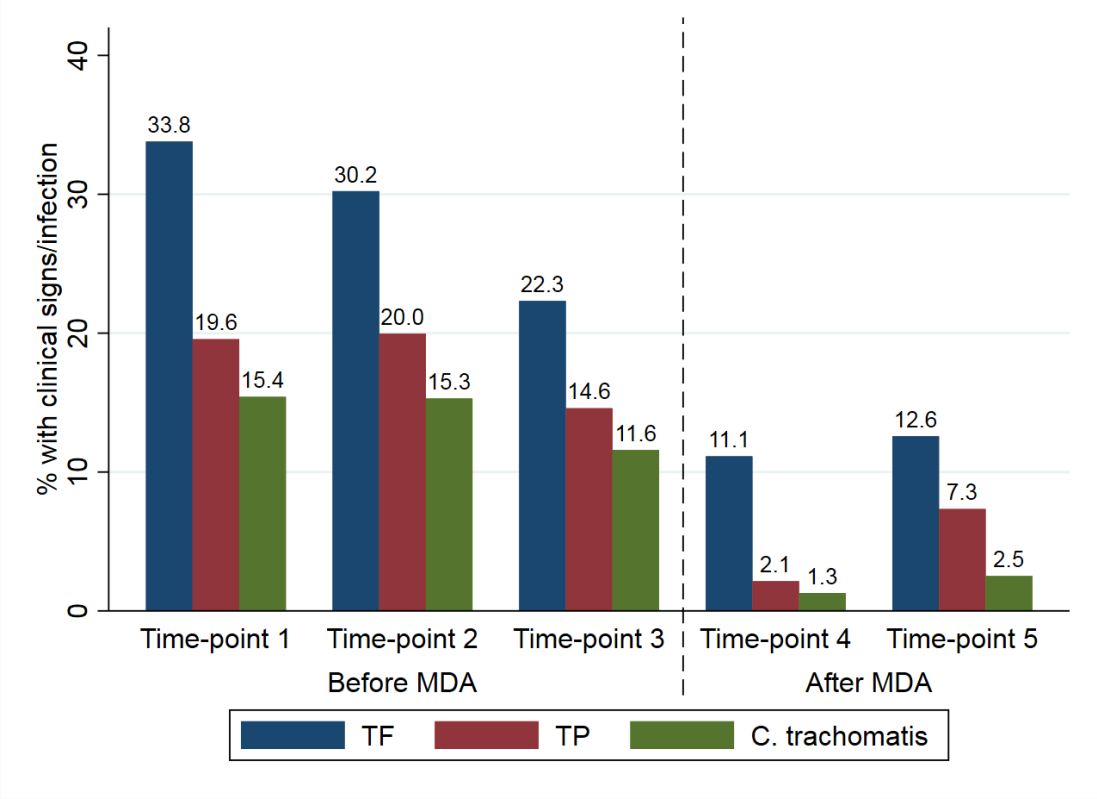
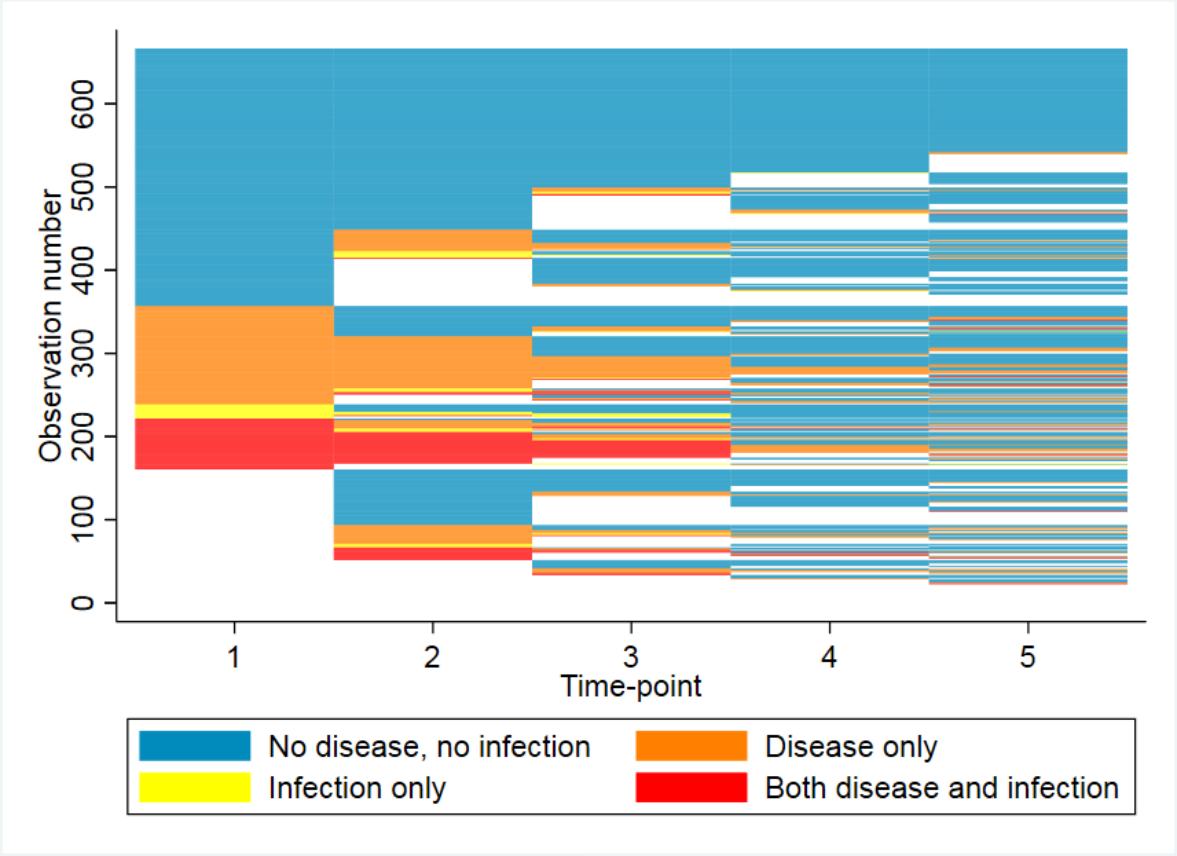
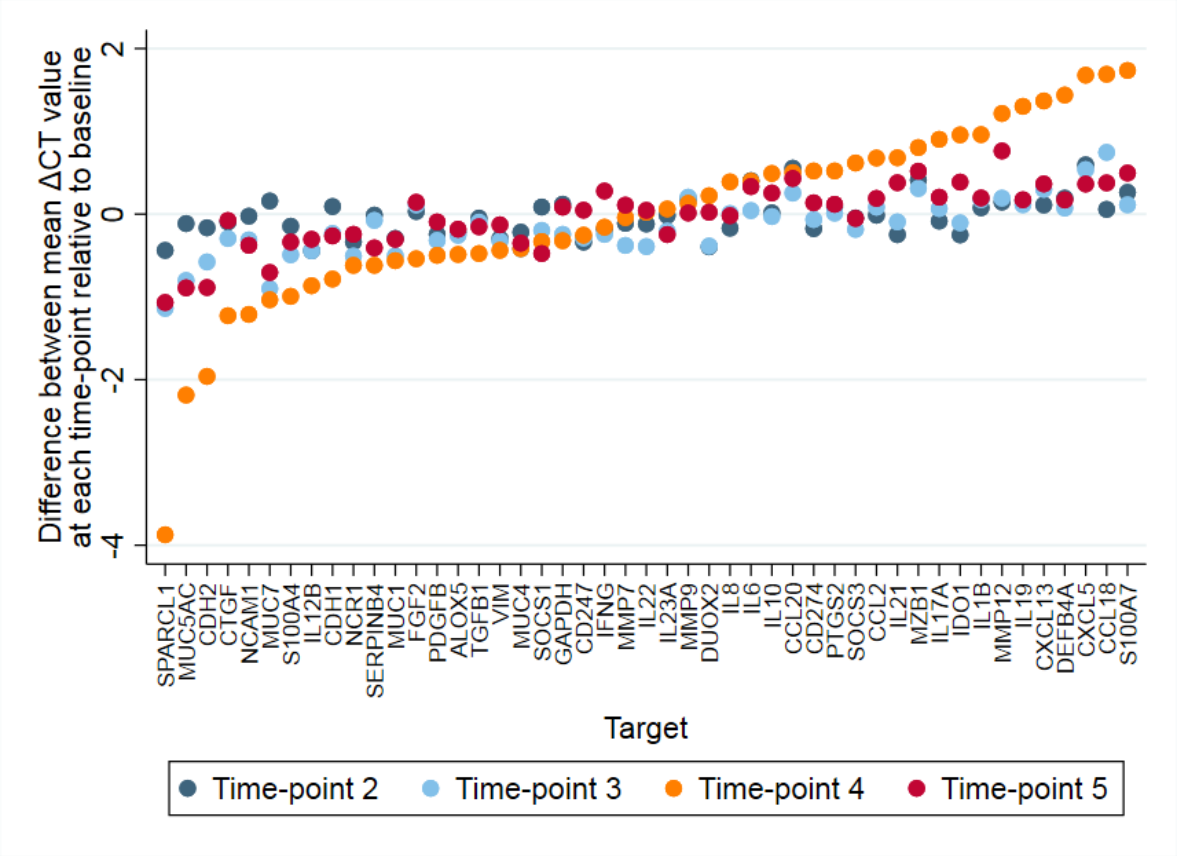
WHO 2018. Weekly Epidemiological Record. ***WHO,*** **93, 12.**


% with clinical signs/infection
0
10
20
30
40
33.8
19.6
15.4
30.2
20.0
15.3
22.3
14.6
11.6
11.1
2.1
1.3
12.6
7.3
2.5
Time-point 1
Time-point 2
Time-point 3
Time-point 4
Time-point 5
Before MDA
After MDA
TF
TP
C. trachomatis


Observation number
0
100
200
300
400
500
600
1
2
3
4
5
Time-point
No disease, no infection
Disease only
Infection only
Both disease and infection



Target description:

Antimicrobial Peptides: *DEFB4A, S100A7*
Cytokines/Chemokines: *CCL2, CXCL5, CXCL13, CCL18, CCL20, IDO1, IFNG, IL1B, IL6, IL8, IL10, IL12B, IL17A, IL19, IL21, IL22, IL23A, PTGS2,*
EMT Markers: *CDH1, CDH2, S100A4 and VIM,*
Matrix Modifiers: *CTGF-1, FGF2, MMP7, MMP9, MMP12, PDGFB, SPARCL1 and TGFβ1*
Microbiota Response: *ALOX5, DUOX2*
Mucins: *MUC1, MUC4, MUC5AC, MUC7*
NK Cell Markers: CD247, *NCAM1 and NCR1*
Regulators/Signalling Pathways: *CD274, MZB1, SERPINB4, SOCS1 and SOCS3*

Supplementary Table 1a. **2 X 2 table showing agreement between qpcr and ddpcr assays for *c. trachomatis* detection in dna extracted from conjunctival swabs at time-point 2.**

		ddPCR		
		+	-	Total
qPCR	+	71 (13.6%)	7 (1.3 %)	78
	-	15 (2.9%)	430 (82.2%)	445
	Total	86	437	523

Sensitivity = 82.6% (95% CI 72.8 – 89.9), Specificity = 96.7% (95% CI 96.7 -99.4), NPV = 96.6% (95% CI 94.8 – 97.6%); PPV = 91.0% (95% CI 82.9 – 95.5%); Cohens Kappa = 0.84, Accuracy: overall probability that a sample will be correctly classified 95.8% (95% CI 93.7 – 97.4),for these samples at this prevalence (16.4%) with ddPCR as the reference standard.

Supplementary Table 1b. Agreement between field and photo grading at baseline (time-point 1) for follicular and papillary inflammation in the conjunctiva. Kappa scores between field and photographs grading were 0.92 for TF and 0.68 for TP.

Field Grading	Photo Grading			
	No TF (%)	***TF (%)***		
No TF	317 (94.6%)	18 (5.4%)		
TF	19 (11.1%)	152 (88.9%)		
	No TP (%)	***TP (%)***		
No TP	345 (84.8%)	62 (15.2%)		
TP	1 (1.0%)	98 (99.0%)		
	F0 (%)	***F1 (%)***	***F2 (%)***	***F3 (%)***
F0	140 (96.6%)	4 (2.8%)	1 (0.7)	0 (0.0%)
F1	61 (32.1%)	112 (59.0%)	17 (9.0%)	0 (0.0%)
F2	0 (0.0%)	16 (21.3%)	49 (65.3%)	10 (13.3%)
F3	0 (0.0%)	3 (3.1%)	14 (14.6%)	79 (82.3%)
	P0 (%)	***P1 (%)***	***P2 (%)***	***P3 (%)***
P0	173 (60.5%)	99 (34.6%)	13 (4.6%)	1 (0.4%)
P1	6 (5.0%)	67 (55.4%)	41 (33.9%)	7 (5.8%)
P2	1 (1.9%)	0 (0.0%)	38 (70.4%)	15 (27.8%)
P3	0 (0.0%)	0 (0.0%)	4 (8.9%)	41 (91.1%)

S2 Table. The relationship between sex and (i) clinical signs (from field grading) and (ii) *c. trachomatis* infection of each of the 5 time-points. The number of individuals with each clinical phenotype or infection is shown as a proportion of the total number of males and females at each time-point. Associations between sex and clinical phenotypes or infection were tested using logistic regression.

Time-point 1				Time-point 2				Time-point 3				MDA	Time-point 4				Time-point 5			
Male (%)	**Female (%)**	**OR (95%CI)**	**p-value**	**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**	**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**		**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**	**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**
Follicular Trachoma (TF)																				
77/251 (30.7)	94/255 (36.9)	1.32 (0.9-1.9)	0.142	72/258 (27.9)	90/278 (32.4)	1.24 (0.9-1.8)	0.261	44/218 (20.2)	60/248 (24.2)	1.28 (0.8-2.0)	0.278		23/216 (10.7)	29/251 (11.6)	1.10 (0.6-2.0)	0.756	27/229 (11.8)	34/248 (13.7)	1.19 (0.7-2.0)	0.531
Papillary Inflammation (TP)																				
41/251 (16.3)	58/255 (26.8)	1.51 (1.0-2.4)	0.070	45/258 (17.4)	63/278 (22.7)	1.39 (0.9-2.1)	0.133	28/218 (12.8)	41/248 (16.5)	1.34 (0.8-2.3)	0.264		6/216 (2.8)	4/251 (1.6)	0.57 (0.2-2.0)	0.384	12/229 (5.2)	23/248 (9.3)	1.85 (0.9-3.8)	0.096
Chlamydia trachomatis																				
34/252 (13.5)	44/255 (17.3)	1.34 (0.8-2.2)	0.241	32/258 (12.4)	48/278 (17.3)	1.47 (0.9-2.4)	0.116	31/218 (14.2)	24/248 (9.7)	0.65 (0.4-1.1)	0.131		1/216 (0.5)	4/251 (1.60)	3.48 (0.4-31)	0.266	6/229 (2.6)	6/248 (2.4)	0.92 (0.3-2.9)	0.889

Supplementary Table 3: Multivariable linear regression models for conjunctival gene expression associated with clinical signs, *C. trachomatis* , female sex and age. FC = fold change. Using the Benjamini and Hochberg approach to adjust for multiple comparisons, in order to control the false discovery rate <5% only tests with a p-value below 0.027 are considered statistically significant.

	Time-point 1										Time-point 2									
	TF		TP		*C. trachomatis*		Sex		Age		TF		TP		*C. trachomatis*		Sex		Age	
Target	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value
Antimicrobial Peptides																				
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	1.21	0.314	2.2	0.000	1.67	0.024	1.51	0.005	0.89	0.002	1.28	0.089	2.29	0.000	1.89	0.001	1.37	0.010	0.86	0.000
Psoriasin-1 (*S100A7*)	2.41	0.001	3.68	0.000	2.01	0.024	1.6	0.021	0.86	0.004	2.03	0.000	5.25	0.000	2.12	0.002	1.52	0.009	0.79	0.000
Cytokines/Chemokines																				
Chemokine ligand 2 (*CCL2*)	1.49	0.019	2.46	0.000	3.52	0.000	1.31	0.045	0.86	0.000	1.5	0.002	2.52	0.000	3.85	0.000	1.17	0.151	0.86	0.000
Chemokine ligand 5 (*CXCL5*)	1.6	0.019	2.18	0.001	0.97	0.889	0.94	0.697	0.9	0.008	1.76	0.000	2.1	0.000	1.53	0.034	1.04	0.791	0.88	0.000
Chemokine ligand 13 (*CXCL13*)	2.13	0.000	2.7	0.000	2.5	0.000	1.4	0.039	0.86	0.000	2.3	0.000	3.33	0.000	2.79	0.000	1.35	0.037	0.83	0.000
Chemokine ligand 18 (*CCL18*)	1.79	0.004	2.95	0.000	2.25	0.001	1.11	0.521	0.86	0.000	2.2	0.000	4.28	0.000	2.26	0.000	1.07	0.660	0.81	0.000
Chemokine ligand 20 (*CCL20*)	1.76	0.000	1.61	0.004	1.47	0.028	1.12	0.338	0.94	0.048	1.66	0.000	1.52	0.001	1.86	0.000	1.11	0.272	0.9	0.000
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	1.5	0.013	1.73	0.003	2.33	0.000	1.54	0.001	0.88	0.000	1.41	0.014	1.53	0.008	2.58	0.000	1.5	0.001	0.83	0.000
Interferon gamma(*IFNG*)	1.67	0.000	1.36	0.055	7.95	0.000	1.5	0.000	0.9	0.001	1.77	0.000	1.73	0.000	7.19	0.000	1.28	0.011	0.88	0.000
Interleukin 1 beta(*IL1B*)	1.54	0.011	2.09	0.000	1.89	0.002	1.32	0.041	0.9	0.003	1.44	0.005	1.57	0.002	2.46	0.000	1.07	0.531	0.88	0.000
Interleukin 6 (*IL6*)	1.32	0.077	1.82	0.001	1.2	0.000	1.07	0.589	0.93	0.020	1.5	0.001	1.43	0.012	2.46	0.000	1	0.982	0.91	0.000
Interleukin 8 (*IL8*)	1.25	0.085	1.76	0.000	1.55	0.006	1.16	0.154	0.96	0.140	1.38	0.005	1.88	0.000	1.53	0.003	1.04	0.691	0.92	0.001
Interleukin 10 (*IL10*)	1.51	0.003	2.03	0.000	2.12	0.000	1.32	0.012	0.91	0.001	1.72	0.000	2.31	0.000	2.29	0.000	1.15	0.150	0.91	0.000
Interleukin 12 beta (*IL12B*)	1.69	0.000	1.43	0.005	3.71	0.000	1.37	0.001	0.97	0.154	1.36	0.000	1.32	0.019	4.99	0.000	1.11	0.214	0.94	0.007
Interleukin 17A (*IL17A*)	1.73	0.001	1.82	0.001	2.81	0.000	1.37	0.014	0.87	0.000	2.03	0.000	2.23	0.000	2.95	0.000	1.39	0.005	0.87	0.000
Interleukin 19 (*IL19*)	1.56	0.018	2.73	0.000	2.63	0.000	1.65	0.001	0.85	0.000	1.79	0.000	3.04	0.000	2.82	0.000	1.54	0.001	0.84	0.000
Interleukin 21 (*IL21*)	2.22	0.000	1.97	0.000	3.75	0.000	1.37	0.017	0.89	0.001	2.08	0.000	2.66	0.000	3.51	0.000	1.28	0.063	0.88	0.000
Interleukin 22 (*IL22*)	1.31	0.114	1.83	0.001	7.29	0.000	1.4	0.015	0.92	0.020	1.48	0.021	1.71	0.004	6.99	0.000	1.26	0.099	0.98	0.497
Interleukin 23A (*IL23A*)	1.49	0.005	1.98	0.000	2.15	0.000	1.15	0.219	0.92	0.002	1.45	0.000	1.7	0.000	2.47	0.000	1.19	0.040	0.9	0.000
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	1.4	0.025	1.78	0.001	1.47	0.033	1.06	0.534	0.96	0.137	1.34	0.006	1.62	0.000	1.68	0.000	0.97	0.748	0.94	0.007
EMT Markers																				
Epithelial cadherin (*CDH1*)	1.22	0.060	1.28	0.034	0.8	0.081	1.05	0.562	0.99	0.762	1.01	0.901	1.12	0.220	1.1	0.364	1.07	0.295	0.98	0.189
Neuronal cadherin (*CDH2*)	0.97	0.787	1.15	0.295	1.29	0.072	1.03	0.271	1.11	0.218	1.01	0.916	1.04	0.756	1.51	0.002	1.13	0.168	1.03	0.279
S100 calcium binding protein A4 (*S100A4*)	1.08	0.523	1.05	0.755	0.49	0.000	0.98	0.846	1.05	0.061	0.82	0.020	0.81	0.036	0.74	0.007	1.04	0.595	1.02	0.371
Vimentin (*VIM*)	1.52	0.002	1.42	0.019	1.65	0.002	1.02	0.845	1.01	0.836	1.31	0.001	1.43	0.000	2.05	0.000	1.12	0.095	0.93	0.000
Matrix Modifiers																				
Connective tissue growth factor (*CTGF-1*)	1.05	0.701	1.2	0.190	1.47	0.009	0.87	0.142	1.01	0.712	0.97	0.735	1.25	0.023	1.24	0.047	0.91	0.171	1	0.992
Fibroblast growth factor 2 (basic) (*FGF2*)	0.9	0.447	1.83	0.000	1.77	0.001	1.03	0.818	0.99	0.658	1.03	0.823	1.69	0.001	1.71	0.001	0.95	0.696	0.97	0.320
Matrix metallopeptidase 7 (*MMP7*)	1.46	0.009	1.43	0.029	0.46	0.000	1.02	0.849	0.94	0.042	1.17	0.225	1.36	0.032	0.61	0.002	1.06	0.576	0.93	0.006
Matrix metallopeptidase 9 (*MMP9*)	1.91	0.000	2.01	0.000	2.06	0.000	1.14	0.251	0.95	0.064	1.67	0.000	1.54	0.001	2.72	0.000	1.13	0.192	0.92	0.001
Matrix metallopeptidase 12 (*MMP12*)	1.78	0.000	2.16	0.000	2.27	0.000	1.28	0.052	0.92	0.013	1.92	0.000	2.59	0.000	2.67	0.000	1.17	0.165	0.88	0.000
Platelet-derived growth factor beta polypeptide (*PDGFB*)	1.3	0.012	1.5	0.001	1.7	0.000	1.2	0.011	0.9	0.010	1.29	0.002	1.56	0.000	1.93	0.000	1.15	0.029	0.93	0.000
SPARC-like 1 (hevin) (*SPARCL1*)	0.57	0.004	0.54	0.006	0.52	0.006	0.77	0.095	1.18	0.000	0.51	0.000	0.43	0.000	0.47	0.001	0.88	0.384	1.18	0.000
Transforming growth factor, beta 1 (*TGFβ1*)	1.3	0.012	1.25	0.067	1.51	0.001	1.14	0.109	0.98	0.274	1.21	0.013	1.19	0.048	1.76	0.000	1.06	0.371	0.94	0.000
Microbiota Response																				
Arachidonate 5-lipoxygenase (*ALOX5*)	1.17	0.157	1.21	0.125	0.85	0.217	1.08	0.351	0.1	0.893	1.04	0.628	1.1	0.301	1.02	0.848	1.04	0.551	0.95	0.004
Dual oxidase 2 (*DUOX2*)	1.2	0.206	1.71	0.001	1.58	0.010	1.36	0.007	0.91	0.001	1.28	0.028	1.56	0.001	1.58	0.001	1.39	0.000	0.88	0.000
Mucins																				
Mucin 1, cell surface associated (*MUC1*)	1.15	0.227	1.28	0.056	1.08	0.602	1.11	0.220	0.97	0.264	1.12	0.154	1.27	0.012	1.14	0.211	1.12	0.087	0.95	0.003
Mucin 4, cell surface associated (*MUC4*)	1.22	0.089	1.55	0.001	0.83	0.182	1	0.988	0.96	0.056	1.05	0.615	1.32	0.008	1.02	0.856	1.16	0.046	0.91	0.000
Mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*)	0.86	0.266	0.94	0.679	0.71	0.038	1.09	0.437	1.04	0.111	0.87	0.207	0.85	0.214	0.73	0.026	1.04	0.634	1.06	0.009
Mucin 7, secreted (*MUC7*)	0.98	0.902	0.9	0.555	0.52	0.000	0.66	0.001	1.08	0.014	0.79	0.065	0.76	0.053	0.64	0.005	0.77	0.012	1.06	0.021
NK Cell Markers																				
CD247 molecule (CD247)	1.41	0.003	1.32	0.031	2.15	0.000	1.2	0.049	1	0.212	1.32	0.001	1.51	0.000	2.14	0.000	1.12	0.106	0.93	0.000
Neural cell adhesion molecule 1 (*NCAM1*)	0.9	0.413	1.12	0.435	2.13	0.000	1.14	0.210	1.01	0.702	1.02	0.832	1.04	0.683	2.01	0.000	1.08	0.268	0.98	0.240
Natural cytotoxicity triggering receptor 1 (*NCR1*)	1.4	0.003	1.33	0.023	2.86	0.000	1.28	0.005	0.94	0.007	1.43	0.000	1.41	0.001	2.86	0.000	1.22	0.009	0.94	0.001
Regulators/Signalling Pathways																				
CD274 molecule (*CD274*)	1.37	0.029	1.64	0.003	3.36	0.000	1.31	0.020	0.9	0.019	1.46	0.000	1.81	0.000	3.42	0.000	1.18	0.053	0.89	0.000
Marginal zone B and B1 cell-specific protein (*MZB1*)	1.76	0.001	1.33	0.117	2.61	0.000	1.29	0.048	0.96	0.201	1.51	0.008	2.84	0.000	2.92	0.000	1.34	0.020	0.89	0.000
Serpin peptidase inhibitor clade B member 4, (*SERPINB4*)	1.08	0.739	3.72	0.000	5.1	0.000	1.05	0.811	0.97	0.564	1.03	0.878	2.94	0.000	5.77	0.000	1.12	0.514	0.9	0.018
Suppressor of cytokine signalling 1 (*SOCS1*)	1.55	0.000	1.48	0.001	2.26	0.000	1.18	0.057	0.94	0.004	1.36	0.000	1.5	0.000	2.76	0.000	1.1	0.191	0.92	0.000
Suppressor of cytokine signalling 3 (*SOCS3*)	1.56	0.002	1.89	0.000	1.35	0.079	1.17	0.169	0.93	0.015	1.39	0.003	1.6	0.000	1.85	0.000	1.08	0.422	0.88	0.000

	Time-point 3										Time-point 4									
	TF		TP		*C. trachomatis*		Sex		Age		TF		TP		*C. trachomatis*		Sex		Age	
Target	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value
Antimicrobial Peptides																				
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	1.12	0.567	1.84	0.005	2.23	0.001	1.38	0.018	0.87	0.000	3.01	0.000	1.64	0.400	3.04	0.135	1.23	0.224	0.91	0.026
Psoriasin-1 (*S100A7*)	1.58	0.080	4.18	0.000	3.34	0.000	1.38	0.080	0.78	0.000	3.01	0.000	2.08	0.154	10.81	0.000	1.13	0.391	0.87	0.000
Cytokines/Chemokines																				
Chemokine ligand 2 (*CCL2*)	1.42	0.039	2.28	0.000	4.1	0.000	1.2	0.124	0.84	0.000	2.43	0.000	1.17	0.646	5.45	0.000	0.94	0.525	0.91	0.000
Chemokine ligand 5 (*CXCL5*)	1.19	0.387	1.72	0.019	2.03	0.004	1.04	0.771	0.82	0.000	4.38	0.000	1.5	0.462	2.51	0.185	1.06	0.731	0.88	0.001
Chemokine ligand 13 (*CXCL13*)	1.68	0.024	2.69	0.000	2.94	0.000	1.43	0.027	0.78	0.000	3.59	0.000	1.41	0.477	10.61	0.000	1.15	0.297	0.84	0.000
Chemokine ligand 18 (*CCL18*)	1.63	0.033	2.92	0.000	3.61	0.000	1.18	0.292	0.83	0.000	2.41	0.000	1.22	0.659	11.77	0.000	0.94	0.635	0.94	0.033
Chemokine ligand 20 (*CCL20*)	1.36	0.059	1.34	0.108	2.05	0.000	1.11	0.367	0.88	0.000	3.12	0.000	1.09	0.844	2.79	0.058	1.13	0.323	0.89	0.000
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	1.39	0.063	1.86	0.002	3.53	0.000	1.61	0.000	0.83	0.000	4.84	0.000	1.49	0.443	6.8	0.004	1.46	0.011	0.88	0.000
Interferon gamma(*IFNG*)	1.43	0.019	1.95	0.000	9.71	0.000	1.26	0.032	0.88	0.000	2.79	0.000	1.08	0.836	13.43	0.000	1.28	0.022	0.88	0.000
Interleukin 1 beta(*IL1B*)	1.27	0.173	1.71	0.006	2.53	0.000	1.15	0.240	0.89	0.000	2.86	0.000	1.78	0.183	3.29	0.030	1.09	0.502	0.94	0.048
Interleukin 6 (*IL6*)	1.03	0.875	1.4	0.087	2.71	0.000	1.05	0.684	0.93	0.018	2.04	0.001	0.72	0.476	3.73	0.022	1.22	0.124	0.91	0.003
Interleukin 8 (*IL8*)	0.93	0.649	1.72	0.003	2.18	0.000	1.12	0.312	0.92	0.006	2.2	0.000	1.63	0.273	1.71	0.335	1.1	0.462	0.98	0.598
Interleukin 10 (*IL10*)	1.25	0.154	2.25	0.000	2.38	0.000	1.17	0.145	0.89	0.000	2.11	0.000	1.38	0.273	4.81	0.000	1.02	0.769	0.91	0.000
Interleukin 12 beta (*IL12B*)	1.44	0.007	1.43	0.018	5.47	0.000	1.16	0.121	0.92	0.000	1.88	0.000	1.06	0.844	10.34	0.000	1.11	0.265	0.92	0.000
Interleukin 17A (*IL17A*)	1.65	0.008	2.18	0.000	3.25	0.000	1.43	0.007	0.83	0.000	4.15	0.000	1.57	0.309	10.54	0.000	1.27	0.055	0.88	0.000
Interleukin 19 (*IL19*)	1.24	0.283	2.73	0.000	3.83	0.000	1.57	0.002	0.83	0.000	3	0.000	2.09	0.110	6.86	0.001	1.23	0.119	0.91	0.004
Interleukin 21 (*IL21*)	1.66	0.015	2.62	0.000	4.41	0.000	1.37	0.029	0.83	0.000	3.49	0.000	1.67	0.236	13.11	0.000	1.2	0.164	0.87	0.000
Interleukin 22 (*IL22*)	1.7	0.014	1.54	0.066	8.75	0.000	1.19	0.252	0.96	0.282	1.41	0.148	1.63	0.332	13.13	0.000	1.17	0.321	0.99	0.736
Interleukin 23A (*IL23A*)	1.11	0.440	1.8	0.000	2.65	0.000	1.11	0.291	0.89	0.000	1.93	0.000	1.02	0.957	5.04	0.000	0.97	0.778	0.91	0.000
Prostaglandin-endoperoxide synthase 2	1.02	0.515	1.42	0.041	1.84	0.001	1.16	0.172	0.9	0.000	2.4	0.000	1.85	0.109	1.83	0.211	0.97	0.778	0.94	0.022
EMT Markers																				
Epithelial cadherin (*CDH1*)	1.02	0.797	1.14	0.212	1.22	0.079	1.06	0.359	0.94	0.000	1.55	0.020	0.88	0.763	1.49	0.441	1.05	0.663	1	0.873
Neuronal cadherin (*CDH2*)	1.03	0.808	0.99	0.947	1.24	0.181	1.13	0.189	1.03	0.317	1.02	0.892	0.59	0.158	1.55	0.344	1.05	0.645	0.99	0.644
S100 calcium binding protein A4 (*S100A4*)	0.96	0.738	0.9	0.447	0.72	0.028	1	0.987	1	0.912	1.52	0.060	0.86	0.746	0.84	0.777	0.87	0.318	1.02	0.483
Vimentin (*VIM*)	1.18	0.102	1.3	0.024	2.17	0.000	1.07	0.360	0.92	0.000	2.17	0.000	1.01	0.978	3.4	0.017	1.03	0.794	0.97	0.216
Matrix Modifiers																				
Connective tissue growth factor (*CTGF-1*)	0.99	0.915	0.98	0.872	1.4	0.012	0.9	0.173	0.97	0.089	1.44	0.045	0.59	0.174	2.22	0.105	0.91	0.397	1.02	0.492
Fibroblast growth factor 2 (basic) (*FGF2*)	1.2	0.182	1.34	0.044	1.85	0.000	0.97	0.725	0.95	0.041	1.46	0.035	1.04	0.922	1.96	0.179	0.96	0.756	0.97	0.263
Matrix metallopeptidase 7 (*MMP7*)	1.36	0.052	1.17	0.366	0.68	0.040	0.97	0.754	0.89	0.000	2.3	0.000	1.14	0.760	1.06	0.917	0.97	0.777	0.94	0.056
Matrix metallopeptidase 9 (*MMP9*)	1.78	0.000	1.18	0.332	2.51	0.000	1.06	0.580	0.9	0.000	2.75	0.000	1.55	0.268	3.44	0.014	1.16	0.187	0.9	0.000
Matrix metallopeptidase 12 (*MMP12*)	1.37	0.097	2.16	0.000	2.89	0.000	1.36	0.020	0.83	0.000	3.5	0.000	1.65	0.332	6.38	0.005	1.18	0.256	0.89	0.002
Platelet-derived growth factor beta polypeptide	1.12	0.247	1.48	0.001	2.08	0.000	1.14	0.066	0.92	0.000	1.8	0.000	1.15	0.690	2.98	0.014	1.1	0.319	0.97	0.270
SPARC-like 1 (hevin) (*SPARCL1*)	0.7	0.142	0.58	0.042	0.42	0.003	0.73	0.066	1.12	0.010	0.36	0.000	0.32	0.025	0.32	0.077	0.85	0.252	1.05	0.178
Transforming growth factor, beta 1 (*TGFβ1*)	1.1	0.338	1.15	0.179	1.81	0.000	1.02	0.741	0.93	0.000	2	0.001	0.94	0.878	3.55	0.020	1.04	0.728	0.99	0.621
Microbiota Response																				
Arachidonate 5-lipoxygenase (*ALOX5*)	0.95	0.633	1.17	0.193	1.1	0.430	1.01	0.845	0.94	0.002	1.75	0.008	1.17	0.726	1.28	0.663	1.03	0.837	0.99	0.729
Dual oxidase 2 (*DUOX2*)	1.16	0.315	1.55	0.008	2.13	0.000	1.5	0.000	0.84	0.000	2.74	0.000	1.95	0.186	4.26	0.023	1.36	0.031	0.91	0.010
Mucins																				
Mucin 1, cell surface associated (*MUC1*)	1.08	0.496	1.26	0.054	1.35	0.021	1.14	0.074	0.9	0.000	2.04	0.001	1.09	0.857	1.69	0.366	1.1	0.453	0.98	0.497
Mucin 4, cell surface associated (*MUC4*)	0.96	0.766	1.19	0.243	1.47	0.016	1.04	0.686	0.89	0.000	1.46	0.056	1.64	0.254	1.65	0.362	1.15	0.257	0.95	0.075
Mucin 5AC, oligomeric mucus/gel-forming	0.84	0.185	0.84	0.229	0.77	0.083	1.09	0.315	1.05	0.036	0.79	0.317	0.6	0.314	0.3	0.059	1.2	0.200	1.08	0.032
Mucin 7, secreted (*MUC7*)	1.02	0.913	0.71	0.064	0.78	0.199	0.7	0.002	0.99	0.819	1.52	0.055	0.66	0.375	0.76	0.653	0.76	0.039	0.95	0.111
NK Cell Markers																				
CD247 molecule (CD247)	1.24	0.061	1.43	0.006	2.8	0.000	1.12	0.178	0.92	0.000	2.07	0.000	0.92	0.812	5.16	0.000	1.07	0.469	0.95	0.019
Neural cell adhesion molecule 1 (*NCAM1*)	0.96	0.719	1.09	0.468	1.83	0.000	0.92	0.271	0.96	0.050	1.57	0.007	0.59	0.150	2.2	0.085	0.9	0.312	0.98	0.539
Natural cytotoxicity triggering receptor 1	1.35	0.011	1.41	0.008	3.01	0.000	1.09	0.291	0.92	0.000	2.46	0.000	1.14	0.704	5.33	0.000	1.17	0.097	0.9	0.000
Regulators/Signalling Pathways																				
CD274 molecule (*CD274*)	1.27	0.090	1.88	0.000	3.56	0.000	1.22	0.047	0.87	0.000	2.64	0.000	1.16	0.683	9.87	0.000	1.14	0.223	0.9	0.000
Marginal zone B and B1 cell-specific protein	1.58	0.015	1.68	0.013	2.3	0.000	1.03	0.811	0.86	0.000	2.7	0.000	1.09	0.867	10.7	0.000	1.16	0.307	0.88	0.000
Serpin peptidase inhibitor clade B member 4,	1.36	0.205	2.46	0.001	3.69	0.000	0.93	0.672	0.87	0.003	0.88	0.736	1.11	0.892	7.78	0.021	0.75	0.220	0.93	0.211
Suppressor of cytokine signalling 1 (*SOCS1*)	1.26	0.032	1.43	0.003	2.97	0.000	1.13	0.105	0.89	0.000	2.06	0.000	1.32	0.379	4.65	0.000	1.02	0.793	0.96	0.040
Suppressor of cytokine signalling 3 (*SOCS3*)	1.21	0.180	1.6	0.003	2.08	0.000	1.15	0.170	0.87	0.000	2.68	0.000	1.54	0.244	3.27	0.012	1.04	0.678	0.94	0.012

		Time-point 5									
		TF		TP		*C. trachomatis*		Sex		Age	
Target	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value
Antimicrobial Peptides											
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	0.026	1.98	0.009	3.44	0.000	4.51	0.018	1.52	0.009	0.82	0.000
Psoriasin-1 (*S100A7*)	0.000	3.31	0.000	4.39	0.000	7.39	0.000	2.06	0.000	0.78	0.000
Cytokines/Chemokines											
Chemokine ligand 2 (*CCL2*)	0.000	1.69	0.008	1.71	0.022	8.74	0.000	1.29	0.035	0.92	0.006
Chemokine ligand 5 (*CXCL5*)	0.001	2.5	0.001	2.99	0.001	2	0.215	1.47	0.024	0.82	0.000
Chemokine ligand 13 (*CXCL13*)	0.000	3.26	0.000	2.41	0.003	4.07	0.005	1.59	0.002	0.79	0.000
Chemokine ligand 18 (*CCL18*)	0.033	2.88	0.000	4.07	0.000	7.05	0.000	1.13	0.388	0.84	0.000
Chemokine ligand 20 (*CCL20*)	0.000	2.23	0.000	1.45	0.133	2.61	0.023	1.41	0.007	0.92	0.007
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	0.000	2.49	0.000	3.16	0.000	3.77	0.008	1.46	0.013	0.85	0.000
Interferon gamma(*IFNG*)	0.000	1.44	0.032	1.68	0.011	16.98	0.000	1.41	0.001	0.94	0.013
Interleukin 1 beta(*IL1B*)	0.048	2.1	0.000	2.09	0.003	3.24	0.006	1.19	0.180	0.87	0.000
Interleukin 6 (*IL6*)	0.003	1.44	0.073	0.96	0.866	4.67	0.000	1.16	0.242	0.94	0.048
Interleukin 8 (*IL8*)	0.598	1.61	0.016	1.58	0.051	3.27	0.003	1.08	0.512	0.94	0.035
Interleukin 10 (*IL10*)	0.000	1.96	0.000	1.66	0.007	3.9	0.000	1.2	0.057	0.88	0.000
Interleukin 12 beta (*IL12B*)	0.000	1.36	0.044	0.98	0.909	6.43	0.000	1.2	0.044	1	0.876
Interleukin 17A (*IL17A*)	0.000	2.31	0.000	2.4	0.000	5.98	0.000	1.59	0.000	0.86	0.000
Interleukin 19 (*IL19*)	0.004	2.53	0.000	3.49	0.000	7.06	0.000	1.6	0.001	0.83	0.000
Interleukin 21 (*IL21*)	0.000	2.39	0.000	2.1	0.001	7.25	0.000	1.66	0.000	0.88	0.000
Interleukin 22 (*IL22*)	0.736	1.37	0.109	1.85	0.010	13.77	0.000	1.17	0.209	0.98	0.461
Interleukin 23A (*IL23A*)	0.000	1.77	0.001	1.71	0.011	3.67	0.000	1.12	0.285	0.91	0.000
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	0.022	1.7	0.003	1.66	0.018	2.22	0.029	1.25	0.049	0.87	0.000
EMT Markers											
Epithelial cadherin (*CDH1*)	0.873	1.19	0.207	1.2	0.272	1.24	0.444	1.12	0.188	0.95	0.032
Neuronal cadherin (*CDH2*)	0.644	0.89	0.461	0.8	0.236	1.63	0.126	1.23	0.038	1.06	0.030
S100 calcium binding protein A4 (*S100A4*)	0.483	0.88	0.499	0.87	0.523	0.84	0.641	1.03	0.780	1.02	0.587
Vimentin (*VIM*)	0.216	1.49	0.002	1.38	0.034	2.58	0.000	1.08	0.330	0.95	0.008
Matrix Modifiers											
Connective tissue growth factor (*CTGF-1*)	0.492	1.08	0.661	0.93	0.720	1.93	0.056	1	0.985	0.99	0.690
Fibroblast growth factor 2 (basic) (*FGF2*)	0.263	1.37	0.065	1.04	0.836	2.32	0.010	1.09	0.448	1.02	0.417
Matrix metallopeptidase 7 (*MMP7*)	0.056	1.49	0.043	1.34	0.211	0.91	0.810	1.01	0.923	0.89	0.000
Matrix metallopeptidase 9 (*MMP9*)	0.000	1.87	0.001	1.56	0.036	3.42	0.001	1.11	0.330	0.91	0.001
Matrix metallopeptidase 12 (*MMP12*)	0.002	1.89	0.004	1.63	0.065	5.91	0.000	1.23	0.125	0.87	0.000
Platelet-derived growth factor beta polypeptide (*PDGFB*)	0.270	1.37	0.017	1.55	0.005	2.77	0.000	1.28	0.002	0.95	0.007
SPARC-like 1 (hevin) (*SPARCL1*)	0.178	0.37	0.000	0.21	0.000	0.27	0.017	0.95	0.736	1.25	0.000
Transforming growth factor, beta 1 (*TGFβ1*)	0.621	1.31	0.054	1.31	0.100	1.89	0.025	1.13	0.166	0.96	0.040
Microbiota Response											
Arachidonate 5-lipoxygenase (*ALOX5*)	0.729	1.06	0.719	1.03	0.881	1.43	0.263	1.06	0.576	0.94	0.018
Dual oxidase 2 (*DUOX2*)	0.010	1.96	0.002	2.42	0.001	2.79	0.022	1.32	0.039	0.86	0.000
Mucins											
Mucin 1, cell surface associated (*MUC1*)	0.497	1.12	0.504	1.09	0.673	1.91	0.064	1.08	0.449	0.94	0.013
Mucin 4, cell surface associated (*MUC4*)	0.075	1.26	0.183	1.17	0.442	1.61	0.174	1.17	0.142	0.93	0.010
Mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*)	0.032	0.83	0.293	0.99	0.960	0.76	0.442	1.23	0.062	1.11	0.000
Mucin 7, secreted (*MUC7*)	0.111	0.88	0.533	1.15	0.555	0.7	0.380	0.85	0.174	0.94	0.063
NK Cell Markers											
CD247 molecule (CD247)	0.019	1.32	0.097	1.53	0.033	3.21	0.001	1.22	0.056	0.92	0.002
Neural cell adhesion molecule 1 (*NCAM1*)	0.539	0.89	0.427	1.15	0.418	1.72	0.071	1	0.996	1.02	0.363
Natural cytotoxicity triggering receptor 1 (*NCR1*)	0.000	1.43	0.022	1.53	0.021	2.97	0.001	1.28	0.011	0.91	0.000
Regulators/Signalling Pathways											
CD274 molecule (*CD274*)	0.000	1.74	0.002	2.02	0.001	5.56	0.000	1.29	0.024	0.87	0.000
Marginal zone B and B1 cell-specific protein (*MZB1*)	0.000	1.83	0.005	1.53	0.102	4.2	0.001	1.46	0.005	0.87	0.000
Serpin peptidase inhibitor clade B member 4, (*SERPINB4*)	0.211	1.18	0.600	2.15	0.044	21.14	0.000	0.98	0.940	1	0.965
Suppressor of cytokine signalling 1 (*SOCS1*)	0.040	1.67	0.001	1.52	0.021	2.89	0.001	1.1	0.311	0.9	0.000
Suppressor of cytokine signalling 3 (*SOCS3*)	0.012	1.76	0.003	1.86	0.006	2.47	0.020	1.12	0.345	0.85	0.000

Supplementary Table 4. Estimated Fold Changes (FC) with their respective p-values comparing the expression of each gene between the combined first three time-points (time-points 1, 2 and 3) before MDA and time-point 4 (three months after MDA), not adjusted for *C. trachomatis* infection. A FC of >1 indicates increased expression of the gene at time-point 4. Random effects multivariable linear regression of all individuals (first panel), untreated only (second panel) and treated only (third panel). The final column (p-value for interaction) provides evidence as to whether the fold change from before to after MDA is different in the treated and untreated groups. Results are ordered by FC of ''**All**'' individuals. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control the false discovery rate <5%, only tests with a p-value <0.035 are considered statistically significant.

Target	All		Untreated Only		Treated Only		p-value for
	FC	p-value	FC	p-value	FC	p-value	interaction
SPARCL1	18.65	2.75x10^-250	8.21	3.65x10^-23	21.77	1.32x10^-235	2.69X10^-5
MUC5AC	5.33	7.6x10^-215	3.14	3.06x10^-18	5.88	7.21x10^-206	1.19X10^-5
CDH2	3.92	2.22x10^-169	2.72	3.72x10^-17	4.24	1.9x10^-158	6.84X10^-4
MUC7	2.71	2.52x10^-72	2.45	9.19x10^-11	2.74	5.15x10^-62	0.458
CTGF	2.40	2.04x10^-118	2.29	1.12x10^-19	2.43	1.68x10^-101	0.553
NCAM1	2.34	5.3x10^-90	1.79	8.89x10^-9	2.49	8.21x10^-86	0.003
S100A4	2.30	1.87x10^-143	1.95	5.02x10^-17	2.37	4.66x10^-129	0.026
CDH1	1.94	1.28x10^-122	1.78	1.38x10^-17	1.97	3.13x10^-107	0.173
ALOX5	1.42	1.61x10^-65	1.45	1.37x10^-13	1.41	2.70x10^-53	0.606
MUC1	1.39	7.87x10^-39	1.40	4.01x10^-8	1.40	2.12x10^-33	0.975
FGF2	1.39	6.72X10^-6	1.41	0.046	1.42	1.77x10^-5	0.994
TGFB1	1.34	9.68x10^-46	1.41	5.79x10^-12	1.32	2.41x10^-35	0.272
MUC4	1.30	5.69x10^-19	1.40	2.54x10^-6	1.29	4.82x10^-15	0.273
GAPDH	1.29	6.09x10^-18	1.50	1.65x10^-8	1.25	1.06x10^-11	0.020
IL12B	1.24	7.69x10^-6	1.28	0.035	1.24	5.35x10^-5	0.794
PDGFB	1.22	1.49x10^-12	1.29	1.54x10^-4	1.20	1.01x10^-9	0.351
NCR1	1.21	2.44x10^-8	1.15	0.086	1.22	6.90x10^-8	0.530
VIM	1.13	7.56x10^-8	1.17	0.005	1.13	3.59x10^-6	0.515
SOCS1	1.13	2.42x10^-4	1.36	1.35x10^-4	1.09	0.021	0.012
SX10RPINB4	1.13	0.341	1.34	0.320	1.09	0.565	0.520
MMP7	1.11	0.006	1.17	0.086	1.09	0.028	0.487
CD247	0.96	0.122	1.02	0.770	0.94	0.059	0.297
MMP9	0.84	9.28x10^-5	0.91	0.371	0.83	1.37x10^-4	0.457
IL23A	0.83	2.24x10^-6	0.92	0.369	0.81	2.43x10^-6	0.250
IL6	0.80	2.75x10^-5	0.77	0.050	0.81	3.65x10^-4	0.758
CCL20	0.78	5.98x10^-8	0.88	0.263	0.76	8.80x10^-8	0.232
IFNG	0.76	7.28x10^-8	0.76	0.030	0.77	1.07x10^-6	0.985
DUOX2	0.71	1.51x10^-22	0.87	0.116	0.69	1.05x10^-22	0.011
PTGS2	0.68	1.96x10^-19	0.77	0.011	0.67	3.94x10^-18	0.203
IL8	0.68	1.75x10^-24	0.69	7.73x10^-5	0.68	2.06x10^-20	0.822
IL22	0.65	1.91x10^-6	0.95	0.801	0.60	2.79x10^-7	0.042
IL10	0.60	7.40x10^-35	0.74	0.002	0.58	2.33x10^-33	0.029
SOCS3	0.58	8.67x10^-40	0.73	0.001	0.56	3.03x10^-38	0.015
MZB1	0.56	6.51x10^-26	0.65	0.001	0.55	7.23x10^-24	0.260
CD274	0.54	1.82x10^-57	0.68	3.92x10^-5	0.51	4.37x10^-55	0.008
CCL2	0.48	3.48x10^-37	0.55	1.23x10^-5	0.47	1.01x10^-32	0.341
IL1B	0.46	5.42x10^-59	0.56	7.91x10^-07	0.44	4.31x10^-54	0.054
IDO1	0.41	7.77x10^-102	0.65	2.11x10^-5	0.38	3.28x10^-104	1.56X10^-6
IL17A	0.40	4.45x10^-62	0.57	2.46x10^-5	0.38	2.21x10^-59	0.005
IL21	0.40	1.52x10^-39	0.61	0.003	0.37	6.53x10^-39	0.007
MMP12	0.37	1.06x10^-87	0.51	4.56x10^-8	0.35	3.18x10^-82	0.005
CXCL5	0.36	3.84x10^-72	0.59	2.11x10^-4	0.32	1.17x10^-72	1.04X10^-4
DX10FB4A	0.35	1.46x10^-64	0.50	4.51x10^-6	0.33	5.23x10^-61	0.013
IL19	0.33	1.01x10^-79	0.48	2.57x10^-7	0.31	1.71x10^-75	0.005
CXCL13	0.30	4.71x10^-66	0.44	9.38x10^-7	0.28	3.44x10^-62	0.015
S100A7	0.24	2.33x10^-60	0.47	3.39x10^-4	0.21	3.46x10^-61	4.20X10^-4
CCL18	0.23	1.32x10^-77	0.41	1.17x10^-6	0.21	9.46x10^-76	8.98X10^-4

Supplementary Table 5. Estimated Fold Change (FC) with their respective p-values for the expression of each gene at time-point 4 only (three months post MDA), comparing MDA treated (after time-point 3) to untreated individuals. Multivariable linear regression of all individuals adjusted (first panel) and not adjusted (second panel) for *C. trachomatis* infection. Results are ordered by FC of adjusted data with infection. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control false discovery rate <5%, only tests with a p-value <0.009 are considered statistically significant.

Target	Adjusted for infection		Not adjusted for infection	
	FC	p-value	FC	p-value
SPARCL1	2.06	3.10x10^-04	2.34	2.55 x10^-5
MUC5AC	1.44	0.011	1.62	9.45 x10^-4
MUC7	1.28	0.081	1.35	0.030
CDH2	1.27	0.062	1.30	0.037
NCAM1	1.19	0.055	1.19	0.048
IFNG	1.16	0.225	1.03	0.802
S100A4	1.08	0.238	1.14	0.052
MZB1	1.07	0.660	0.97	0.820
NCR1	1.05	0.568	0.99	0.904
CDH1	1.03	0.701	1.05	0.464
CD247	1.02	0.748	0.97	0.668
IL12B	1.01	0.890	0.92	0.468
VIM	1.00	0.950	0.97	0.599
TGFB1	0.98	0.738	0.96	0.429
SERPINB4	0.97	0.913	0.89	0.700
ALOX5	0.95	0.389	0.98	0.725
CCL2	0.95	0.602	0.89	0.247
PDGFB	0.95	0.440	0.93	0.300
IL6	0.94	0.627	0.91	0.479
MMP9	0.94	0.552	0.90	0.331
CTGF	0.93	0.462	0.94	0.529
IL23A	0.93	0.340	0.88	0.110
GAPDH	0.92	0.330	0.91	0.275
MMP7	0.91	0.370	0.93	0.520
SOCS1	0.90	0.183	0.86	0.057
MUC1	0.88	0.046	0.89	0.071
IL8	0.88	0.208	0.88	0.203
CD274	0.87	0.088	0.80	0.006
CCL20	0.86	0.199	0.84	0.123
PTGS2	0.85	0.100	0.84	0.083
IL10	0.85	0.028	0.80	0.004
IL1B	0.84	0.083	0.81	0.034
CXCL13	0.82	0.199	0.74	0.051
MUC4	0.82	0.015	0.84	0.024
FGF2	0.78	0.134	0.79	0.132
SOCS3	0.78	0.008	0.76	0.003
DUOX2	0.76	0.017	0.73	0.005
IL19	0.74	0.018	0.69	0.003
IL21	0.74	0.041	0.64	0.004
CCL18	0.73	0.042	0.67	0.008
IL17A	0.73	0.013	0.66	0.001
IL22	0.72	0.110	0.64	0.030
MMP12	0.71	0.009	0.66	0.001
DEFB4A	0.71	0.068	0.69	0.042
S100A7	0.70	0.030	0.63	0.005
IDO1	0.64	8.99 x10^-4	0.59	1.03 x10^-4
CXCL5	0.57	4.02E-04	0.56	1.98E-04

Supplementary Table 6. Estimated fold changes (FC) with their respective p-values comparing the expression of each gene between the combined first three time-points (1, 2 and 3) before MDA and separately time-points 4 and 5 (three and six months following MDA treatment), in individuals who were free from infection and/or disease at all 5 time-points. Results are ordered by Fold Change (FC) in pre-MDA time-points vs. time-point 4. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control the false discovery rate <5%, only tests with a p-value <0.035 are considered statistically significant.

Target	Pre-MDA vs time-point 4		Pre-MDA vs time-point 5	
	FC	p-value	FC	p-value
SPARCL1	8.97	1.10 x10^-55	1.57	2.15x10^-3
MUC5AC	3.89	2.69 x10^-50	1.59	3.37 x10^-7
CDH2	3.36	2.64 x10^-46	1.62	3.16 x10^-9
CTGF	2.10	3.97 x10^-28	0.98	0.756
NCAM1	2.01	2.57 x10^-17	1.29	0.002
CDH1	1.77	2.56 x10^-40	1.16	9.50 x10^-4
S100A4	1.72	1.29 x10^-28	1.10	0.063
MUC7	1.53	3.95 x10^-7	1.32	7.86 x10^-4
SERPINB4	1.51	0.059	1.26	0.210
TGFB1	1.36	1.26 x10^-16	1.10	0.007
IL12B	1.35	1.14 x10^-4	1.06	0.467
PDGFB	1.29	4.08 x10^-9	0.97	0.427
MUC1	1.27	3.36 x10^-9	1.03	0.418
SOCS1	1.27	1.08 x10^-5	1.29	6.99 x10^-6
ALOX5	1.25	3.352 x10^-13	1.02	0.471
FGF2	1.24	0.0718	0.87	0.240
GAPDH	1.19	5.74 x10^-4	0.91	0.166
MUC4	1.18	4.17 x10^-4	1.07	0.214
NCR1	1.16	0.0122	0.96	0.500
VIM	1.14	6.14 x10^-4	0.97	0.430
CD247	1.06	0.224	0.86	9.20 x10^-4
IL22	1.03	0.854	0.99	0.942
IL23A	0.97	0.583	1.11	0.080
MMP9	0.96	0.609	1.07	0.382
MMP7	0.95	0.357	0.81	1.10 x10^-4
IFNG	0.94	0.433	0.83	0.009
CCL20	0.76	3.23 x10^-4	0.79	0.004
IL6	0.74	9.71 x10^-4	0.81	0.016
IL10	0.74	2.19 x10^-6	0.84	0.006
CCL2	0.73	2.76 x10^-4	1.02	0.778
IL8	0.72	1.036 x10^-7	0.87	0.019
PTGS2	0.70	6.47 x10^-7	0.87	0.047
DUOX2	0.70	2.64 x10^-9	0.75	2.37 x10^-5
CD274	0.65	5.91 x10^-12	0.86	0.017
MZB1	0.64	6.88 x10^-8	0.83	0.023
SOCS3	0.63	3.10 x10^-10	0.93	0.359
IL1B	0.56	1.57 x10^-12	0.89	0.169
IL21	0.55	2.97 x10^-7	0.78	0.025
IL17A	0.50	1.66 x10^-14	0.89	0.221
IL19	0.49	3.98 x10^-15	0.90	0.254
CCL18	0.47	3.62 x10^-10	0.93	0.505
MMP12	0.44	1.15 x10^-25	0.64	8.38 x10^-8
CXCL13	0.44	3.50 x10^-14	0.87	0.211
DEFB4A	0.42	2.15 x10^-19	0.81	0.035
S100A7	0.41	7.82 x10^-15	0.76	0.018
IDO1	0.41	8.19 x10^-38	0.68	5.38 x10^-8
CXCL5	0.40	1.12 x10^-22	0.90	0.271

8. Progression of scarring trachoma in Tanzanian children: a four-year cohort study

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Athumani M. Ramadhani
Principal Supervisor	Prof. Matthew J. Burton
Thesis Title	Studies on the Development of Scarring Trachoma in Tanzania

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	N/A		
When was the work published?	N/A		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	N/A		
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	PLOS Medicine
Please list the paper's authors in the intended authorship order:	Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Patrick Massae, Elias Mafuru, Aiweda Malisa, Kelvin Mbuya, Chrissy h. Roberts, William Makupa, Tara Mtuy, Robin L. Bailey, David C. W. Mabey, Martin J. Holland and Matthew J. Burton
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed laboratory work and analysed the data with some guidance from Matthew Burton, David Macleod and Tamsyn Derrick. I wrote a first draft of this paper and comments from co-authors were considered. I

	periodically attended field work, doing clinical data entry from the study participants and QA of sample collection process and grading. I was involved in side-by-side photograph grading with Matthew Burton.

Student Signature: [redacted] **Date:** 29.10.2018

Supervisor Signature [redacted] **Date:** 27/10/2018

Progression of scarring trachoma in Tanzanian children: a four-year cohort study

Authors:

Athumani M. Ramadhani[1,2], Tamsyn Derrick[1,2], David Macleod[1], Patrick Massae[2], Elias Mafuru[2], Aiweda Malisa [2], Kelvin Mbuya[2], Chrissy h. Roberts[1], William Makupa[2], Tara Mtuy[1,2], Robin L. Bailey[1], David C. W. Mabey[1], Martin J. Holland[1] and Matthew J. Burton[1]

Affiliations:

1. London School of Hygiene and Tropical Medicine, London, UK
2. Kilimanjaro Christian Medical Centre, Moshi, Tanzania

Corresponding Author:

Athumani M. Ramadhani

International Centre for Eye Health

Clinical Research Department

Faculty of Infectious and Tropical Diseases

London School of Hygiene & Tropical Medicine

Keppel Street

London

WC1E 7HT

Email: Athumani.Ramadhani@lshtm.ac.uk

Running Title:

Progressive scarring trachoma in children

Abstract

Background

Trachoma is a progressive blinding disease initiated by infection of the conjunctiva with *C. trachomatis*. Repeated infections are thought to cause chronic inflammation which drives scarring, leading to in-turning of the eyelids. The relationship between *C. trachomatis,* clinical inflammation and scarring development in children is not fully understood due to a paucity of longitudinal studies with infection data at frequent follow-up.

Methods and Findings

This longitudinal cohort study took place in northern Tanzania. Children aged 6-10 at baseline were eligible for inclusion. Participants were visited every three months for four years. Clinical signs and conjunctival swabs for *C. trachomatis* detection by qPCR were collected at each time-point. Conjunctival photographs from baseline and final timepoints were graded and compared side-by-side to determine scarring incidence and progression.

Of the 666 children enrolled in the study, outcome data was obtained for 448. Scarring progression was detected in 103/448 (23%) children; 48 (11%) of which had incident scarring and 55 (12%) had progression of existing scarring. Scarring was strongly associated with increasing episodes of papillary inflammation. Weaker associations between episodes of infection and TF with scarring progression in univariate models were absent in multivariate analysis after adjusting for TP (multivariate results: *C. trachomatis* p=0.44, TF p=0.25, TP p=<0.0001, age p=0.13, female sex p=0.05). Individuals with TP at 30% or more of the time-points they were seen had an odds ratio of 7.5 (95%CI=2.7-20.8) for scarring progression relative to individuals without any TP detected during the study period.

Conclusions

These data suggest that the effect of infection on scarring progression is mediated through TP, and that other factors contributing to the development of TP in addition to *C. trachomatis* infection are important in driving conjunctival scarring progression in children.

Introduction

Sight loss from trachoma, the leading infectious cause of blindness, is the end result of an inflammatory-scarring process. Starting from early childhood, people growing-up in a trachoma endemic community may be repeatedly exposed to ocular challenge with *Chlamydia trachomatis*, the causative organism. This is thought to trigger inflammatory responses that lead to conjunctival scarring in some individuals.[233] Scarring complications usually develop during adulthood. The eyelids and eyelashes turn in, scratching the ocular surface (trichiasis), resulting in corneal opacification.[233]

Trachoma control rests on the SAFE Strategy: **S**urgery for trichiasis, **A**ntibiotic treatment to treat *C. trachomatis* infection, **F**acial cleanliness and **E**nvironmental improvements to reduce transmission. Endemic countries and the international community have set the ambitious target of 2020 for the elimination of trachoma as a public health problem.[268] There is no specific treatment to halt the progression of scarring, beyond controlling the infection.

Around 3.2 million people are estimated to have trichiasis and 1.9 million are blind or have severe visual impairment.[15] Currently WHO estimates that 158 million people live in districts that require A, F and E interventions.[13] Nearly 90% live in Sub-Saharan Africa.

Longitudinal data sets documenting the incidence or progression of conjunctival scarring are limited.[233] Such studies are complex and can take many years to complete. In this paper we report the clinical signs and infection results of a four-year longitudinal study with 3-monthly follow up. The aim of the study was to determine the contributions of *C. trachomatis* infection, clinical signs, age and sex to scarring incidence and progression in Tanzanian children.

Methods

Ethical statement

This study was reviewed and approved by Ethics Committees of the Tanzania National Institute for Medical Research, Kilimanjaro Christian Medical University College and the London School of Hygiene & Tropical Medicine. It adhered to the tenets of the Declaration of Helsinki. The study was explained in detail in Kiswahili or Maasai; written informed consent from a parent or legal guardian was necessary for enrolment.

Study Design and Population

We recruited a cohort of children from three neighbouring villages in northern Tanzania. They were assessed every three months for four years, totalling 17 time-points. The communities and recruitment have been described in detail.[23] These communities are predominantly comprised of Maasai people. Children aged between 6 and 10 years at baseline (February 2012), who were normally resident in the villages, were eligible for inclusion. This restricted age group was chosen as we anticipated that younger children may not have manifest incident / progressive conjunctival scarring during the four years of follow-up. A census was conducted and eligible children enrolled.

Clinical assessment and sample collection

At each time-point all available children were examined by an experienced ophthalmic nurse. The eye was first anaesthetized with preservative-free proxymetacaine hydrochloride 0.5% eyedrops. The left upper eyelid was everted and tarsal conjunctiva examined (using x2.5 loupes and torch) for signs of trachoma and graded using the 1981 WHO 'FPC' detailed grading system.[26] This grading system corresponds to the Simplified WHO system: F2/F3 equates to *Trachomatous Inflammation-Follicular* (TF), and P3 to *Trachomatous Inflammation-Follicular* (TI).[20] "Clinically Active Trachoma" was defined as presence of TF and/or TI. We also consider that both P2 and P3 represent clinically significant papillary inflammation, and refer to this as "TP".[260] High resolution photographs (Nikon D90 camera with 105mm Macro lens) were taken of the conjunctiva for independent grading.

Two conjunctival swab samples were collected (Dacron polyester, Puritan Medical Products Company, Maine) at each time-point. The first was placed in RNAlater (Thermo Fisher, UK) and the second was stored dry. Clinical swabs and air control swabs were collected and stored as described previously.[23] Samples were stored at -80^{0}C until processed.

Trachoma Control

Following approval from the Ministry of Health and in collaboration with district eye coordinators the SAFE strategy was implemented in study villages by the study field team. Education was provided regarding facial cleanliness and environmental improvements, free trichiasis surgery was offered and MDA was administered according to WHO guidelines in August 2012, August 2013 and August 2014.

***Chlamydia trachomatis* detection**

At the first time-point, DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) from swab samples stored in dry tubes and *C. trachomatis* was detected by droplet digital PCR, as previously described.[23, 263] In all subsequent time-points, DNA was extracted from samples stored in RNAlater using the Norgen RNA/DNA purification kit (Norgen Biotek) and *C. trachomatis* was detected by triplex quantitative PCR (qPCR) for chlamydial chromosomal (*omcB*) and plasmid (*pORF2*) genes and a human endogenous control gene (*RPP30*), as described previously.[258] Time-point 2 Norgen-extracted samples were tested by both detection methods and the kappa score for agreement was 0.84. Samples were tested in duplicate and were defined as *C. trachomatis* positive if *RPP30* and *pORF2* and/or *omcB* amplified in <40 cycles in one or both replicates.

Analysis

We used photographic grading to determine whether there was either development of incident scarring in previously un-scarred conjunctiva or increase in pre-existing scarring. Conjunctival photographs from baseline (time-point 1) were compared to the final time-point (time-point 17). For individuals not seen at time-point 1, the image from time-point 2 was used for baseline. Similarly, if an individual was not seen at time-point 17, the image from time-point 16 was used as the final time-point. The images were assessed by an ophthalmologist experienced in using a detailed scarring grading system.[245] Baseline and final photographs were compared side-by-side to produce a binary outcome variable "scarring progression", defined as evidence of incident scarring or worsening of pre-existing scarring. We subdivided individuals with "no scarring progression" into (1) no scarring at either baseline or final; (2) scarring unchanged between baseline and final. We subdivided individuals with "scarring progression" into (3) incident scarring (no scarring at baseline and new scarring at final); (4) increasing scarring (some scarring at baseline and more at final).

All field data were handled in Access (Microsoft). Data were merged and analyzed in STATA v14. The total number of time-points at which participants were seen varied due to absence or

refusal. We excluded from the analysis individuals who were seen on fewer than four occasions or did not have outcome data (time-point 16 or 17 assessments). A proportion variable was generated for each of TF, TP and *C. trachomatis* infection: number of time-points with each factor as a proportion of the total number of time-points that individual was seen. Proportions were subsequently categorized.

Separate mixed effects logistic regression models were used to determine the association between (1) TP, (2) TF, and (3) *C. trachomatis* infection with sex and baseline age, using data from all time-points in the longitudinal dataset. Mixed effects regression was also performed to assess the relationship between (1) TF and *C. trachomatis* infection, and (2) TP and *C. trachomatis*, again using data from all time-points in the longitudinal dataset (adjusting for age at baseline and sex). These analyses were limited to the 448 individuals with outcome data.

To identify risk factors for scarring progression, analysis was initially performed using logistic regression to assess the association between categorized proportions of TF, TP or *C. trachomatis* infection with overall scarring progression. Each of these were initially included as exposures separately in a logistic regression using scarring progression as the outcome variable and adjusting for age at baseline and sex. Following this, all three were included in a final multivariable model (adjusting for baseline age and sex), and likelihood ratio tests were performed between models including versus excluding each exposure to determine its overall *P* value. The analyses were subsequently repeated to identify risk factors for incident scarring and progression of pre-existing scarring separately. In the first set of univariate and multivariate analyses (using the same exposures as above) the analysis was restricted to individuals without scarring at baseline (incident scarring versus no scarring). In the second set, analysis was restricted to individuals with scarring at baseline (progression of existing scarring versus no progression of existing scarring).

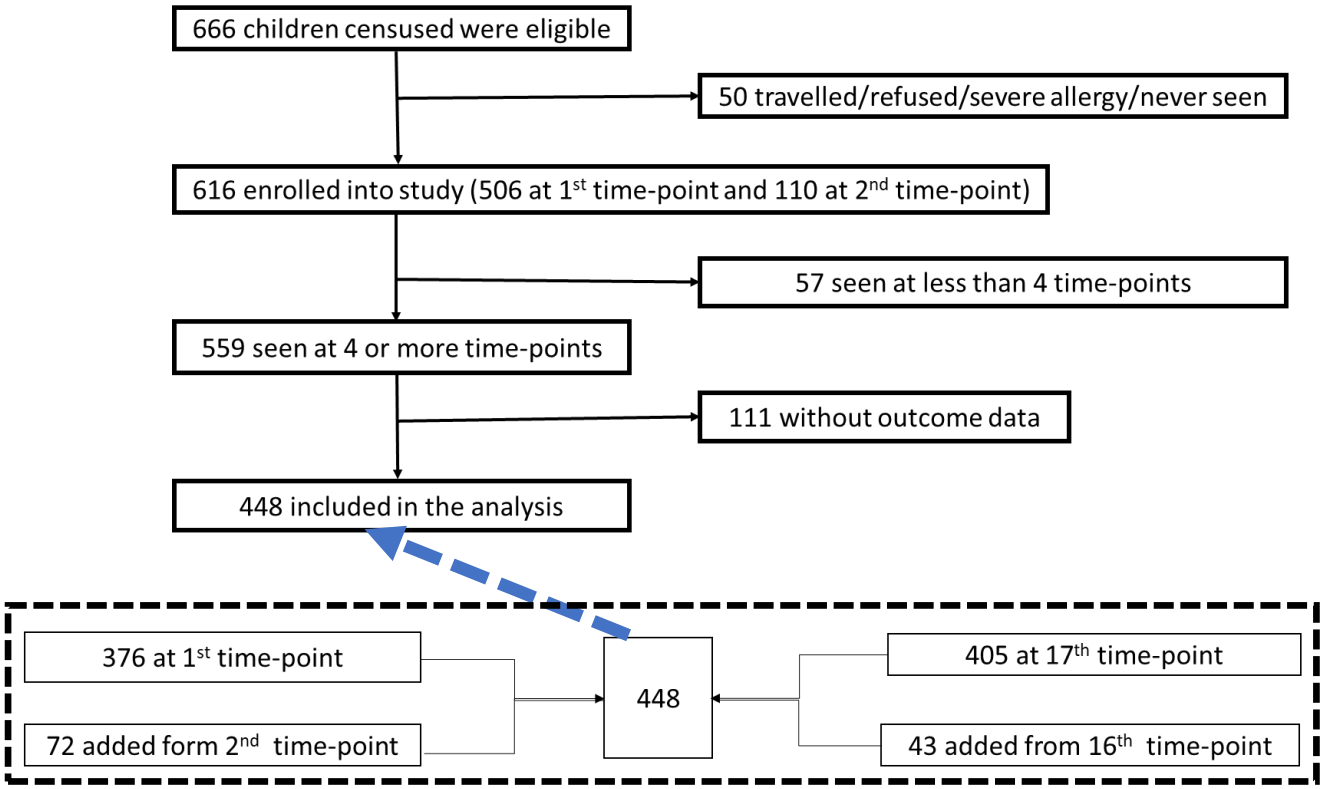
Chlamydial load was calculated by extrapolating from a standard curve. *OmcB* in copies/μl was log10 transformed to normalize the distribution. In the longitudinal dataset random effects linear regressions were performed to look for associations between a) chlamydial load and scarring progression (adjusting for MDA-period, age at baseline and sex), and b) chlamydial load and age at time-point (in years) in *C. trachomatis* positive individuals (adjusting for sex and MDA-period. Age at time-point was split into four groups; <7.5 years, ≥7.5 - <10 years, ≥10 - <12.5 years, and >12.5 years. A random effects linear regression was then performed to assess for association between chlamydial load, age group and progression, including an interaction term between age group and progression in order to determine whether the association between

chlamydial load and progression was modified by age. MDA-period (pre-MDA, post first MDA, post second MDA, post third MDA) was adjusted for to avoid confounding.

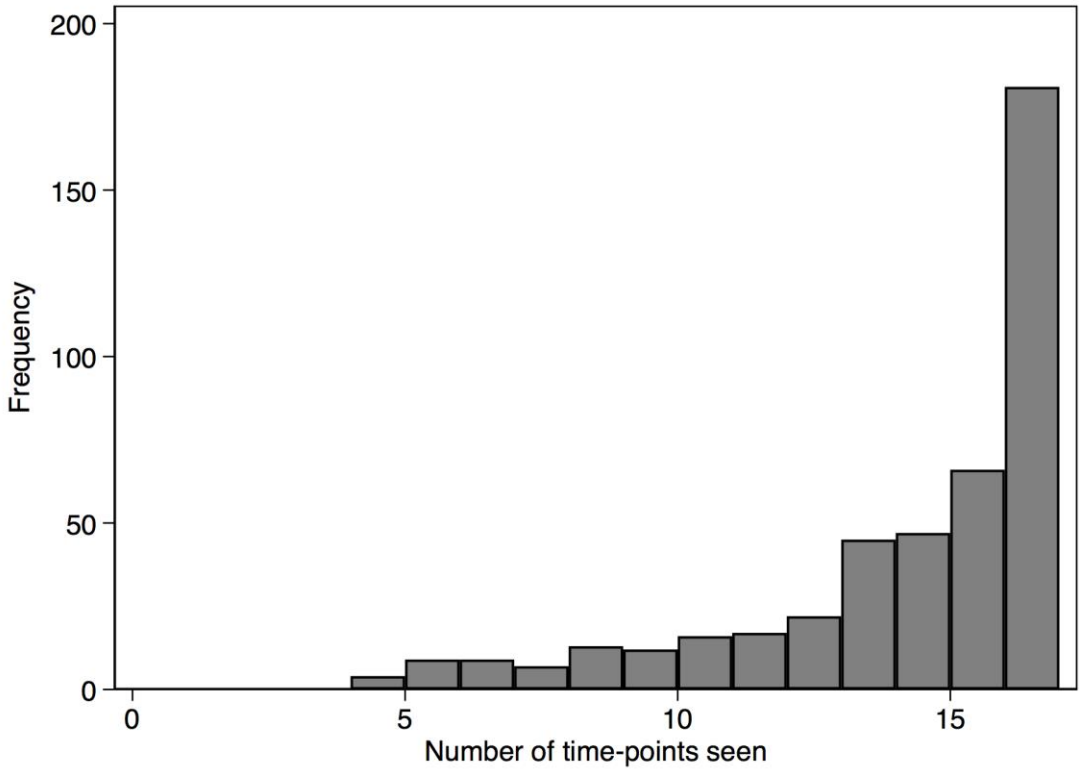
Study participants

The participant flow is shown in Figure 1. There were 666 eligible and 616 enrolled. Enrolment into the cohort was permitted at only the first or second time-point. We had this restriction as we wanted to ensure that children were followed up for sufficient time, with a consistent approach to be able to observe scarring progression at the end of study in the fourth year. There were a few entries after the second time-point which were not included in the analysis. There was naturally variation of number of children available at each time-point and who these were, with some missing due to various reasons (highlighted in figure 1).Fifty either refused or were absent. We excluded 57 who were examined on less than four occasions, and 111 without scarring progression outcome data (no time-point 16 or 17 assessment). This left 448 in the analysis, who were seen at a median of 15 time-points (1^{st} – 3^{rd} quartiles = 13-16, Supplementary Figure 1).

Figure 1. Participant flow chart showing the number of individuals enrolled, excluded and included in the analysis of scarring progression.



Supplementary Figure 1. Histogram showing the number of time-points participants were seen (448 participants with outcome data).



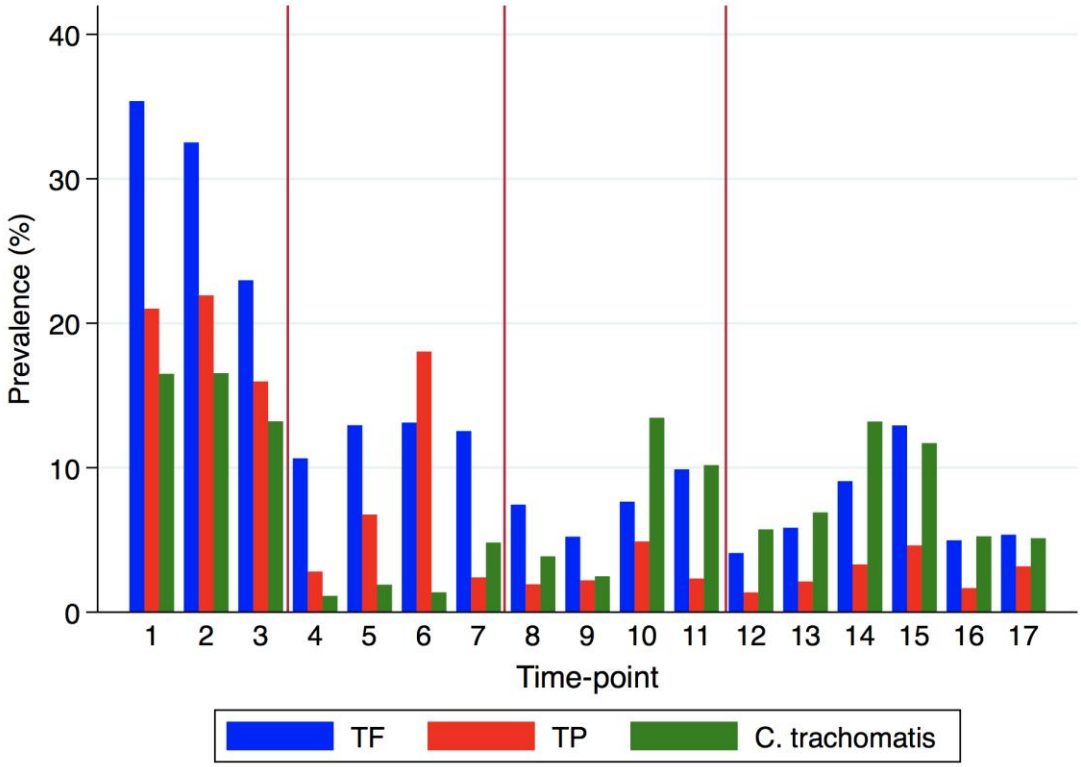
The demographic characteristics of the entire cohort were described in the baseline report.[23] Of the 448 children included in these analyses, 242 (54.0%) were female, mean age at baseline was 6.8 years, and 438 (97.8%) were Maasai. Of the 218 children not included, 92 (42.2%) were female (OR=1.61, 95%CI=1.16-2.23, p=0.004), mean age was 7.4 years (OR=0.86,95% CI=0.79-0.93, p<0.0002) and 214 (98.2%) were Maasai. Younger children and females were therefore more likely to be included in this study analysis.

Antibiotic coverage of the 448 children included in scarring progression analysis was 355 (79.2%) in 2012, 374 (83.5%) in 2013 and 344 (76.8%) in 2014. The estimated community-wide MDA coverage in 2012, 2013 and 2014 were 68.7%, 42.9% and 72.9%, respectively.

Clinical Disease

Of the 448 participants, 240 (53.6%) had TF (F2/F3), 185 (41.3%) had TP (P2/P3)), and 248 (55.4%) had clinically active trachoma (F2/3 and/or P3) at one or more time-point. The prevalence of TF and TP is shown for each time-point in Figure 2. There was a significant reduction in inflammatory disease following MDA, although TP prevalence was particularly high at time-point 6.

Figure 2. **The prevalence of *C. trachomatis* infection and clinical signs at each time-point in the 448 individuals with outcome data. Red vertical lines indicate MDA treatment.**



Examination of inflammation and infection prevalence by village revealed that this peak in TP was found in only two of the three villages ("A" and "C"), and did not appear to correlate with infection (Supplementary Figure 2 & Figure 3).

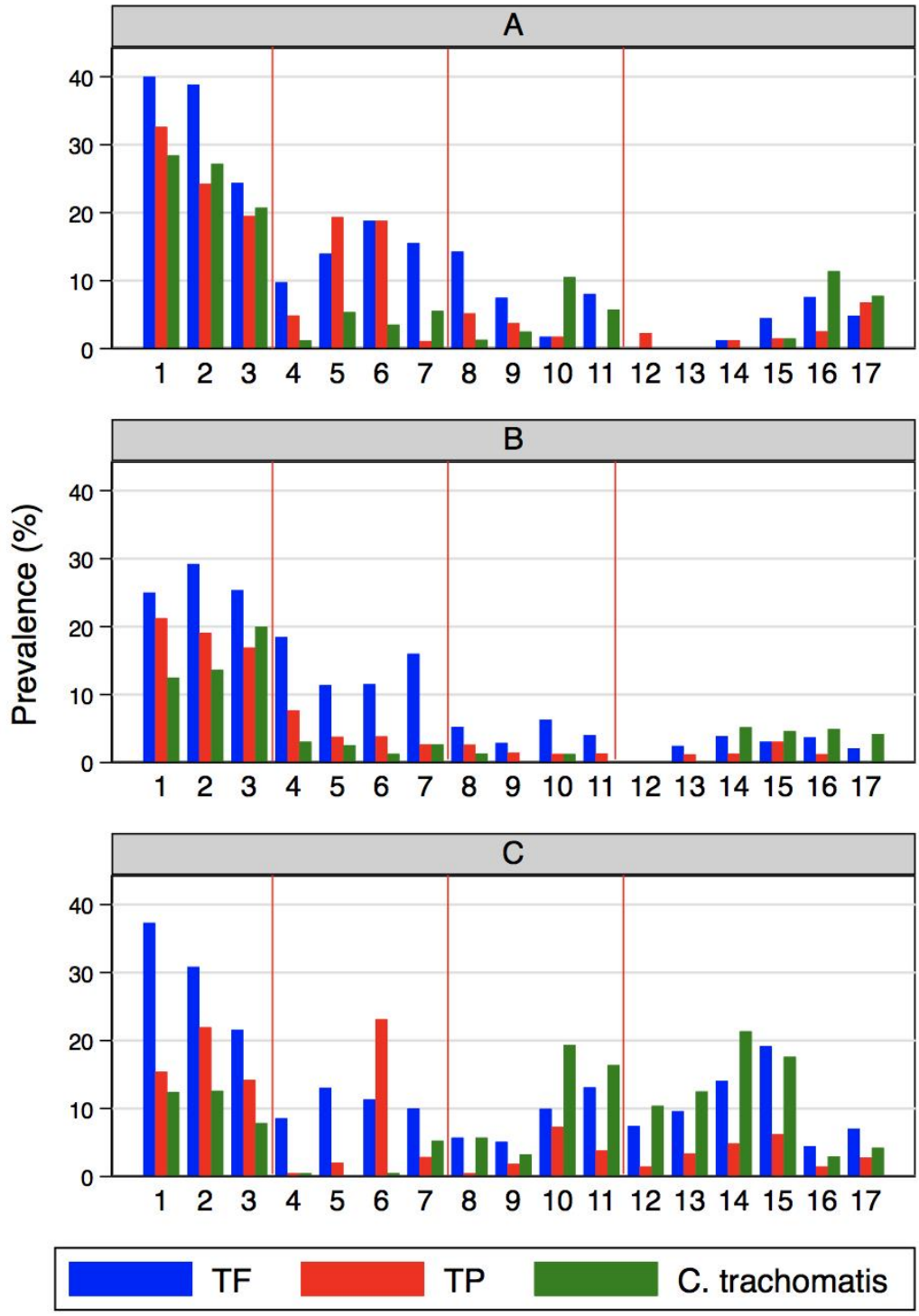
The number of individuals with categorized proportions of time-points with TF, TP and *C. trachomatis* infection is shown in Table 1. At baseline, 93 (20.8%) had some degree of conjunctival scarring.

Table 1: The number of individuals with *C. trachomatis* infection, TF and TP, categorized by the proportion of time-points at which *C. trachomatis* infection, TF and TP were detected as a percentage of the total number of time-points that individual was seen.

Proportion of time-points	*C. trachomatis*		TF		TP	
	n	(%)	n	(%)	n	(%)
None	229	(51.1)	208	(46.4)	263	(58.7)
<10%	78	(17.5)	52	(11.6)	71	(15.8)
10-19%	79	(17.6)	71	(15.9)	48	(10.7)
20-29%	43	(9.6)	56	(12.5)	41	(9.2)
30% +	19	(4.2)	61	(13.6)	25	(5.6)

The odds of TF were estimated to be higher in females (OR=1.49, 95%CI=1.05-2.11, P=0.025) and lower with each additional year of age (OR=0.65, 95%CI=0.59-0.71, p<0.0001) in the longitudinal dataset. The odds of TP were also estimated to reduce with age (OR=0.79, 95%CI=0.71-0.88, p<0.0001) but the evidence of an association with sex was much weaker (OR=1.39, 95%CI=0.92-2.09, p=0.119. There was no difference between children included and those excluded in the analysis in terms of sex, baseline TF (OR=1.32, 5%CI=0.86-2.04, P=0.203), baseline TP (OR=1.46, 5%CI=0.85-2.50, P=0.165) and baseline infection (OR=1.41, 5%CI=0.78-2.54, P=0.257), although those excluded tended to be slightly older on average than those included (mean age 7.4 vs 6.8, p<0.001).

Supplementary Figure 2. The prevalence of *C. trachomatis* infection and clinical signs at each time-point in all 17 time-points in the 448 individuals with outcome data, split by village. Red vertical lines indicate MDA treatment.



***C. trachomatis* infection**

C. trachomatis was detected in 219/448 (48.9%) at one or more time-points. The prevalence of infection is shown for each time-point in Figure 2. The proportion of time-points that each individual had infection is shown in Table 1. The median proportion of time-points infected

among the 219 individuals who had *C. trachomatis* detected on at least one occasion was 12.5%, which was equivalent to ~2 time-points if someone had been seen on all 17 visits.

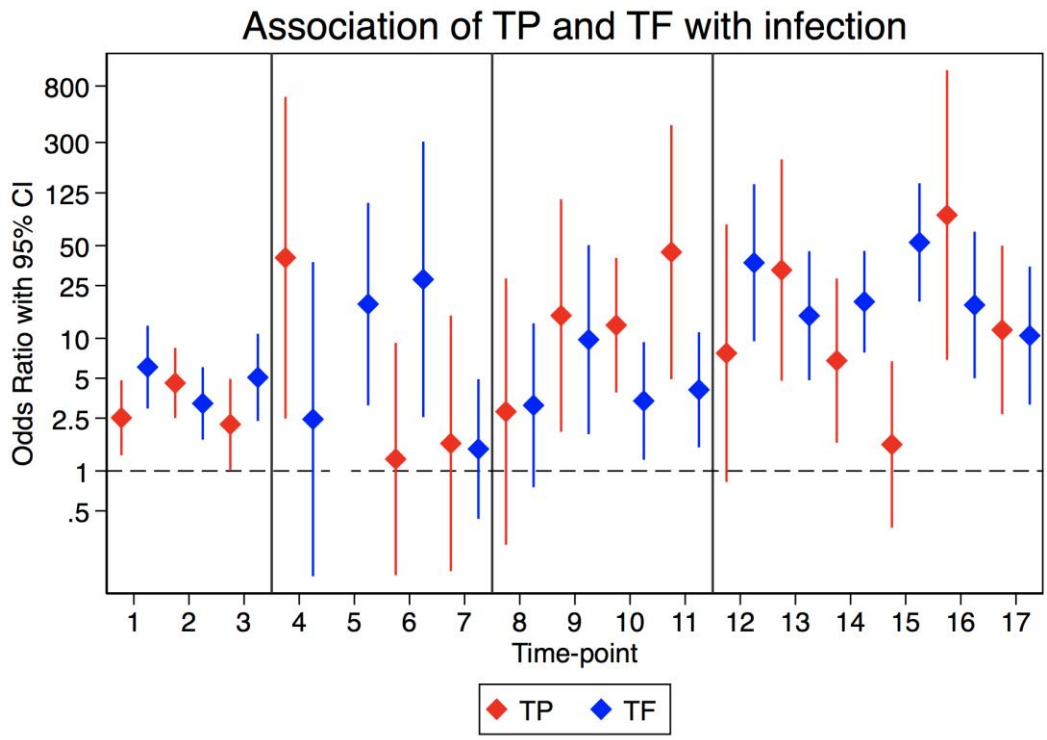
Infection prevalence declined following each round of MDA, however, at time point 10 (9 months after second MDA) it had increased and at time-point 14 (9 months after third MDA) infection prevalence had returned to pre-MDA levels (10-15%). Infection prevalence dropped again by time-points 16 and 17. Further examination of infection and clinical sign prevalence in each of the three villages showed that the majority of infection and TF at later time-points was found in only village "C" (Supplementary Figure 2). This village is located in a different administrative district, which received government-administered MDA treatment in 2015, possibly explaining the drop in infection prevalence at time-points 16 and 17 (Supplementary Figure 2).

In a mixed effects logistic regression of infection at any time-point, female sex (OR=1.7, 95%CI=1.25-2.34, p=0.001) and younger baseline age (OR=0.82, 95%CI=0.75-0.99, p<0.0001) were significantly associated with *C. trachomatis* infection.

Clinical disease and infection

Overall, there was a strong association between *C. trachomatis* infection and TF (OR=11.6, 95%CI=8.9-15.0, P<0.0001) and TP (OR=9.6, 95%CI=7.1-12.8, P<0.0001) in the longitudinal dataset (adjusted for baseline age and sex). The odds ratios for TP and TF as predictors of *C. trachomatis* infection (adjusted for TP/TF, age at baseline and sex) at each time-point were generally similar to or slightly higher after the initiation of MDA, however, confidence intervals were much wider (Supplementary Figure 3).

Supplementary Figure 3. The association between *C. trachomatis* infection and TF (blue points) and TP (red points) at each timepoint. Odds ratios with 95% confidence intervals are plotted. Vertical red lines indicate MDA. The OR for TP at time-point 5 is missing as there was insufficient data to generate a result.



Scarring progression

Overall, scarring progression was observed in 103/448 (23.0%) participants (Table 2). There were 307 (68.5%) who had no scarring; 38 (8.5%) with unchanged scarring; 48 (10.7%) with incident scarring; and 55 (12.3%) with increasing scarring.

Table 2: Scarring progression category by presence of *C. trachomatis* infection and clinical features, detected at one or more time-points.

		Overall scarring		Scarring subgroups			
Clinical phenotype	Total	Progression	No progression	No scarring	Unchanged scarring	Incident scarring	Increasing scarring
Total	448	103 (23%)	345 (77%)	291 (65%)	54 (12%)	48 (10.7%)	55 (12.3%)
No infection/TP/TF	122	17 (13.9%)	105 (86.1%)	95 (77.9%)	10 (8.2%)	11 (9%)	6 (4.9%)
Any TF	240	67 (27.9%)	173 (72.1%)	136 (56.7%)	37 (15.4%)	31 (12.9%)	36 (15%)
Any TP	185	67 (36.2%)	118 (63.8%)	89 (48.1%)	29 (15.7%)	28 (15.1%)	39 (21.1%)
Any *C. trachomatis*	219	55 (25.1%)	164 (74.9%)	137 (62.6%)	27 (12.3%)	29 (13.2%)	26 (11.9%)

The relationships between scarring progression and proportion of time-points when *C. trachomatis* was detected or signs of inflammation (TF and TP) seen, adjusting only for age and sex, are shown in Table 3. In these models strong evidence was found of an association between

progression and both TP and TF, but the association between infection and progression was weaker. There was also evidence of a greater odds of progression in females compared to males.

Table 3: Univariate associations between overall scarring progression and categorized proportions of time-points with *C. trachomatis* infection, TF and TP, adjusted for age and sex. The overall *P* value for each model is shown in line with the variable name.

	n/N	(%)	OR	95% CI	P value
C. trachomatis					0.041
None	48/229	(20.9)			
<10%	12/78	(15.4)	0.64	0.3 - 1.3	
10-19%	21/79	(26.6)	1.29	0.7 - 2.4	
20-29%	15/43	(34.9)	1.91	0.9 - 3.9	
30% +	7/19	(36.8)	2.20	0.8 - 6.0	
TF					0.0004
None	36/208	(17.3)			
<10%	8/52	(15.4)	0.93	0.4 - 2.2	
10-19%	18/71	(25.4)	1.96	1.0 - 3.9	
20-29%	15/56	(26.8)	1.92	0.9 - 4.0	
30% +	26/61	(42.6)	4.41	2.2 - 8.8	
TP					<0.0001
None	36/263	(13.7)			
<10%	17/71	(23.9)	2.14	1.1 - 4.2	
10-19%	15/48	(31.3)	3.08	1.5 - 6.4	
20-29%	21/41	(51.2)	7.26	3.5 - 15.0	
30% +	14/25	(56.0)	8.41	3.5 - 20.2	
Age at baseline			1.01	0.9 -1.1	0.853
Sex					
Male	38/206	(18.5)			
Female	65/242	(26.9)	1.62	1.0 -2.6	0.036

In a multivariable model (Table 4) for scarring progression (retaining infection, TF, TP, age and sex), the strong relationship between increasing proportion of time-points with TP and scarring progression remained. Female sex was marginally associated. There was no association with either TF or infection, suggesting that the univariate associations between TF and infection with scarring were mediated through TP.

Table 4: Multivariable logistic regression model for overall scarring progression, retaining categorized proportions of time-points with *C. trachomatis* infection, TF and TP, adjusted for age and sex. The overall P value for each variable is shown in line with the variable name, derived from a likelihood ratio test of the model including versus excluding that variable.

	OR	95% CI	P value
C. trachomatis			0.4396
None			
<10%	0.49	0.2 - 1.1	
10-19%	0.90	0.5 - 1.8	
20-29%	0.80	0.3 - 1.9	
30% +	0.72	0.2 - 2.5	
TF			0.2535
None			
<10%	0.85	0.3 - 2.1	
10-19%	1.07	0.5 -2.3	
20-29%	0.84	0.3 - 2.0	
30% +	2.09	0.9 - 5.0	
TP			<0.0001
None			
<10%	2.19	1.1 - 4.4	
10-19%	2.94	1.3 - 6.7	
20-29%	6.67	3.0 - 14.9	
30% +	7.48	2.7 - 20.8	
Age at baseline	1.11	1.0 -1.3	0.128
Sex (Female)	1.65	1.0 -2.7	0.051

The analysis was repeated, restricted to individuals with (a) no scarring at baseline, and (b) some scarring at baseline, in order to differentiate between factors associated with incident scarring and progression of pre-existing scarring, respectively. In univariate models there was evidence for associations between episodes of *C. trachomatis* infection, TF and TP with incident scarring (Supplementary Table 1). In the multivariate model however, only TP was significantly associated with incident scarring, again suggesting that the effect of infection and TF was mediated through TP (Supplementary Table 2). Neither infection, TF nor TP were significantly associated with progression of pre-existing scarring in either univariate models or multivariate models. There was a trend for increasing risk of progressive scarring with increasing episodes of TP however, this effect was not statistically significant, possibly due to the relatively small sample size in this group with pre-existing scarring at baseline. These data from children with

pre-existing scarring did not demonstrate that additional episodes of *C. trachomatis* infection were associated with further progression of scarring. Female sex was associated with an increase in pre-existing scarring but not with incident scarring. There were no associations with age.

Supplementary Table 1: Univariate logistic regression models for a) incident scarring and b) increase in pre-existing scarring, in individuals with (a) no scarring at baseline or (b) some scarring at baseline. Univariate associations between scarring and infection, TF and TP were adjusted for age at baseline and sex.

A) INCIDENT SCARRING	n/N	%	OR	95% CI	P value
	48/339	14.16			
C. trachomatis					0.0307
None	19/173	10.98			
<10%	5/67	7.46	0.68	0.24 - 1.91	
10-19%	10/53	18.87	1.98	0.84 - 4.64	
20-29%	9/32	28.13	3.40	1.33 - 8.68	
30% +	5/14	35.71	4.75	1.42 - 15.90	
TF					0.0013
None	17/172	9.88			
<10%	2/37	5.41	0.62	0.13 - 2.86	
10-19%	6/47	12.77	1.63	0.58 - 4.58	
20-29%	8/43	18.60	2.54	0.96 - 6.71	
30% +	15/40	37.50	7.35	2.91 - 18.54	
TP					<0.0000
None	20/222	9.01			
<10%	4/49	8.16	0.99	0.32 - 3.09	
10-19%	5/29	17.24	2.40	0.80 - 7.19	
20-29%	15/28	53.57	13.42	5.34 - 33.73	
30% +	4/11	36.36	6.03	1.61 - 22.63	
Age at baseline			0.99	0.84 - 1.15	0.859
Sex					
Male	24/168	14.29			
Female	24/171	14.04	0.98	0.53 - 1.80	0.947

B) PROGRESSIVE SCARRING	n/N	%	OR	95% CI	P value
	55/109	50.46			
C. trachomatis					0.3973
None	29/56	51.79			
<10%	7/11	63.64	0.93	0.21 - 4.18	
10-19%	11/26	42.31	0.51	0.18 - 1.46	
20-29%	6/11	54.55	0.80	0.20 - 3.18	
30% +	2/5	40.00	0.62	0.09 - 4.36	
TF					0.5382
None	19/36	52.78			
<10%	6/15	40.00	0.68	0.19 - 2.40	
10-19%	12/24	50.00	1.01	0.33 - 3.09	
20-29%	7/13	53.85	1.19	0.31 - 4.60	
30% +	11/21	52.38	0.87	0.26 - 2.91	
TP					0.1783
None	16/41	39.02			
<10%	13/22	59.09	2.17	0.73 - 6.46	
10-19%	10/19	52.63	1.79	0.57 - 5.63	
20-29%	6/13	46.15	1.44	0.40 - 5.23	
30% +	10/14	71.43	3.63	0.94 - 14.04	
Age at baseline			1.02	0.84 - 1.24	0.839
Sex					
Male	14/38	36.84			
Female	41/71	57.75	2.34	1.04 - 5.27	0.039

Supplementary Table 2: Multivariable logistic regression models for a) incident scarring and b) increase in pre-existing scarring, in individuals with (a) no scarring at baseline or (b) some scarring at baseline.

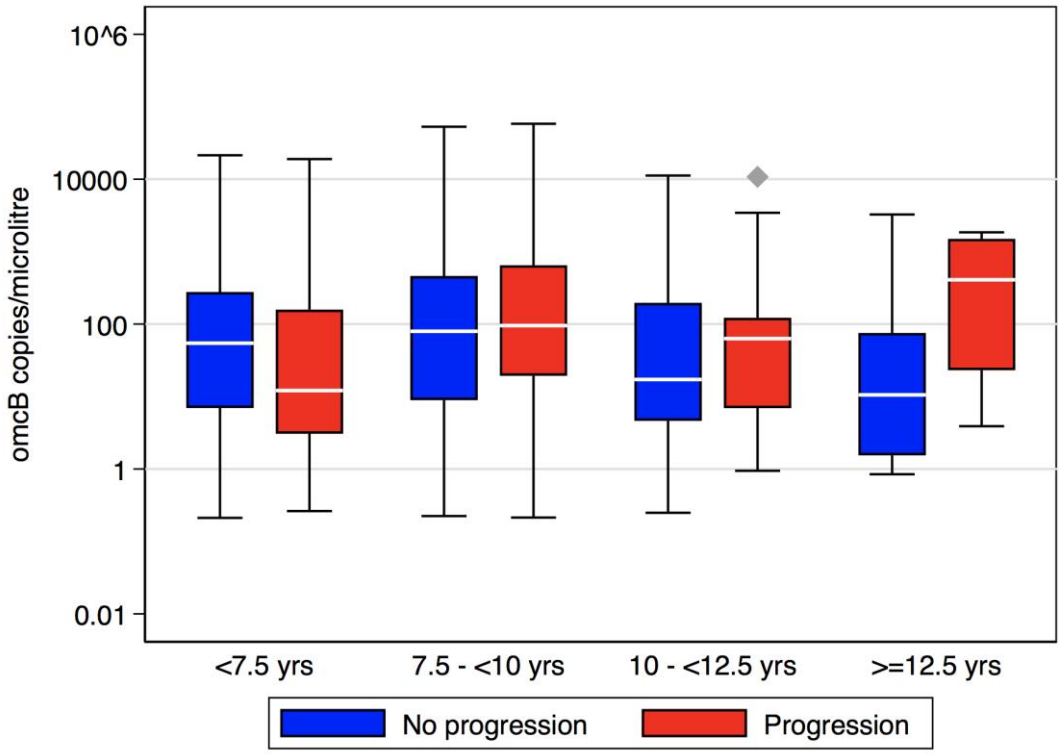
A) INCIDENT SCARRING	OR	95% CI	P value
C. trachomatis			0.3477
None			
<10%	0.46	0.14 - 1.48	
10-19%	1.52	0.57 - 4.04	
20-29%	1.19	0.36 - 3.93	
30% +	1.90	0.38 - 9.46	
TF			0.1472
None			
<10%	0.70	0.14 - 3.41	
10-19%	0.71	0.20 - 2.49	
20-29%	0.75	0.20 - 2.75	
30% +	2.77	0.79 - 9.65	
TP			0.0003
None			
<10%	1.04	0.30 - 3.56	
10-19%	2.00	0.57 - 7.08	
20-29%	11.97	3.74 - 38.31	
30% +	3.81	0.76 - 19.22	
Age at baseline	1.16	0.95 - 1.42	0.149
Sex (female)	0.95	0.46 - 1.94	0.885

B) PROGRESSIVE SCARRING			
C. trachomatis			0.5287
None			
<10%	0.89	0.16 - 4.87	
10-19%	0.42	0.13 - 1.38	
20-29%	0.41	0.07 - 2.35	
30% +	0.31	0.03 - 3.26	
TF			0.9483
None			
<10%	0.76	0.19 - 3.07	
10-19%	1.01	0.28 - 3.62	
20-29%	0.84	0.16 - 4.46	
30% +	0.59	0.13 - 2.63	
TP			0.1777
None			
<10%	2.26	0.73 - 6.98	
10-19%	2.95	0.76 - 11.48	
20-29%	1.51	0.40 - 5.79	
30% +	6.30	1.23 - 32.26	
Age at baseline	0.95	0.74 - 1.22	0.678
Sex (female)	2.54	0.91 - 7.03	0.074

C. trachomatis infection load

Bacterial load in *C. trachomatis* positive individuals was equivalent between people with and without scarring progression, using data from all time-points (adjusting for age at baseline, sex and pre/post-MDA period) (OR=1.1, 95% CI=0.86-1.42, p=0.45). There was evidence of an association between age at time-point (in years) and infection load among *C. trachomatis* positive individuals, with lower loads in older individuals (OR=0.91, 95%CI=0.86-0.97, p=0.004) (adjusting for sex and MDA period). Bacterial load in *C. trachomatis* positive progressors and non-progressors was plotted across different age groups (derived from age in years at time-point) to determine whether the association between scarring progression and load varied by age. In the oldest age group, progressors had a slightly higher infection load relative to non-progressors, Figure 4, which was supported by evidence for an interaction between age group and progression in their association with bacterial load (p=0.012). The model including the interaction explained the data better than the model without the interaction (p=0.016).

Figure 4. Distribution of *omcB* load in scarring progressors and non-progressors, split by age group at time-point.



Discussion

The development of trachomatous scarring is probably the result of a complex interaction between *C. trachomatis* infection, variation in the host immune response and possibly other pro-inflammatory stimuli. However, long-term data exploring the relative contributions of these factors to the development of scarring in children are limited.

In this cohort of Tanzanian children aged 6-10 years old at baseline we found that 23% had trachomatous scarring progression over the course of four years. Roughly half of this was new scarring whilst the other half involved progression of pre-existing scarring. Scarring progression was strongly associated with increasing frequency of conjunctival papillary inflammation (TP).

We found only weak evidence of an association between *C. trachomatis* infection and scarring progression. Increasing proportions of infection episodes were associated with incident scarring, however, multivariable analysis showed that this effect was mediated through TP. These data suggest that other factors, in addition to *C. trachomatis* infection, are important determinants of the development of TP and progression of scarring. The data also indicate that the clinical sign of TF has no association with scarring progression after adjusting for TP, suggesting that TF is not a direct cause of, nor the best prognostic marker for scarring progression. For this purpose, TP prevalence might be a more informative clinical marker, and control programs could consider using TF in combination with TP prevalence to predict future risk of scarring.

Our finding that increasing frequency of papillary inflammation is strongly associated with scarring progression has consistently been reported by other studies,[233] however, the relationship between chlamydial infection and scarring progression is less definitive.

The first longitudinal study to try to investigate scarring progression was conducted by Dawson et al in Tunisia, starting in the late 1960's. They examined a group of children and younger adults (n=213) on two occasions about 14 years apart; no tests for *C. trachomatis* infection were performed. TI (P3) was the strongest risk factor for developing severe scarring (RR=18), whilst TF had a weaker association (RR=2.8).[269] Interestingly, there also appeared to be increased scarring risk associated with P2.

West et al investigated the relationship between "constant severe trachoma" (TI diagnosed on more than half examinations) in a group of children (n=190) examined on four occasions during the baseline year and again at 7 years.[270] Constant severe trachoma (TI) was associated with

increased risk of developing scarring by 7 years. TF alone was not associated with development of scarring.

Only one study, by Wolle et al, has previously examined the relationship between *C. trachomatis* infection and the subsequent development of scarring in children.[271] They reported a five-year cohort of Tanzanian children (n=189) that were examined on five occasions during the first 18 months and again at 5 years. They found that incident scarring over the five years was associated with constant inflammation and/or constant *C. trachomatis* infection during the first 18 months (OR 5.74, 95%CI 2.39-13.8). However, the effects of infection and inflammation were not modelled independently of each other, possibly due to sample size limitations, therefore, the contribution from infection remained unclear.

Burton et al found a strong association between progression of pre-existing scarring in adults and increasing episodes of papillary inflammation (P2/P3) in Ethiopia (n=585; OR 5.93, 95%CI 3.31–10.6, p<0.0001) and Tanzania (n=577; OR 5.76, 95%CI 2.60–12.7, p<0.0001).[260] The study examined and sampled participants on a 6-monthly basis for two years. Episodes of *C. trachomatis* infection were very infrequent, and they were not associated with disease progression.

Our finding that *C. trachomatis* infection was marginally associated with incident scarring (before adjustment) but not progressive scarring suggests that chlamydial infection may be important in initiating the scarring disease process. However, once scarring is established, other factors driving TP are perhaps increasingly important in scarring progression. However, the analysis of progression of pre-existing scarring was limited by a smaller sample size.

Conjunctival inflammation may be associated with other bacterial or viral infections, or with allergic conjunctivitis.[68, 70]. Several cross-sectional studies have found associations between non-chlamydial ocular bacterial infections and active trachoma (TF/TP), conjunctival scarring, trichiasis/recurrent trichiasis and corneal scarring.[3, 69, 245, 246, 272-274] A recent longitudinal study in 452 Tanzanian adults found that ocular commensal and pathogenic non-chlamydial bacterial infections were more common in scarring progressors relative to non-progressors and that, after adjusting for other factors, ocular infections were marginally associated with 2-year scarring progression.[248]

C. trachomatis infection might damage the barrier function or homeostasis of the conjunctival epithelium, such that external stimuli cause inflammation where they would not have done

otherwise. The expression of mucins has consistently been found to be dysregulated in active and scarring trachoma, supporting this hypothesis.[23, 166] Long-term exposure to cooking smoke has also been linked to conjunctival inflammation,[275] particularly affecting women, however, a Tanzanian longitudinal study did not find any association between exposure to cooking fires and incident scarring.[276] Use of traditional medicines might also have a role, as could differences in diet or coinfections that lead to variation in host immune responses.

Genetic or epigenetic factors might also contribute to differences in host inflammatory responses. Several studies have reported associations between genetic differences and risk of trachoma, including one genome-wide association study.[101, 102, 277-281] Further detailed investigations of ocular microbial infections and host genetics are required to establish their roles in this disease.

There was a marginal association between female sex and overall scarring progression. Further analysis revealed that female sex was associated with progression of pre-existing scarring but not with incident scarring. Female sex was also associated with *C. trachomatis* infection, TF and very marginally with TP. These findings are difficult to reconcile; there were associations between infection and incident (but not progressive) scarring, and between infection and female sex, whereas there was no association between infection and progressive scarring and only a marginal link between TP and female sex, yet female sex was associated with scarring progression but not incidence. Our findings also contrast those of previous longitudinal studies (reviewed in where female sex was associated with scarring incidence but not progression.[233] The numbers in our analysis of pre-existing scarring were relatively small, therefore a larger sample size might explain these findings. Nevertheless, overall females were at greater risk of scarring after adjustment for *C. trachomatis* infection and TP, suggesting that this effect was mediated by another mechanism.

We found no association between age and scarring progression, which may be due to the limited age range of our study participants. Age was strongly associated with *C. trachomatis* infection, TF and TP, all of which were more common in younger participants, perhaps suggesting that some level of protective immunity develops. There was some evidence that in the oldest age group, scarring progressors had a higher chlamydial load relative to non-progressors, perhaps suggesting that their immunity was impaired, although this evidence was very weak.

A strong association was seen between infection and TF or TP. In contrast to previous reports the association did not diminish after MDA,[282] however, *C. trachomatis* infection was still

relatively common and the effect estimates had very wide confidence intervals. Infection prevalence recovered after MDA to near pre-MDA levels, whereas TP and TF prevalence remained low post-MDA. A study from central Tanzania has shown a significant reduction of both *C. trachomatis* infection and TF after each of Four annual rounds of MDA in children aged 1 year and above, where MDA coverage was >80%.[283] The overall TF prevalence was reduced from 27.3% at baseline to 9.4% in fourth year (one year after final MDA) and infection prevalence was reduced from >20% in 1-9-year olds to <10% in the fourth year. The recovery of infection prevalence in our study may be due to inadequate MDA coverage and interaction with untreated neighboring communities. Community members are mainly pastoralists and often away from their homes during the day. These results highlight the need for high MDA coverage to effectively bring *C. trachomatis* prevalence under control. However, the sustained reduction in TP prevalence may be promising in terms of reducing scarring risk.

Conclusion

Progressive scarring trachoma was strongly associated with papillary inflammation in this longitudinal study. *C. trachomatis* infection was no longer associated with scarring progression after adjustment for TP, suggesting that the effect of infection is mediated through TP, and that other factors contributing to TP in addition to *C. trachomatis* infection are important determinants of disease progression. Further research is required to understand what these factors are; they might include other ocular or non-ocular infections, genetic variation in host immune responses or environmental factors. Females were at greater risk of *C. trachomatis* infection, clinical inflammation and scarring progression. The addition of TP to trachoma control programs might provide a more accurate indication of the need for future trichiasis interventions, which are likely to be needed for many years to come in this community.

9. Discussion

This chapter discusses all the findings in this thesis, implications for trachoma control, study limitations and proposed areas for future work

9.1. Implications for Trachoma Control

Rate of Scarring Progression

Evidence of scarring progression was found even at this very early stage of life in children in this trachoma endemic setting. A total of 103 (23.0%) out of 448 children had scarring progression. Of those who progressed, 48 (10.7%) had incident scarring and 55 (12.3%) had progression of pre-existing scarring into more severe scarring. Incident and progressive scarring rates per year were 2.7% and 3.1%, respectively. These were broadly similar to the rates reported in earlier studies discussed below.[260, 269-271, 284] The rate of progression in this community was lower than expected (23% versus 30%); that was probably because of our previous prediction which was relied on microscopic scarring detectable by IVCM which is not clinically apparent.

One hundred and eleven children were not seen during the last two time-points. Of these, 63 children were seen during time-points 13 to 15. We compared the baseline image to the latest (timepoint 13, 14 or 15) to determine whether they had progressed. We found a similar rate of scarring progression among these 63 individuals, compared to the overall scarring progression in children who had outcome data in the last two time-points. Fourteen (22.2%) out of 63 had scarring progression, in which 2 (3.2%) were new cases while 12 (19.0%) had progression of pre-existing scarring.

There are several studies that have reported incidence and progression of scarring from different geographical locations and ages. A 14 year study from Tunisia, which included children and younger adults (<30 years), found 17.1% (14/82) had incident severe scarring (developed from normal to scarring) with an incident scarring rate of 1.2% per year.[269] Individuals reported to progress from pre-existing scarring into more severe scarring were 53.8% (70/130), with a progression rate of 3.8% per year. In the Tunisian study there were no observations made between the baseline and 14-year follow-up. No tests for infection were performed at any stage.

Three longitudinal studies have been conducted in central Tanzania. The first, a 7 year study of children aged 1-7 years, found incident scarring developed in 29.2% (28/96) of those who were documented to have constant severe trachoma and in 9.6% (9/94) of children who did not have constant severe trachoma. Constant TI was diagnosed if children were found to have at least 3 out of 4 examinations with TI. This gives incidence rates of 4.2%/year and 1.4%/year, respectively.[270] In the second study of a cohort of children followed for five years, 17.4% (32/183) showed incident scarring with rate of 3.5%/year.[271]

Neither of these two studies from central Tanzania reported on progression of previous pre-existing scarring. A third, separate 5 year study of all ages from central Tanzania examined both incident and progression of pre-existing scarring: 20.4% (75/367) had incident scarring, (rate of 4.1%/year) and 47.1% (40/85) had progression of pre-existing scarring (rate of 9.4%/year).[284]

Two similar studies each conducted for two years in adults from Ethiopia and Tanzania found respectively 23.1% (135/585) and 30.0% (173/577) of individuals progressed from pre-existing into more severe scarring, with yearly progression rates of 11.6% and 15.0%.[260]

Collectively all the studies referred to above, including the data presented in this thesis, show an ongoing process of scarring progression in trachoma endemic community settings. The rates are consistently higher for the progression of pre-existing scarring, compared to the development of incident scarring. This suggests that, scarred/compromised tissues are easier to progress into severe than unscarred one.

S*carring progression and active trachoma (TF/TP) and C. trachomatis infection*

In this cohort study we found only relatively weak evidence directly linking detection of *C. trachomatis* infection to scarring progression. In contrast, we found evidence of a strong association between increasing numbers of TP inflammatory episodes and the development of scarring.

Previous cohort studies have had fewer, less frequent observations and fewer or no laboratory testing for *C. trachomatis.* However, a consistent pattern has emerged from these, that similarly links the development and progression of scarring to diffuse, papillary conjunctival inflammation characterised by P2 or P3 (TI / TP).

In the Tunisian study TI was reported as the main predictor of scarring progression.[269] Interestingly the authors emphasised the importance of the P2 sign in addition to P3 as a precursor to development of scarring. TF and household density were also reported to associate with scarring progression, but their association was far weaker than TI. The study did not report association of sex or infection.

As outlined above, the rate of scarring progression in the first study from central Tanzania was found to be strongly associated with constant TI, in which incident scarring was more than three times higher in the constant TI group than in the controls.[270] Children were matched for age, sex and neighbourhood. In the two later studies from central Tanzania, incident scarring was

strongly associated with TI and moderately with age, female sex and TF in children.[270, 271] None of these studies reported a clear association with scarring progression and *C. trachomatis* infection. The studies in Ethiopia and Tanzania in adults found strong association between scarring progression and P2/P3, but not chlamydial infection.[260]

These studies all seem to point to TP / TI being the clinical correlate of the disease process that leads to scarring, rather than TF. One interpretation of this observation might be that in an environment in which many people are repeatedly exposed to *C. trachomatis,* whether or not they progress to develop scarring depends much more on how they react to the infection. If they react with a very severe inflammatory phenotype (TP/TI) they are at substantially increased risk of scarring, and it could be that this is determined by the immunogenetic background of the individual. Alternatively, other environmental (such as diet, allergy, dust) or non-chlamydial infectious factors causing TP/TI might contribute to driving the scarring process after initiation by *C. trachomatis*.

We found a stronger association between TF and *C. trachomatis* infection than TP and infection. This difference might be because TF was mainly caused by *C. trachomatis* infection while TP can have other causes. At time-points 5 and 6 we observed a peak in the prevalence of TP in two of the three study villages which did not correlate with *C. trachomatis* infection. This TP may have been caused by another infection and further study is required to find out the cause, and subsequently, whether this can also contribute to driving scarring.

Presence of scarring progression even at a young age, as observed, implies that many years later development of scarring complications will persist in this community or others even when infection was formerly brought under control. There is evidence from The Gambia in which *C. trachomatis* infection and active trachoma were reported to have been at very low levels for some years but there was ongoing incidence of trichiasis.[285, 286]

Interestingly, progression of scarring was observed in some children who were never diagnosed as having either infection or any clinical signs of active trachoma throughout the four years of study. Previous studies which categorized children by age (0-4, 5-14 and ≥15 years) reported clinical signs of disease and infection in children 5 years and above that were resolved within three months.[27-29] The age range of the present cohort study was between 6 and 10 years at baseline. This finding of progression of scarring in the absence of a single documented episode of infection or clinical sign throughout the study time is perhaps most likely due to the observation intervals. For cost and logistical reasons, the study reassessed the cohort at intervals

of three months. This gap between examinations may well be long enough for someone to be infected or develop clinical signs and then for these to fully resolve.

An alternative explanation might be that scarring progression in individuals without documented infection or disease might be due to the background of high infection endemicity, during the years of their earlier childhood. Children who had been repeatedly exposed to infection before joining the cohort may already have initiated an ongoing immunological process that led to scarring progression.

What is driving TP leading to scarring?

Several studies have reported other ocular bacteria, rather than chlamydia, to be associated with trachomatous inflammation. The most commonly reported pathogenic and nonpathogenic bacteria found to be associated with active trachoma are *Haemophilus influenzae*, *Haemophilus aegyptius*, *Streptococcus pneumoniae, Moraxella spp*, *Neisseria spp*, *Staphylococcus aureus, Streptococcus viridians, Corynebacterium xerosis, Staphylococcus pyogenes, Escherichia coli* and diphtheroids.[22, 239-241, 246] Recently Hu and colleagues have reported an association between trachomatous scarring progression and non-chlamydial ocular bacterial infection in adults.[248] Progression of scarring was assessed by comparing photographs of baseline and two years and microbial results were obtained through culture. An association was seen between progression of scarring and the presence of pathogens with or without commensal organisms, however, the association was no longer statistically significant when the analysis included only commensal microorganisms. This association between progression of scarring and other ocular bacteria increases confidence that other factors may contribute to TP, however, the relationship between conjunctival inflammatory signs and ocular bacteria was not evaluated in Hu et al's study. However inversely, cross sectional study in central Tanzania in women aged between 18 and 47 years found no association between scarring and non-chlamydial bacterial carriage.[249]

The interaction between host and other bacterial communities (microbial dysbiosis), loss of epithelial homeostasis/integrity, host immunogenetics and epigenetics might also cause persistence of TP previously triggered by *C. trachomatis*. These factors are discussed in detail below in the 'future work' subsection. Furthermore, TP can be triggered by many other things such as allergy, viruses, smoke, dust and probably type of diet.

Immune responses, clinical signs and C. trachomatis infection

Several animal model and human studies from other groups investigated associations between trachoma and host immune responses which support our main findings. Studies from chlamydial genital tract infection in mice found IL-22, IL-23 and IFN**γ** were associated with reduction of the infection pathogenesis and suggested pathogenic potential of IL-17;[287, 288] these cytokines where also highly expressed in infected individuals in our study. In human studies IL-1β, TNFα and TGFβ1 were significantly associated with both C. trachomatis infection and trachomatous disease;[289, 290] these proinflammatory cytokines were similarly expressed in our participants. Generally our main findings are consistent with that published elsewhere.[169, 287, 288, 290-296] The differential expression of cytokines associated with Th1 cell and NK cell pathways including *IFN-γ*, indoleamine-2,3-dioxgenase-1 (IDO1) and *IL-12* were diminished after the clearance of infection, suggesting that their activity is tightly regulated in relation to infection. In contrast, Th17 associated cytokine genes were upregulated across all clinical signs and in response to infection. IFN-γ induces IDO1, an enzyme which catabolizes tryptophan.[297] Tryptophan is an amino acid required for ocular chlamydial replication, therefore this mechanism likely inhibits chlamydial growth.

Studies in animal models found that both Th1 and Th17 cells were involved in clearance of *C. trachomatis* infections; however, they were also associated with pathogenic inflammatory disease.[298] The role of Th17 associated mediators in the pathogenesis of ocular *C. trachomatis* infections is not fully understood, however, the same dual protective and pathogenic roles might also be active in humans, perhaps with different contributions during early and late stages of disease. Many studies in animal models reported protective behaviour of Th17 cytokines mostly in extracellular infections, but they are thought to have a pathological effect during *Mycobacterium tuberculosis* infection (discussed in the introduction). However, when IL-17 is deficient in humans, such as in hyper-IgE syndrome with STAT3 gene mutations they are highly susceptible to a number of infections such as *S. aureus*, *Streptococcus pneumoniae* and *C. albicans* .[299] Th17 cells are important in recruiting neutrophils to the site of infection, which clear infection but also cause significant tissue damage. Inflammation (TF/TP) was strongly associated with upregulation of antimicrobial peptides, chemokines, cytokines some EMT markers and matrix modifiers (with the exception of *SPARCL1* which was strongly downregulated). Inflammation was also associated with downregulation of genes associated with cell survival and apoptosis (*TNFRSF1A, TNFRSF1B, CD40, ALOX5 and DUOX2*), and mucins (*MUC5AC* and *MUC7*). A previous study conducted in a neighboring area to this study site found that TF and TP in children were significantly associated with increased expression of *S100A7*, *IL1-7A*, *CCL18*, *CXCL5*, and *CTGF.*[259] Another study from Gambia found higher expression of *TNF-α, IL-1, IL-10 and MMP-9* in children with active trachoma.[164] Studies in adults from Ethiopia and Tanzania

found strong association between inflammation and increased expression of *S100A7, IL-1B, IL-17A, CXCL5, CTGF, CEACAM5, MMP-7, CD83* and reduced *SPARCL1*.[260] Increased expression of *TNF, IL-1β and MMP-9* was also associated with inflammation in adults following trichiasis surgery.[300] These findings and those of this study show consistent upregulation of *IL-17* even after clearance of infection. It is possible that Th17 cells and associated cytokines in the absence of *C. trachomatis* infection might be associated more with pathogenesis than protection.

Increased expression of some EMT markers and reduced expression of *MUC5AC* and *MUC7* may be associated with loss of epithelial homeostasis and barrier function, possibly due to the influx of immune cells. The presence of macrophages and neutrophils likely contributes to the increased expression of MMPs. Initiation of the wound healing process possibly results in the reduced expression of *SPARCL1*, contributing to matrix remodeling, collagen deposition and subsequent conjunctival scarring. This gene expression data implies that inflammation (TP) is associated with an ongoing epithelial-derived innate inflammatory response, perhaps with some interaction with Th17 cells. A loss of epithelial homeostasis due to inflammatory damage and exposure to foreign antigens or microbes could promote a positive inflammatory feedback loop, sustaining chronic TP and leading to accumulating scar tissue. A similar profile of genes were expressed in both TP and TF, however, expression tended to be higher in TP probably because of increased immune cell recruitment to the conjunctiva, which was seen in our previous studies.[259] However, *IL-12β*, *IFN-γ* and *TNF* were consistently more expressed in TF than TP, cytokines which stimulate Th1 cell differentiation and proliferation from naïve CD4 T cells.

Most frequently reported interleukins influencing allergic inflammatory reactions are IL-4 and IL-13 (cause a switch to IgE production by differentiating B cells and mast cell growth factors), IFN-γ (inhibits switching and prevention of specific IgE production), IL-10 (inhibits the activity of IFN-γ and allowing the original IL-4 to proceed in the IgE cascade) and Th2 differentiation factors.[301] However, from this finding (excluding *IFN-γ*) there was no direct evidence of host immune responses (gene expression) related with allergy associated with ocular inflammation. Studies in animal models found strong association between Th2 associated factors with conjunctival allergy.[302-305] Contribution of allergy in trachoma complication is not clearly known.

Impact of Azithromycin on Chlamydia infection, inflammation (TF and/or TP) and gene expression

Prior to MDA treatment the prevalence of infection and clinical signs and host immune responses appeared to be relatively stable, suggesting there was a consistent rate of infection/re-infection in this community. Following MDA, TF, TP and *C. trachomatis* infection

prevalence were strongly reduced, which was reflected by a reduction in the expression of pro-inflammatory cytokines and other genes associated with chlamydial disease three months later. Infection and clinical signs remained at a lower prevalence for most of the remaining study period, with some fluctuation. There was evident variation by village, with one village, Tingatinga, harbouring the majority of infection at later time-points.

Azithromycin treatment reduces infection prevalence if coverage guidance is properly implemented. MDA coverage in this study was not particularly high: First MDA: 72.7% community-wide, increasing to 79.2% (355/448) when considering study participants with sufficient outcome data (retained in the analysis); Second MDA: 42.9% community-wide increasing to 83.5% (374) of study participants; Third MDA, 72.9% community-wide increasing to 76.8% (344) of study participants. Between baseline and the final time-point (18 months post third and last MDA) TF was reduced from 33.8% to 3.5%, TP from 19.6% to 6% and infection prevalence from 15.4% to 4.9%. Low MDA coverage village-wide and being surrounded by untreated communities with similar patterns of life might have significantly contributed to the observed disease and infection prevalences.

Azithromycin was delivered by the field team under the supervision of the district eye coordinator. This was probably more effective because it was directly witnessed that the drug had been taken by the community members. However, this was not the case for tetracycline eye ointment which was given to the community members to be given to infants and pregnant women twice a day for six weeks by themselves. It was not practical for tetracycline eye ointment to be administered by our field team, therefore we are uncertain how good the adherence was, and might represent a source of reinfection in that community if was not properly applied.

A study from central Tanzania showed a significant reduction of both *C. trachomatis* infection and TF after each of three annual rounds of MDA in children aged 1 year and above, where MDA coverage was >80%.[283] The overall TF prevalence was reduced from 27.3% at baseline to 9.4% at the fourth year (one year after finishing MDA). Infection prevalence was reduced from >20% in 1-9-year old to <10% at the fourth year. Another study from the same area in Tanzania reported reductions in infection prevalence from 20.1% and 23.8% at baseline to 4% and 5% at 36 months respectively, one year after the third round of MDA, in two separate cohorts. Likewise TF prevalence was reduced from 30.4% and 30.9% at baseline to 6.1% and 9% at 36 months.[306] No differences in prevalence were found between the cohorts, despite one achieving >90% coverage whereas the other received the standard 80%.

In The Gambia, at a time when trachoma was meso-endemic, Burton and co-authors showed a significant reduction of infection prevalence from 7% to 2% and active trachoma from 8% to 4%, 17 months after finishing three years of MDA with a coverage of 83%.[307] Studies from The Gambia and Tanzania found that communities with extensive contact with untreated populations and immigrants were strongly associated with re-emergence of infection.[308, 309] These studies illustrate that a sustained reduction in TF and infection prevalence can be achieved if a high MDA coverage is maintained with a wider geographical coverage.

The MDA coverage achieved in this study was relatively low (WHO guidance requires no less than 80% MDA coverage of the total population), however, the reduction of infection, TF and TP prevalence was comparable to the other studies reported over a similar timescale, despite those studies reporting >80% coverage. Government-administered MDA was provided to Tingatinga in 2015, which likely contributed to the overall reduction in disease. We were unable to achieve high coverage due to several reasons including individuals being absent in the village during the day (due to taking care of their livestock away from home) and some refused due to cultural beliefs or bad personal history of MDA. Due to budgetary reason and study design, only the three study villages were offered MDA as the rest of the district was not, therefore there was high risk of re-infection due to contact with neighbouring communities.

Other findings have reported variation of azithromycin treatment effectiveness in different disease endemicities. A study in children under 10 years *C. trachomatis* was found to cluster by household after first and second rounds of MDA but was not seen after the third round. Infection was brought under control, however, active trachoma did not cluster at any round of MDA.[310] Hyperendemic areas with an active trachoma prevalence >50% have been reported to require over 7 annual rounds of MDA to bring the prevalence down to 5%,[311] similarly, a study from the same (hyperendemic) area, 2 rounds of annual MDA was administered with an average coverage of >80%, however, an impact survey three years after the last MDA found trachoma was not eliminated.[312] In low trachoma endemic setting both one and three annual rounds of azithromycin MDA with a coverage of >80% at baseline, 1st and 2nd years were enough to bring down both TF and *C. trachomatis* infection prevalence in children below 10 years from 6.5% and 0.8% respectively at the baseline to 2.8% and 0.5% after 36 months.[313] Low trachoma prevalence communities require only a few rounds of MDA, whereas high prevalence communities need several more rounds of MDA to bring the disease under control. In this cohort study, three annual MDAs were sufficient to bring TF from >30% to <5%.

MDA resulted in a reduction of pro-inflammatory gene expression responses that was evident three months post treatment, however, this effect was mostly lost at six months post MDA. The effect was observed in individuals without infection or inflammatory disease, and even in individuals that did not receive treatment, though the effect size was less. These results suggest that azithromycin may act to reduce pro-inflammatory gene expression through some or all of the following mechanisms; through killing *C. trachomatis* and possibly other species, through directly modulating pro-inflammatory signalling pathways, and/or by reducing transmission and exposure to *C. trachomatis* and other species.

Azithromycin has a long half-life in the tissues, though previous findings from several diseases have reported uncertainty of azithromycin's effects on immunomodulation after six months.[314, 315] Direct immunomodulatory effects of azithromycin have previously been reported in the absence of disease. In 12 healthy human volunteers who received azithromycin (500mg/day) for 3 days, blood samples were taken 1 hour (h) before treatment and then 2.5h, 24h and 28 days after treatment.[316] Neutrophil activity was strongly reduced shown by a decrease in azurophilic granule enzyme activities in cells as azithromycin concentration increased in serum and neutrophils. This was associated with a reduction in IL-6 concentration in the serum, delayed oxidative burst and an increase in apoptosis of neutrophils.

Azithromycin immunomodulation occurs through interaction with structural cells such as epithelial or endothelial cells, smooth muscle cells or fibroblasts, and leukocytes.[317] In these immune and non-immune cells azithromycin interacts with phospholipids and Erk1/2, which modulate transcription factors AP-1 and NFκB, resulting in reduced production of pro-inflammatory cytokines in the acute phase, promoting the resolution of chronic inflammation in the late phase, and reduction of mucin (MUC5AC) production in epithelial cells.[317-320] Azithromycin has also been reported to reduce all-cause childhood mortality in <5 year olds, though the mechanism is unclear.[134, 321]

Many studies reported an anti-inflammatory effect of MDA on gene expression (discussed in chapter 7). This might be beneficial in reducing the TP that drives scarring progression, independently of its role in reducing *C. trachomatis* infection. Wolle and colleagues offered azithromycin MDA twice, annually to the community, however, there was no difference in progression of scarring between those who received MDA once and those who received it twice.[284] Currently there is no therapy to halt conjunctival scarring progression and the potential benefits of azithromycin warrant further investigation.

Sex and Age

Trachoma is strongly associated with female sex and increasing age. According to a recent WHO report, females requiring trichiasis surgery outnumber males by four times.[12] In this study, despite the early age group we found a significant increase in scarring progression in females relative to males. This might be caused by more frequent exposure of females to *C. trachomatis,* as they spend more time at home looking after younger siblings (who are most likely to be infected), while males are more likely to spend time taking care of their livestock away from home.

The natural history of trachoma is strongly associated with age. Infection and active trachoma (TF/TP) are commonly diagnosed during childhood, with very few cases of scarring and almost no trichiasis cases. Some degree of natural immunity is thought to develop, leading to little infection/TF detected in adults. The most common features during adulthood are TP, scarring, trichiasis and corneal scarring. This highlights the difference again between TF and TP, with TF mostly associated with *C. trachomatis* infection. The factors causing TP in adults are not known.

Summarizing Implications of this work for trachoma control

In this trachoma endemic community progression of scarring was apparent at a very early age. Progression of scarring complications will therefore persist for many years in this community as this cohort and their peers age. Even after *C. trachomatis* is controlled in this community, trichiasis surveillance and interventions will be required for many years beyond GET2020.

A strong association was found between TF and *C. trachomatous* infection; this was somewhat stronger than the association between TP and infection. Azithromycin MDA was followed by a reduction in both TF and TP. However, infection started to increase again, probably due to low MDA coverage (discussed in chapter 7). In agreement with reported literature (Chapter 4), the reduction in TF prevalence lagged behind the reduction in *C. trachomatis* infection prevalence after the first round of MDA. Therefore, as a proxy marker for *C. trachomatis* infection prevalence in the community, TF is a better clinical sign to use than TP, although the relationship is weakened after MDA with TF overestimating infection prevalence. A cost-effective and simple test for *C. trachomatis* infection in communities for use in trachoma control programs remains desirable.

The strong association between TP and scarring progression and the weak associations between TF and *C. trachomatis* infection with scarring progression raise important questions for trachoma surveillance and predicting future disease burden. The district prevalence of TF alone

is unlikely to provide reliable estimates of future need for trichiasis intervention, whereas TP alone is poorly specific for trachoma. The use of combined TF and TP prevalence in trachoma control programs might be more suitable for predicting future need for trichiasis interventions.

In addition to *C. trachomatis* clearance azithromycin MDA reduces inflammation through its immunomodulatory activities and is used to treat other chronic inflammatory diseases. Given the strong association between TP and scarring progression, azithromycin treatment may be beneficial in reducing risk of scarring progression.

Among the challenges encountered in this community during our visits was the implementation of 'FE' from SAFE strategy. Some of community members responded negatively following our campaign on face washing and improving environmental hygiene. In the future another approach should be implemented, such as conducting a short training of more educated individuals among the community members about the nature of trachoma, mode of transmission and its control, and to ask them to share with their neighbours. Furthermore, to conduct several education meetings with community leaders discussing trachoma so they can discuss with their community members during their formal village meetings. This could be easy to convey the message to the community through their leaders as they are already built trust in them. Printing big coloured pictures showing different stages of clinical trachoma may help to in the explanation.

Limitations of this work

This work has several limitations. The age group of 6-10 years was chosen as it was expected to be the age group in which we were most likely to detect incident and progressive scarring during the four years of study. However, selection of only this age group might have led us to miss some important information as we did not know the participant's clinical and infection histories prior to commencing the study.

Progression of trachoma occurs throughout an individual's life-time, thus, the four year duration of this study is relatively limited and might have led to missing some important features of scarring progression and its relation to clinical signs and infection. To further investigate incident scarring and the role of *C. trachomatis* infection in initiating trachoma it might be necessary to study a younger age group.

Previous findings have reported the clearance of infection and resolution of active trachoma clinical signs within three months, especially for the children aged 5 years and above, hence the

three-month interval used in this study might have also led us to miss some intermediate infection and clinical signs.

We did not record environmental and behavioural variables in this study. These risk factors might have important roles in the development of TP and scarring and might further explain the differences between males and females.

A few genes did not amplify in some samples during qPCR and were therefore not included in the analysis. This might be due to several reasons such as poor choice of primer/probe sets for this population, perhaps due to genetic variation within the population, or low expression of the target genes. We did not examine whether they were associated with infection or clinical signs in a binary (on/off) capacity.

The delta delta CT (2-ΔΔCT) method has been extensively used for relative quantification of qPCR data analysis. However, this method does not consider the different amplification efficiency of various primer pairs, different uses of probes, presence of PCR inhibitors or enhancers and enzymes as it assumes that the amplification efficiency is 100%. 2-ΔΔCT subtracts an unknown background automatically. This may result in generating inaccurate data (and probably misleading interpretation) hence may not be the most suitable calculation method. An alternative approach was reported by Rao et al, Pfaffi et al, and others, in which improved relative quantification of nucleic acid (Individual Efficiency Corrected Calculation) method overcomes all the mentioned weakness of 2-ΔΔCT[322-324]. In this method, PCR efficiency for each individual sample is considered through serial dilution of estimated initial nucleic acid. Note that, qPCR data reported here are MIQE compliant (The protocols used in our analyses are accessible on protocols.io website using the accession number https://dx.doi.org/10.17504/protocols.io.zyhf7t6).

9.2. Future Work

This study has reported on the association between gene expression profiles and presence of clinical signs of trachoma in children at the baseline time-point of this cohort study. It would be desirable to investigate whether any of these genes are also associated with progression of scarring in the longitudinal dataset. This would help to improve our understanding of the molecular immunopathogenic processes that lead to scarring. qPCR data for the expression profile of 46 genes has already been generated for the first five time-points and time-points 7,

9, 11, 13, 15 and 17. It was not within the scope of this thesis to present or analyse these data. Identifying genes associated with resistance and susceptibility to TP/scarring disease would increase our understanding of the immunopathogenesis of trachoma and might inform *C. trachomatis* vaccine design.

It was not feasible to present further data on the immunomodulatory effect of MDA in this cohort after the second and third annual rounds. It would also be of interest to examine whether study participants who received MDA were less likely to develop TP and scarring than their counterparts who did not receive treatment; it was not possible to analyse these data and to be included in this thesis.

Genetic variation in host immune responses might explain why some individuals appear to be resistant to disease while others are highly susceptible (for example some individuals respond with more TP while others do not). There is some evidence that variation in host single nucleotide polymorphisms (SNP) have been found between scarring cases and controls. Several previous studies, mostly from The Gambia, used candidate gene approaches and suggested that scarring and/or trichiasis were associated with a number of HLA alleles, cytokine genes, transcription factors and receptors.[102, 277-281, 325-328] A recent genome wide association study from The Gambia found 27 SNPs which were strongly associated with scarring in adults; however, these did not reach genome wide significance.[101] These studies were case-control and in all age groups, and none assessed the association between these SNPs and longitudinal progression of scarring. It was not possible to investigate the association between genetic variation and development of disease sequalae in this thesis, however, the samples might be valuable for future studies to investigate this question.

During infection, *C. trachomatis* might cause changes to host gene expression that are heritable through cell division (known as epigenetics), resulting in the triggering of chronic inflammation which persists even once infection has been cleared.[102] *C. trachomatis* has a protein nuclear effector that is a histone methylase which might cause chromatin changes to host cells.[329] *C. trachomatis* was reported to induce EMT in cell lines, which was associated with a reduction in E-cadherin through changing DNA methylation status of the E-cadherin promoter and a concurrent increase in the expression of fibronectin and α-SMA, resulting in changes from epithelial to mesenchymal phenotype.[330] The authors suggested that this process might be an early event in the initiation of the scarring process, however, this study was based on laboratory cell lines. Further investigation is required to understand the role of epigenetics in chronic trachomatous inflammation and scarring.

In this study we only tested the expression of selected genes. Whether differences in gene expression persisted into differences in protein levels is not known and would require further examination, as post-transcriptional regulation by miRNA or other mechanisms might have downstream effects as previously reported.[331-333]

It was outside the scope of this project to investigate the role of non-chlamydial infections in driving inflammation. Several studies have reported differences in non-chlamydial infections in trachoma cases and controls. It is unclear however, if these microbes cause disease pathogenesis or whether they are just bystanders, expanding to fill an altered (diseased) mucosal environment. Further questions include whether only pathogenic species promote TP, or whether commensal organisms can become pathogenic in certain circumstances, and whether they initiate the process or they are secondary infections. Only one longitudinal study has investigated the association between non-chlamydial infections and progression of trachomatous scarring in adults.[248] This sample set will have great value for investigating the role of non-chlamydial infections in the incidence and progression of scarring in children.

An imbalance of normal conjunctival microbial communities (dysbiosis) might be initiated by infection with *C. trachomatis* infection. Large-scale studies have been conducted in the intestine and have presented associations between changes of microbial composition and pathogenesis of bowel disease.[334] There has been some recent research in healthy and diseased eyes on polymicrobial communities colonizing the conjunctival epithelia, which have suggested that certain species have a protective immunological role in preventing colonization with pathogenic species.[235, 236 272] Thus far there is just a single conjunctival microbiome study using culture independent 16S-deep sequencing in adults with trachomatous scarring versus controls. This study found a dysbiosis in the conjunctival microbiome that occurred in trachomatous scarring increasing with trachomatous trichiasis.[272] There were no changes in richness and diversity in children with TF compared endemic healthy controls. Changes following *C. trachomatis* infection or disturbed ocular homeostasis resulting from inflammation might lead to microbial dysbiosis and outgrowth of pathogens. Recently a case-control study of conjunctival surface infection in children and adults with trachoma in Sudan found presence association between chlamydia-like organisms and old age but not with clinical signs.[335] Longitudinal investigation of microbial communities in these samples would enhance our understanding of the pathogenesis of trachoma.

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Appendices

Appendix 1: Participant Information and Consent Sheet

The immunological determinants of protective immunity and immunopathology in trachoma

KCMC / LSHTM Trachoma Research Programme
London School of Hygiene & Tropical Medicine

What is this study about?
Trachoma is one of the main causes of blindness in Tanzania. It starts in childhood when children get redness and soreness under their eyelids, caused by infection with a bacteria called chlamydia. As people get older they may develop eyelid scarring causing the eyelashes to turn inwards and rub on the eyeball. This is called trichiasis. This can cause the clear part of the eye to become white leading to blindness and the trichiasis is often painful. Currently, this disease process is only poorly understood and the treatments for it are only partially effective. We believe that the reason some people develop a problem from trachoma is because of the nature of the way their bodies defence systems respond to this infection. In the long term it is hoped that it will be possible to develop a vaccine for this disease. However, before that can happen we need to find out much more about the way the body fights the infection. We would like to follow a group of 650 children aged 6 to 10 years. They would be assessed every three months for

Whose help do we need?
We need the help of some about 650 children of families living in the community.

What will we ask you to do?
We would conduct a census of the village. We will come to visit you at your home. If you agree to participate we will ask you several questions about yourself, such as your age and where you live. The children aged 6-10 years would be recruited into a four year study in which we would come back to see you every three months. We will examine the children's eyes and collect one swab sample by gently rubbing it on the surface of the eye. We would take a photograph of the eyes on each occasion. At the beginning and again at two and four years we would use a special microscope ("camera") to take a photograph of the very fine detail of the surface of the eye.

Every year, in partnership with the regional trachoma control programme the whole community would be offered antibiotic treatment to control the infection, according to the Tanzanian National guidelines.

What benefits are there to taking part in the study?
It is only poorly understood how this disease causes blindness. You will be helping us to answer this question.
We will check the general health of your eyes and refer you to receive appropriate eye care through the Eye Clinic in Moshi or an alternative clinic if it is more convenient.

Are there any risks caused by taking part in the study?
There is no known risk from taking swabs samples, and has been done in many studies conducted in Tanzania and elsewhere. Azithromycin is a very safe medicine which is widely used in Tanzania and elsewhere for trachoma control, as part of the National Trachoma Control Programme's work to stop blindness from this disease.

What tests will we do on the sample?
The eye swab samples will be tested in various ways to try to help improve the understanding of trachoma. Samples will be tested in laboratories in Moshi and London. This will involve tests for chlamydial infection. We will also study the human body's response to the infection to try to understand better how it fights the infection and how the scarring problems develop.

What will happen to the records and photos we keep about your eye?
All the information we collect will be kept confidential. It will be kept securely and only the people organising the study will have access to it.

Do I have to take part in this study?
You do not have to take part in this study, it is entirely voluntary.

Who is doing this study?
This project is a collaboration between the KCMC / LSHTM Trachoma Research Project, International Centre for Eye Health, London School of Hygiene & Tropical Medicine, UK and Kilimanjaro Centre for Community Ophthalmology, Kilimanjaro Christian Medical Centre, Moshi, Tanzania. The study will be coordinated in Moshi by Dr Matthew Burton. The examinations will be conducted by a small team of eye nurses or an eye doctor.
If you have any questions please ask us:

Athumani Mchana Ramadhani
Patrick Massae
KCMC / LSHTM Trachoma Research Project,
KCMC Hospital, Moshi, Tanzania.

Participant Consent Form

The immunological determinants of protective immunity and immunopathology in trachoma

KCMC / LSHTM Trachoma Research Programme
London School of Hygiene & Tropical Medicine

Name of Child: …….
Study Reference Number: | | | | |

I …… (Name of Parent / Guardian) have read / had explained to me the information about the research study. I understand what will be involved in taking part in the study.

…….(field worker) has answered all my questions about the study.
I agree to the above named child to be enrolled in this study.

Signed…….. Date……..

Name:……. Relationship:……

I have explained the purpose of the study to the above subject and I am satisfied that he/she willingly agrees to participate

Signed…….. Date……..

Name:…….

In the event that the patient can not read the above information, an additional witness is required:
I have witnessed the explanation and informed consent to this study of above named patient

Signed…….. Date……..

Name:…….

Appendix 2: Clinical Assessment and Photography Protocol

Clinical assessment will be performed at baseline and at every three months after that. This will involve the following:

1. Examination of conjunctiva and eyelids and grading signs as below
2. Photograph
3. Swabs

Initially on arriving in a Boma, ask for all the children on the enumeration list.
Identify the individual child from the enumeration list. Fill in the card with name and number on it.
Enter the number and date of examination into the database on the field laptop computer.

4.1. Examination:
Patrick Massae and Aiweda Malisa performed all the examinations and grade using the WHO "FPC" system.

Only the left eye to be examined, photographed and swabbed.
Use 2.5x loupe and bright torch, under the Land Rover canopy.
Wear a fresh pair of non-sterile latex examination gloves. These will be changed between each examination, to minimise the risk of cross contamination.

Instill one or two drops of proxymetacaine anaesthetic into the eye conjunctiva. If the dropper touches skin / conjunctiva then discard it.

Step 1: Grading Trichiasis/Entropion and the Cornea:
Inspect the left eye (without touching the eyelids) for trichiasis. Then examine the cornea for signs of scarring. Grade T and C according to the following system. Enter the data into the access database.

a. Trichiasis and/or entropion (T):

T/E 0	No trichiasis and/or entropion.
T/E 1	Lashes deviated towards the eye, but not touching the globe.
T/E 2	Lashes touching the globe but not rubbing the cornea.
T/E 3	Lashes constantly rubbing the cornea.

b. Corneal scarring (C):

C 0	Absent
C 1	Minimal scarring or opacity but not involving the visual axis, and with clear central cornea.
C 2	Moderate scarring or opacity involving the visual axis, with the papillary margin visible through the opacity.
C 3	Severe central scarring or opacity with the papillary margin not visible through the opacity.

Step 2: Lid Eversion and Tarsal Plate grading:
The left upper lid will be everted and the conjunctival surface examined and graded as follows. Record the data in the database.

b. Upper Tarsal Follicles (F):

F 0	No follicles.
F 1	Follicles present, but no more than 5 in zones 2 and 3 together. (1-4)

F 2	More than 5 follicles in zones 2 and 3 together, but less than 5 in zone 3. (5-9)
F 3	Five or more follicles in each of the three zones. (10+)

c. Upper tarsal papillary hypertrophy and diffuse inflammation (P):

P 0	Absent: normal appearance
P 1	Minimal: individual vascular tufts (papillae) prominent, but deep subconjunctival vessels on the tarsus not obscured.
P 2	Moderate: more prominent papillae, and normal vessels appear hazy, even when seen by the naked eye.
P 3	Pronounced: conjunctiva thickened and opaque, normal vessels on the tarsus are hidden over more than half of the surface.

d. Conjunctival scarring (S):

S 0	No scarring on the conjunctiva
S 1	Mild: fine scattered scars on the upper tarsal conjunctiva, or scars on other parts of the conjunctiva.
S 2	Moderate: more severe scarring but without shortening or distortion of the upper tarsus.
S 3	Severe: scarring with distortion of the upper tarsus.

Step 3 – Photography of the tarsal conjunctival:
The tarsal conjunctiva on the left upper lid will be photographed.

Check camera settings:

1. Put the battery and memory card in.
2. Turn camera on.
3. Press "**Info**" button. On the display:

1) Check the battery is fully charged.
2) Check the memory card has enough photos left. You should have space for at least 500 photos.

4. Check that the lens is properly and securely attached to the camera.
5. Set the camera to manual **focus**: (**M**) on the lens
6. Set camera to "**full**" on the lens
7. Set the **lens** to **1:1** (0.314)
8. Set the camera to **aperture** (A) on the dial
9. Press "**Info**" button. Using the display, set the **F-stop** to **F57** by turning the "wind on" dial.
   - Sometimes, it won't let you go to F57. One reason for this may be because the lens is not at 1:1.
10. Press "**menu**" button. Using arrows, move up and down and through the menu options.
    - By pressing the left arrow, you can move up and down through the different menus.
    - Once in the menu you want (e.g. set-up menu), press the right arrow to go to setting you want (e.g. ISO sensitivity, image quality, or image size).
        a.) **ISO sensitivity:** Turn camera on → Press "menu" → Arrow left → Go to set-up menu → Go down to ISO sensitivity → arrow to right → arrow down to choose **400** → OK
        b.) **Image quality:** In set-up menu, go down to image quality → arrow to right → arrow down to choose **JPEG normal** → OK
        c.) **Resolution:** In set-up menu, go down to image size → arrow to right → arrow down to choose **large** → OK

When taking the photo:
Materials: camera, batteries, photo logbook, labels.

This is a combined effort between the photographer and examiner. The photographer can ask the grader to help improve the photo, and vice versa.

a) Focus in on the child's eye
b) Try and aim for the following for the photo:
    a.) Make sure the eye is **centred**
    b.) Try and avoid getting glare on the photo
    c.) Try and **avoid gloved finger** – if the grader's finger is covering the lid, ask them to pull it back
    d.) Make sure the grader hasn't everted the lid too aggressively **to not get blanching**. Tell the grader to release some pressure if this is the case.
    e.) Make sure the **cornea is not showing**. Ask the grader to re-evert to correct this.
    f.) Ask the grader to **avoid "folding"** the lid.
c) Take **one** photo of the left eye.
d) Check the quality of the photo. Check that the lid appears
    a) well everted
    b) not "blanched"
    c) in focus
    d) well centred in the frame
    e) filling the frame
e) If you think the photo isn't good, take another.
f) Record the DSC photo number in the Database.

Step 4 – Conjunctival Swabs:

Assistant to open will open the swab packet carefully so not to touch the end of the stick, and will hold it so that Patrick can take one of the swabs from the packet.

Patrick will then swab (sterile Dacron Polyester tipped swabs) the left upper tarsal conjunctiva with two swabs, one after the other.

1. ***RNA swab – CR#-###***
2. ***DNA swab – CD#-###***

RNA swab – CR#-###

a. Sweep swab horizontally four times with a quarter turn of swab after each pass.
b. Place swab into RNAlater tube, break end off the swab and seal tube. The end will be broken off without touching any part of the swab that is in the tube
c. Label tube
d. Check labelling is correct
e. Record swab number in the database.
f. Place tube in cool box
g. On return to Moshi place tube into fridge over night.
h. Place in -80°C freezer the following morning.

DNA swab – CD#-###

a. Sweep swab horizontally four times with a quarter turn of swab after each pass.
b. Place swab into RNAlater tube, break end off the swab and seal tube. The end will be broken off without touching any part of the swab that is in the tube
c. Label tube
d. Check labelling is correct
e. Record swab number in the database.
f. Place tube in cool box
g. On return to Moshi place tube immediately into -80°C freezer.

Step 5 - Control Swabs

After every 50 subjects collect a pair of air control swabs.
To do this:

a. Collect air swabs under the examination canopy (same place as all examinations)
b. put on fresh gloves.
c. open swab packet.
d. hold swabs in the air in front of you for 15 seconds.
e. Place one swab in a tube containing RNA later and the second in a dry tube.
f. Label tubes with a sequential number indicating control swab 1, 2, 3, etc.

Appendix 3: DNA/RNA Extraction Protocol

RNA/DNA Purification Kit (Norgen)-Aimed to extract both genomic DNA and total RNA
Kit storage
Store proteinase K at -20^{0}C
Other reagents have to be stored at room temperature
Note; unopened reagents are stable for a year

Precautions
Please wear gloves, Lab coat and goggle during work or visit at *www.norgenbiotek.com* for further details

Reagents and equipment
Microcentrifuge
Pipettes
96 - 100 % ethanol
Molecular biology grade water (Milli-Q® water)

Preparation of working reagents
Note; label on the bottle indicating that ethanol has added
RNA washing solution;
Add 50 mL of 96 – 100% ethanol for a final volume of 72 mL

gDNA washing solution I;
Add 20 mL of 95 – 100% ethanol for a final volume of 30 mL

gDNA washing solution II;
Add 15 mL of 95 – 100% ethanol for a final volume of 30 mL

A. Cells lysis

a. Transfer the swab samples with RNA later solution into nuclease free 2ml tubes
b. Add 300 µl of the LYSIS solution into 2ml tube
c. Vortex for 15-20 seconds and ensure the entire swabs part is in fluid
d. Squeeze the swabs against the wall of tube to express as much of the fluid out of the swab back into the tube
e. Discard the swabs

B. Genomic DNA purification

1. **Binding DNA to gDNA purification column**
   a) Add up to about 300µl of the lysate in the column
   b) Centrifuge at **14,000 x *g* (~14,000 RPM)** for 1 minute
   c) **Retain the flowthrough for RNA purification**
   d) Reassemble **DNA Purification spin Column** to collection tubes
   e) Store the DNA purification spin column on ice for DNA extraction

C. Total RNA Purification

Note*; It is important to work quickly when purifying RNA.*

2. **Binding RNA to column**
   a) Add 180µl of 96-100 % Ethanol in the RNA flowthrough from **Step 1c**,
   b) Mix by pipetting
   c) Assemble an **RNA Purification Column** with collection tubes.
   d) Add up to about 480µl of lysate with ethanol onto the column

e) Centrifuge at **14,000 x *g* (~14,000 RPM)** for 1 minute.
f) Discard the flowthrough
g) Reassemble an **RNA Purification Column** to collection tubes

3. RNA Wash

a) Add 400µl of RNA wash solution to the column
b) Centrifuge at **14,000 x *g* (~14,000 RPM)** for 1 minute
c) Discard the flowthrough
d) Reassemble **RNA Purification Column to** collection tubes
e) Repeat the procedures above (a-d)
f) Again repeat the procedures above (a-d)
g) Spin the column at **14,000 x *g* (~14,000 RPM)** for 2 minutes to thoroughly dry the resin
h) Discard the collection tube

4. RNA Elution

a) Place the column into a fresh 1.7 ml Elution tube provided with the kit.
b) Add 50µl of **RNA Elution Solution** to the column.
c) Centrifuge for **2 minutes** at **200 x *g,***
d) Then centrifuge for **1 minute** at **14,000 x *g* (~14,000 RPM).**

5. Store purified RNA samples at -20^{0}C for few days or at -70^{0}C for long term storage

6. Genomic DNA Wash from DNA purification spin column (from 1e above)

a) Add 500µl of **gDNA Wash Solution I** to the column
b) Centrifuge at **14,000 x *g* (~14,000 RPM)** for 1 minute.
c) Discard the flowthrough.
d) Add 500µl of **gDNA Wash Solution II** to the column
e) Centrifuge at **14,000 x *g* (~14,000 RPM)** for 1 minute.
f) Discard the flowthrough.
g) Spin the column at **14,000 x *g* (~14,000 RPM)** for 2 minutes to thoroughly dry the resin.
h) Discard the collection tube.

7. Genomic DNA Elution

a) Place the column into a fresh 1.7 ml Elution tube provided with the kit.
b) Add 50 µl of **gDNA Elution Buffer** to the column.
c) Centrifuge for **2 minutes at 200 x *g* (~2,000 RPM),**
d) Then centrifuge for **1 minute** at **14,000 x *g* (~14,000 RPM).**

8. Store purified DNA at 4^{0}C for short time or at -20^{0}C for long term storage

Appendix 4: *Chlamydia trachomatis* Detection Protocol

(A) Droplet Digital PCR

Chlamydia Research programme

Summary

This protocol describes a method for droplet digital PCR of using the Biorad dx100 instrument PCR products should be 22 µL, from which a 20 µL reaction will be used for droplet generation. The assay detects either the chlamydial plasmid or the chlamydial OMCB gene. Both implementations use the 30 kDa subunit of H.s. RNAseP gene as the endogenous control

Molecular targets

Endogenous control

gi|13937783|gb|BC006991.1| Homo sapiens ribonuclease P/MRP 30kDa subunit,

*Forward (HURNASE-P-F) 5'AGA TTT GGA CCT GCG AGC G 3'

*Reverse (HURNASE-P-R) 5'GAG CGG CTG TCT CCA CAA GT 3'

^Probe (HURNASE_HEX_BHQ1) 5'FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3'

AMPLICON and priming sites

agatttggacctgcgagcgggttctgacctgaaggctctgcgcggacttgtggagacagccgctc

TARGET ONE : Chlamydia Trachomatis cryptic plasmid pLGV440 (X06707; SV 3; circular; genomic DNA; STD; PRO; 7500 BP.)

*Forward (Ct-Plasmid-F) 5'cagcttgtagtcctgcttgagaga3'

*Reverse (Ct-Plasmid-R) 5'caagagtacatcggtcaacgaaga3'

^Probe (Ct-plasmid-FAM-BHQ1) 5'HEX-ccccaccatttttccggagcga-BHQ1-3'

AMPLICON and priming sites

cagcttgtagtcctgcttgagagaacgtgcgggcgatttgccttaaccccaccatttttccggagcgagttacgaagacaaaacctctt cgttgaccgatgtactcttg

TARGET TWO : Chlamydia Trachomatis (Serovar A) OMCB gene.

*Forward (Ct-OMCB-F) 5'gacaccaaagcgaaagacaacac3'

*Reverse (Ct-OMCB-R) 5'actcatgaaccggagcaacct3'

^Probe (Ct-OMCB-HEX-BHQ1) 5'FAM-aagcaaaaaagcaagaaaaaaccacagcaaagag-BHQ1-3'

AMPLICON and priming sites

gacaccaaagcgaaagacaacacttctcataaaagcaaaaaagcaagaaaaaaccacagcaaagagactcccgtagaccgtaaag aggttgctccggttcatgagt

Preparation of primers and probes

ALL STOCK SOLUTIONS should be at 100 µM

Primer/probe supermix A (CtPLASMID/HURNASEp)

2 µM Human RNAse P gene primers and probes

2 µM Chlamydia plasmid primers and probes

NOTE: This can be scaled for smaller volumes

To prepare 1100 µL (enough for 500 reactions plus 10%)

	volume
100 µM HURNASE-P-F	22 µL

100 µM HURNASE-P-R	22 µL
100 µM HURNASE_HEX_BHQ1	22 µL
100 µM Ct-Plasmid-F	22 µL
100 µM Ct-Plasmid-R	22 µL
100 µM Ct-Plasmid-FAM-BHQ1	22 µL
1X TE buffer (sterile/nuclease free)	968 µL
Total	1100 µL

Primer/probe supermix B (Ct_OMCB/HURNASEp)

NOTE: This can be scaled for smaller volumes

2 µM Human RNAse P gene primers and probes

2 µM Chlamydia OMCB primers and probes

To prepare 1100 µL (enough for 500 reactions plus 10%)

	volume
100 µM HURNASE-P-F	22 µL
100 µM HURNASE-P-R	22 µL
100 µM HURNASE_HEX_BHQ1	22 µL
100 µM Ct-OMCB-F	22 µL
100 µM Ct-OMCB-R	22 µL
100 µM Ct-OMCB-FAM-BHQ1	22 µL
1X TE buffer (sterile/nuclease free)	968 µL
Total	1100 µL

PCR set up

Work In a PCR Clean & DNA Free Environment

Each assay uses EITHER primer/probe supermix A OR primer/probe supermix B.

CRITICAL STEP : MIX AND INVERT THE SUPERMIX AND PCR MIXES EXTREMELY WELL, THEY ARE VISCOUS AND THE TESTS WILL FAIL IF YOU DON"T DO THIS PROPERLY.

Composition per reaction

	1X
2X ddPCR supermix	11 µL
Primer/probe supermix (2 µM each of six oligos)	2.2 µL
Nuclease free H2O	3.85 µL
SAMPLE DNA	4.95 µL
Total	22 µL

1. To prepare sufficient ddPCR MASTERMIX to run one 96 well plate (100 reactions)

In A 2 mL, conical based microtube, add the following

	100X
2X ddPCR supermix	1100 µL (2 X 550 µL)
Primer/probe supermix (2 µM each of six oligos)	220 µL
Nuclease free H2O	385 µL
Total	1705 µL

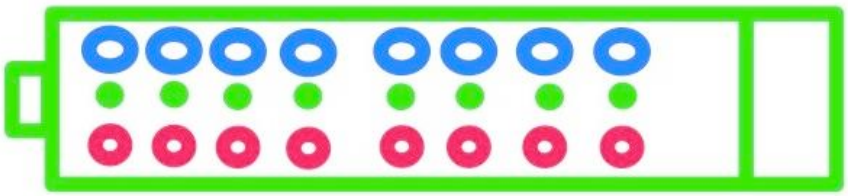
Move to DNA loading/PCR clean area

2. To each well of a 96 well SKIRTED EPPENDORF twin-tec plate (in retainer), aliquot 17.05 µL ddPCR MASTERMIX

3. Aliquot 4.95 µL of DNA from samples to each well of the plate.

4.| Seal with microamp cover film (ensure close seal) and vortex gently (1000 rpm) to mix reagents for 30 seconds
5. Centrifugate at 1000 rcf for 1 minute to pool PCR mix at bottom of plate.
6. Proceed to droplet generation

Droplet generation
Turn on the heat sealer
7. Place a new, clean and EPPENDORF TWIN-TEC **SEMI-SKIRTED** 96 well plate in a retainer
8. Carefully remove the microamp film, without disturbing the PCR mixes
9. Place a new droplet generation (DG8) cartridge in the droplet cassette, the notch should face left (SEE FIGURE). Close the cassette by applying gentle pressure to the short edges.



10. Transfer 20 μL of each PCR mixture from row 1 in to each sample well of the cartridge (green on figure, marked "sample" on the cassette).
Take care to avoid introducing air by putting pipette tips to the lowest point where the wall of the vessel meets the base. Hold pipette at around 20 degree angle to wall, actuate slowly and raise tips from the base as the wells continue to fill
11. Transfer 70 μL droplet generation oil to each oil well of the cartridge (red on figure, marked "oil" on the cassette")
12. Attach a rubber gasket to the notched teeth on the cassette. Take care not to move the rubber across the wells as this could lead to cross-contamination. Ensure that the four teeth are properly engaged with the gasket as failure to do this will prevent a good seal forming.
13. Transfer the cassette to the droplet generation machine. Holding the cassette gently at the top and bottom, place on the magnetised platform. A green light will indicate proper engagement.
14. Activate droplet generation by pressing the button.
15. Proceed to prepare the next cassette
16. When the first droplets are complete, exchange the first cassette for the second in the droplet generator. Reactivate the device
17. Carefully transfer 45 μL of droplets from the droplet wells (blue on figure) to row 1 of the SEMI-SKIRTED plate. Ensure that aspiration is performed slowly (around 10 s) when drawing and expelling droplets as failure to do this will lead to droplet instability
18. Use a short length of orange lab-tape to temporarily seal the wells of row 1
19. Prepare the droplet cassette for row 3, then transfer the droplets from row 2
20. Continue this process until the plate is completed.
21. Remove all orange lab-tape and replace with an easy-pierce, heat seal, ensuring that it is the right way up
22. Place tray in the heat sealer, pull down the heat-block and press down again, hard for 5 seconds. Release, turn the plate through 180 degrees, then repeat sealing for another 5 seconds.
23. Put plate in Bio-Rad thermal cycler and run the program "DDPCR"
Thermal cycler parameters are
The PCR reaction is performed on a thermo cycler (stage 1, 95^{O}C for 10 minutes, stage 2, 95^{O}C for 10 seconds and 60^{O}C for 30 seconds for 40 cycles and stage 3, 98^{O}C for 12 minutes)**024 |** When the cycle is complete, droplets can be stored for up to 48 hours, or counted immediately.

Droplet reading

If this is the first read of the day, start by performing a prime/flush/prime operation
25. Transfer the plate to the special retainer and place this device in the droplet reader. A green light will indicate correct engagement with the platform. Close the door by pressing the button.
26. Open the droplet reader software and load the appropriate template "plasmid" or "omcb"
27. Select all wells on the plate and double click on the plate diagram to edit the details.
28. For sample names, all wells should be labelled with the plate identifier number (1-16)
29. Start the droplet count by pressing the run button

Analysis

(B) Detection of *Chlamydia trachomatis* from human eyes collected by swabs

Preparation of Primer-Probe Mix
Note; *All this work should be done in the pre-PCR room inside the UV hood.*

1. **Primer Rehydration to 100µM stock:**
   a) Primers come dehydrated in the tube
   b) Rehydrate the primer to a concentration of 100µM, using sterile water (sigma)
   c) The volume of water to use is found on the quality assurance document.
   d) Write the abbreviated name of the primer and whether it is forward or reverse, on lid.
   e) This stock can be frozen at -20^{0}C

2. **Probe Rehydration to 100µM stock:**
   a) Probes come dehydrated in the tube.
   b) Rehydrate the probe to a concentration of 100µM, using sterile water (sigma)
   c) The volume of water to use is found on the quality assurance document.
   d) Write the abbreviated name of the probe on the lid.
   e) This stock can be frozen at -20^{0}C

3. **Preparation of 500µl of 10x Primer-Probe Mix for each individual target SEPARATELY:**

Equipment:
   a) Pipette 1000µl + tips
   b) Pipette 20µl + tips
   c) Sterile 1.5ml tube

Procedures:
   a) Add 470µl of sterile water
   b) Add 10µl of Forward Primer from 100µM stock
   c) Add 10µl of Reverse Primer from 100µM stock
   d) Add 10µl of Probe from 100µM stock
   e) Gentle / Brief vortex – make sure it is mixed
   f) Keep in fridge and use in short term.
      Note*; probes must be protected from light as this will degrade them or will bleach dye*

4. **Preparing master mix for multiplex PCR**
   Note*; All this work should be done in the pre-PCR room inside the UV hood*.

Equipments
   a) Pipette 1000µl + tips
   b) Pipette 200µl + tips
   c) Tube 5ml

Reagents
   a) Multiplex PCR Master Mix

b) Water (from kit)
c) Primer and Probe mixes for all THREE targets of the set being tested

Procedures
Add the following to the 5 ml tube, as tabulated below (aiming 400 reactions)

a) Master Mix 2000µl
b) Water 1516µl
c) Primer/Probe mix 84µl

Components	Quantity per single sample (µl)	Quantity per X samples (µl)
PCR master mix	**5**	**5× X**
Primers and Probes	**0.21**	**0.21 × X**
H_2O	**3.79**	**3.79 × X**
Samples	**1**	
Total	**10**	**(10-1 (samples)) × X**

d) Gentle vortex

Take the master mix to the DNA/RNA extraction room/PCR preparation room

5. Preparing PCR reaction
Equipments and materials

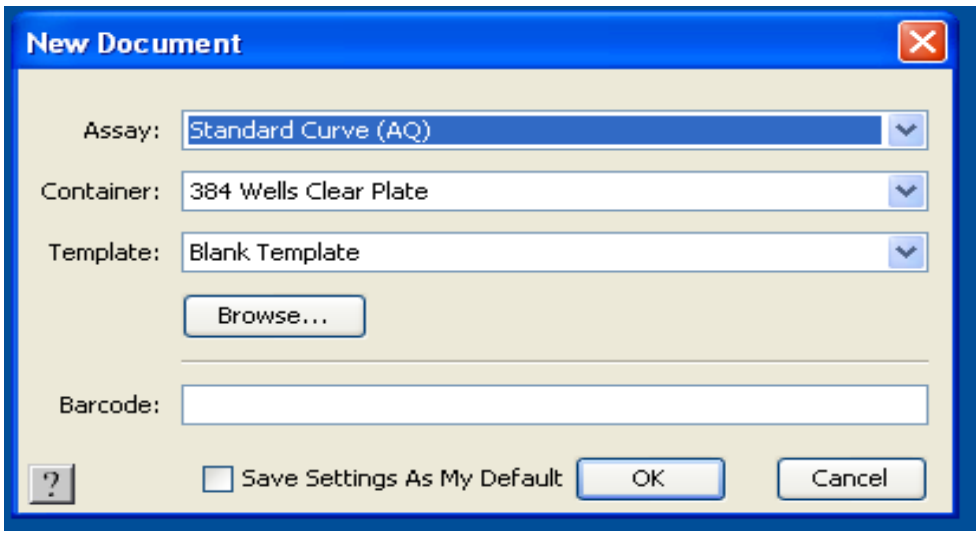
a) Prepared master mix
b) Pipette 10µl + tips
c) Pipette 2µl + tips (*option*)
d) DNA samples (defrosted and spun)
e) Water (negative control)
f) Ct positive control

Procedures
Add the following to the 384 wells plate

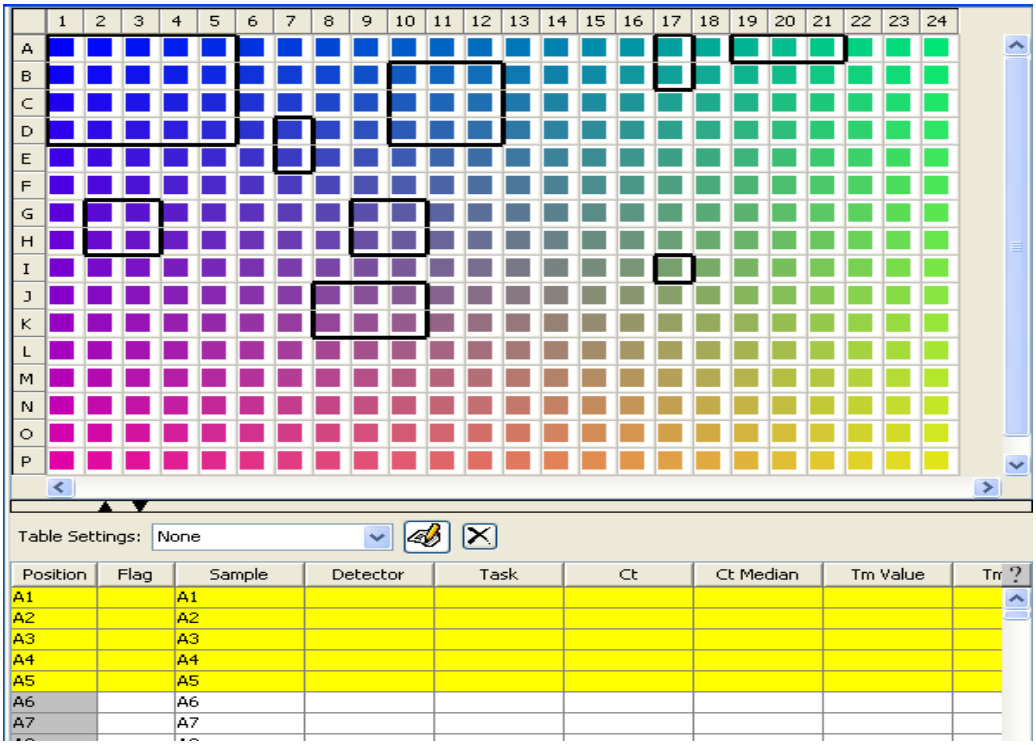
a) Dispense 9µl of master mix in 384 wells
b) Add 1µl of DNA samples in 380 wells
c) Add 1µl of water in 2 wells
d) Add 1µl of positive control in 2 wells
e) Seal the plate with the sealer plate

6. Loading in ViiA7

a) Open SDS 2.4 program
b) Escape password
c) Open new document (from file)
d) Assay (standard curve)
    i. Container 384 well clear plate
    ii. Template-blank template
    iii. Barcode-type from the plate



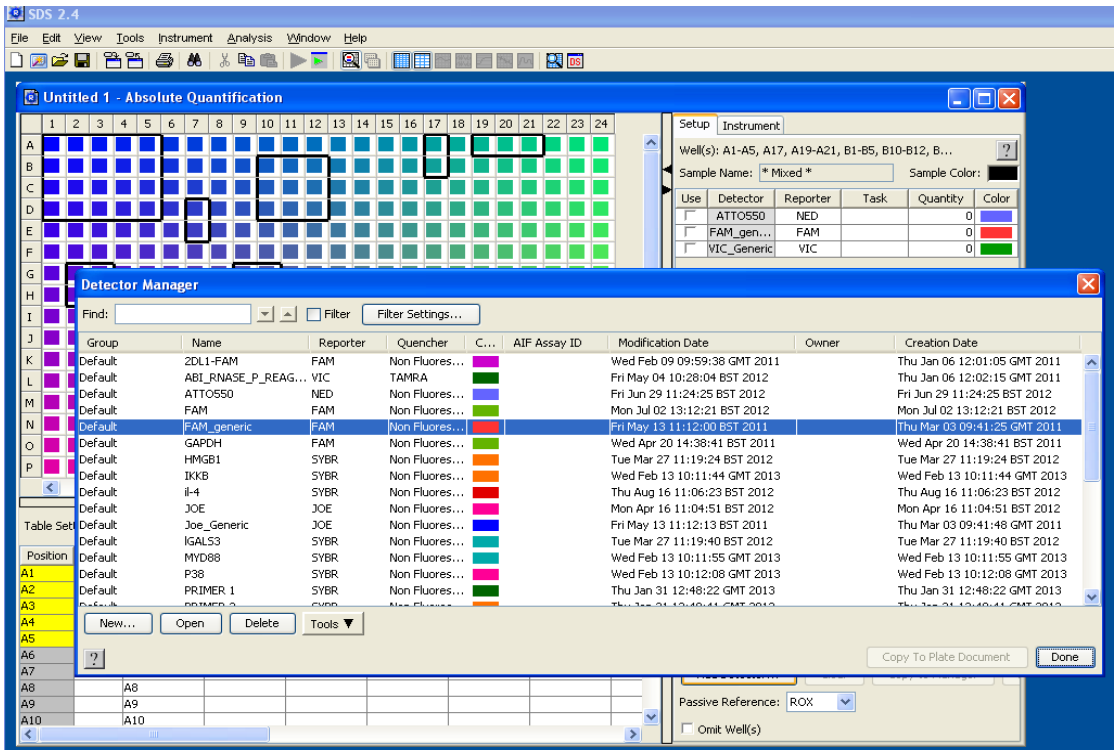
e) Select the field



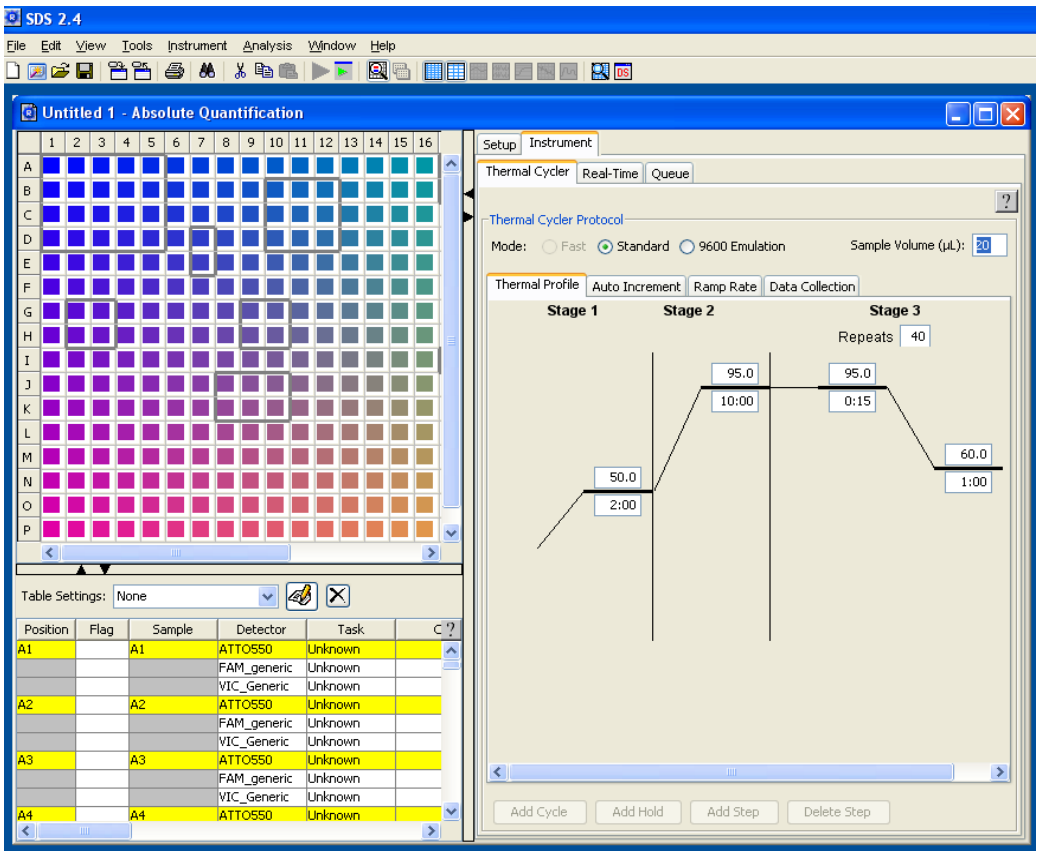
f) Set up-Add detector

g) List of detector such as;

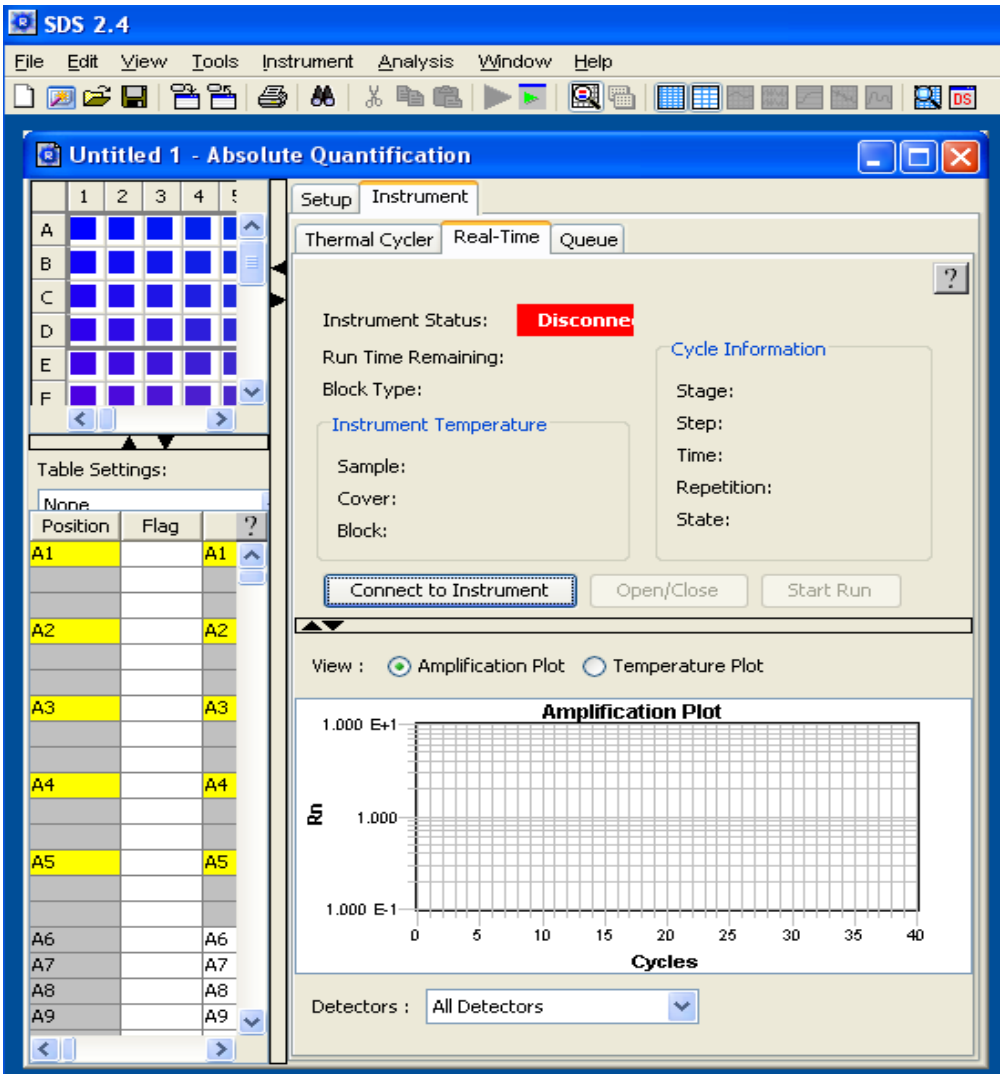
i. ATTO550 (detect Ct plasmid)----------NED
ii. RPP30 (detect human genome)----------VIC
iii. OMC B (detect Ct genome)----------FAM



h) Select each one and then click “copy to plate document”
i) Done
j) To select (cross) the boxes
k) Instrument
l) Sorting thermo cycler (stage 1,2,3)
m) Adjust volume (accordingly), usually 10µl



n) Real time-connect to instrument
o) Open the plate (from open/close button)
p) Close
q) Start the run
r) Save



7. Preliminary analysis

a) Click “click analyse” (top tool bar)
b) Result tab
c) Select the field
d) Detect (at the bottom)-to see the channels
e) Select plot (Rn vs cycle) etc,
f) Analyse settings (to tool bar)-select detector with ATTO550 etc
   i. Auto baseline (**NO**)
   ii. Manual baseline (**YES**)
   iii. Click apply
      1) Change in Rn vs cycle
      2) Repeat the procedures from 2 for FAM and VIC
   iv. Add sample name
   v. Save
   vi. Export- Tab delimited text
   vii. Analysis

8. **Analysis**

Appendix 5: cDNA Synthesis Protocol

SuperScript® VILO™ cDNA Synthesis Kit

Aim: Reverse transcription of Total RNA**.**

Storage: Reagents are stored at -20^{0}C

Preparation of samples and reagents

- ✓ Thaw extracted RNA on ice in the molecular lab
- ✓ Thaw reagents in RNA/DNA free room
- ✓ Prepare RT master mix on ice in UV hood in RNA/DNA free room

Procedures

Add the following to the 5 ml tube on ice, as tabulated below (e.g aiming 100 reactions)

Components	Quantity per single sample (µl)	Quantity per 96 samples (µl)
5X VILO™ Reaction	8 µl	800 µl
10X SuperScriptR Enzyme	4 µl	400 µl
RNA	Add **28 µl** in molecular lab	
Total	**40 µl**	

a) Gently mix the tube contents

b) incubate at **25^{0}C for 10 minutes**
c) Then incubate tubes at **42^{0}C for 1 hour**
d) Terminate the reaction at **85^{0}C at 5 minutes**
e) Store cDNA at **-20^{0}C** until use or at **-80^{0}C** for long storage

Appendix 6: Host Gene Expression Protocol

Host gene expression detection protocol (Taqman Array Card-TAC)

Aim

To identify different host genes expressed in different individual children with different stages of trachoma progression in endemic area.

Materials

- ✓ Vortex
- ✓ Microcentrifuge
- ✓ Pipettes (20µl, 200µl/100µl and 1000µl)
- ✓ Life technologies real time system (ViiA7 with TAC block)
- ✓ Compatible Heraeus Centrifuge
- ✓ Nuclease free water
- ✓ 1.5ml microcentrifuge tubes
- ✓ Array card
- ✓ cDNA from RNA samples extracted from eye's swabs

PCR Reagents preparation

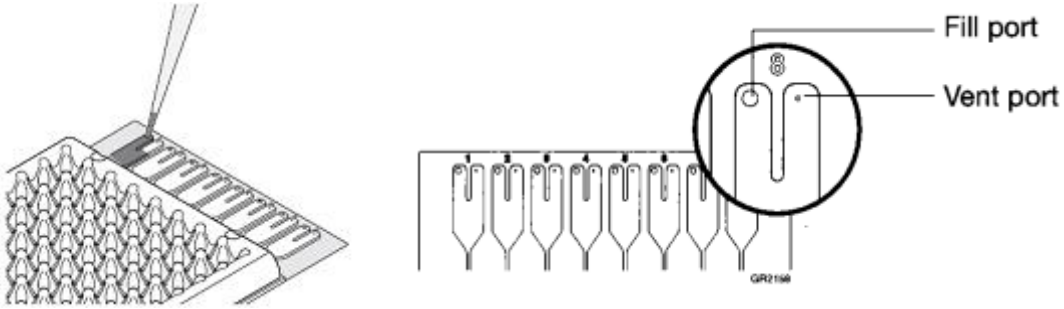
- ✓ Keep all the reagents on ice during assay set up
- ✓ Prepare master mix as follows;

Components	A single sample	9 samples
RT-PCR buffer (Taqman universal master mix II with UNG	50µl	450µl
Nuclease free water	30µl	270µl
cDNA Samples	20µl-**in molecular lab**	
Total	**100µl**	

a) Label all 8 microcentrifuge tubes
b) Aliquot 80µl of master mix into each of **8** 1.5ml microcentrifuge tubes
c) Add 20µl of cDNA to each tube
d) Vortex gently
e) Centrifuge the tubes to eliminate any air bubbles from the mixtures

Loading samples into TAC

a) Thaw the TAC to room temperature
b) Carefully remove TAC from its packaging
c) Place the TAC on a lab bench, with the foil side down
d) Transfer 100µl of each PCR reaction mix into the fill port-the large hole, hold pipette in an angle and slowly dispense the mix into sweeps in and around to fill reservoir toward the vent port without introducing air bubbles.



Important; *Put new strip to the plate with remaining cDNA samples*

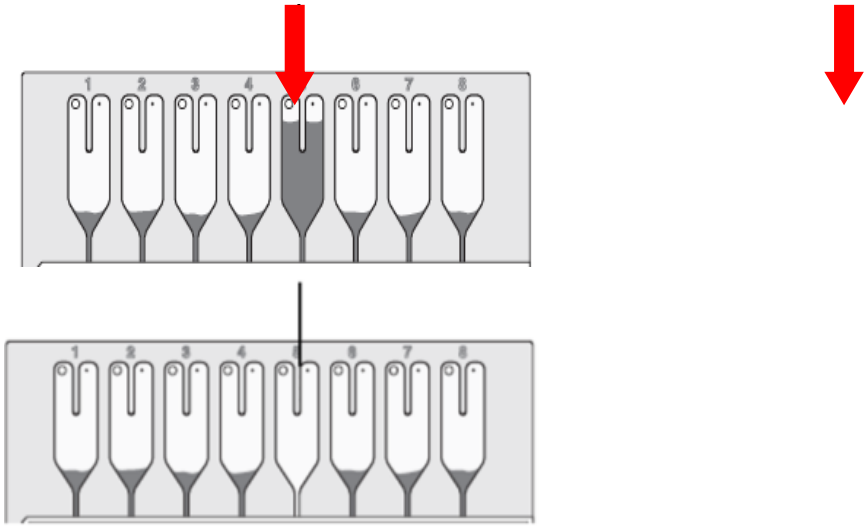
Centrifugation of the TAC

a) Insert TAC into the array holder, please make sure,
   i. The fill reservoirs project upwards out of the array holder
   ii. The reaction wells face the same direction as the ''this side out'' label
   iii. Use blank balance arrays or used arrays to fill any remaining positions in the array holder

Note*; sample loading will be impaired if inserting procedures will not be accomplished*.

b) Place the filled array holder in the bucket, labelled ''This Side Out'' facing away from the centre of centrifuge

c) Centrifuge at 1200 RPM for one minute, (buckets and contents must be balanced)
d) Remove the buckets from the centrifuge, and then remove array holders
e) Gently remove the TAC by their carrier sides
f) The filling reservoirs should be uniform and consistent with the amount of reaction mix
g) If is inconsistence proceeds with next step but avoid results of affected sample



Sealing the TAC

***Note**; Sealer isolates the wells of TAC after it loaded with reaction mix. Proper operation of sealer such as slow, steady and deliberate motion is critical for successful use of TAC.*

a) Place sealer on the table near to centrifuge (used for TAC)

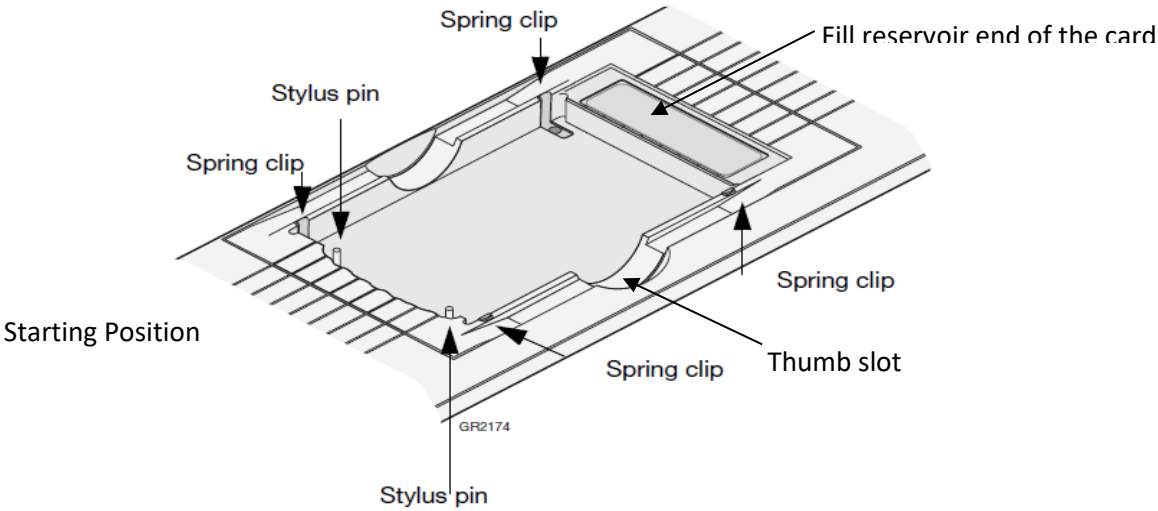
b) Place the carriage sits in the starting motion which is at your side.

***Note**; never insert the TAC into the sealer if carriage is NOT in its starting motion, otherwise will cause irreparable damage*

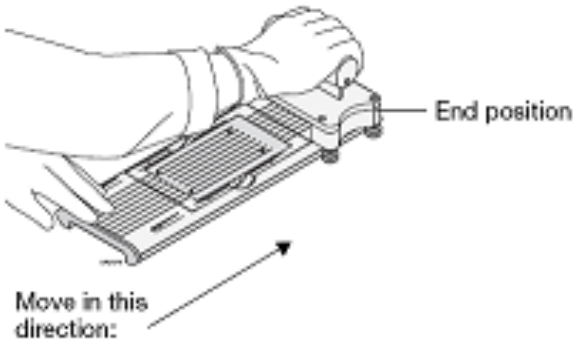
c) Insert TAC into the sealer

   i. Orient the TAC in the proper direction over the sealer insert plate, foil side up and the fill reservoir end should be end closest to the far end of the sealer base.

   ii. Line the card's rear pin groves to the stylus pins on the sealer



iii. Gently place the TAC on top of the insert plate and gently push until is securely fitted and foil surface is level with the base sealer. 4 spring clips ensure the card is held in proper position



iv. Push the carriage in slow motion in the direction of arrow to far end labelled with ''Push to stake'' until the carriage reaches mechanical stop

v. Remove the sealed TAC. Use the thumb slot at the middle of sealer for easy access

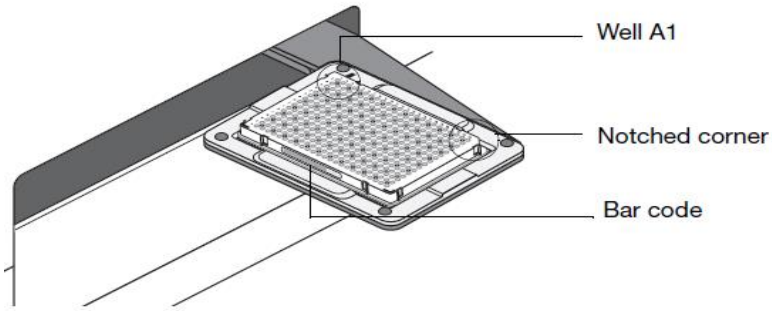
vi. Inspect the TAC for proper sealing. If indentations do not match up or if foil is anyway damaged do not use the TAC.

vii. Return the carriage to its staring position on the base of the sealer

d) Use scissors to trim the fill reservoir from the TAC

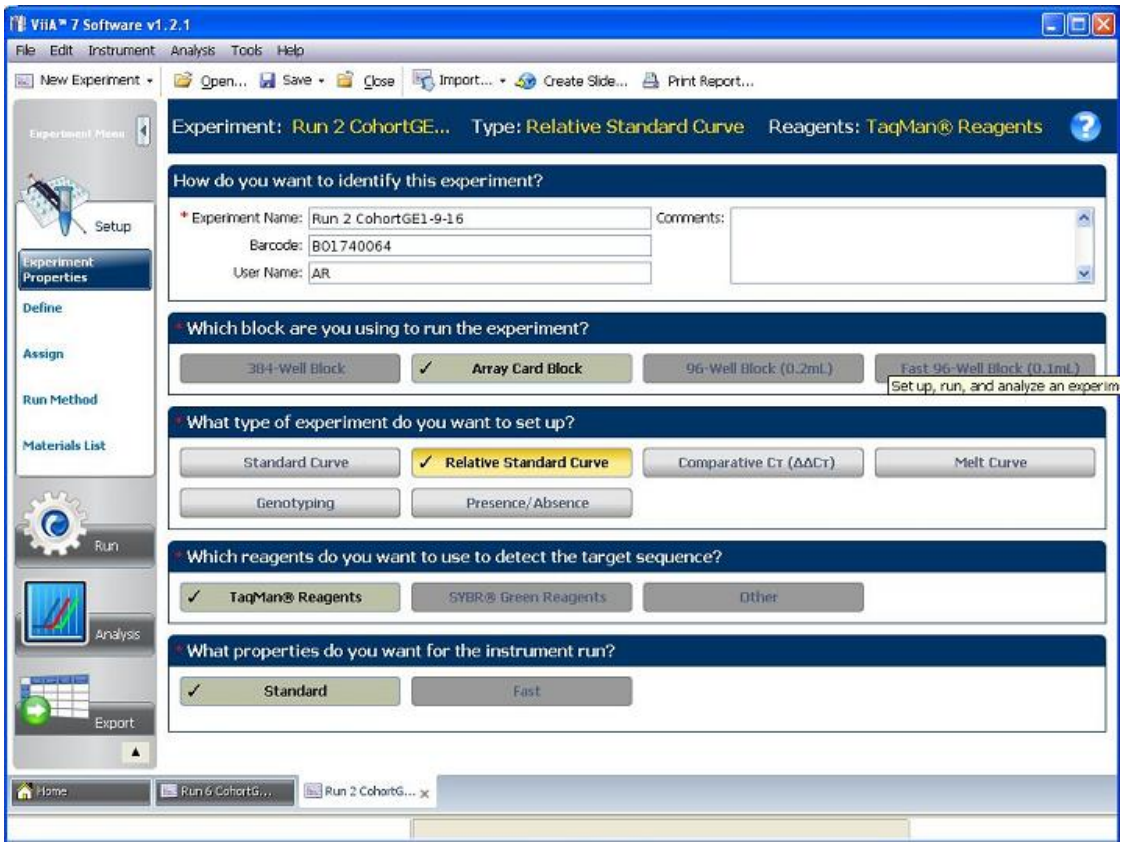
TAC set up;

- Turn on ViiA7 and its computer
- Make sure Block, Adaptor and heated cover are matching
- Open instrument's tray and insert TAC with well A1 at the top left corner and notched corner at the top right and barcode toward front of the instrument

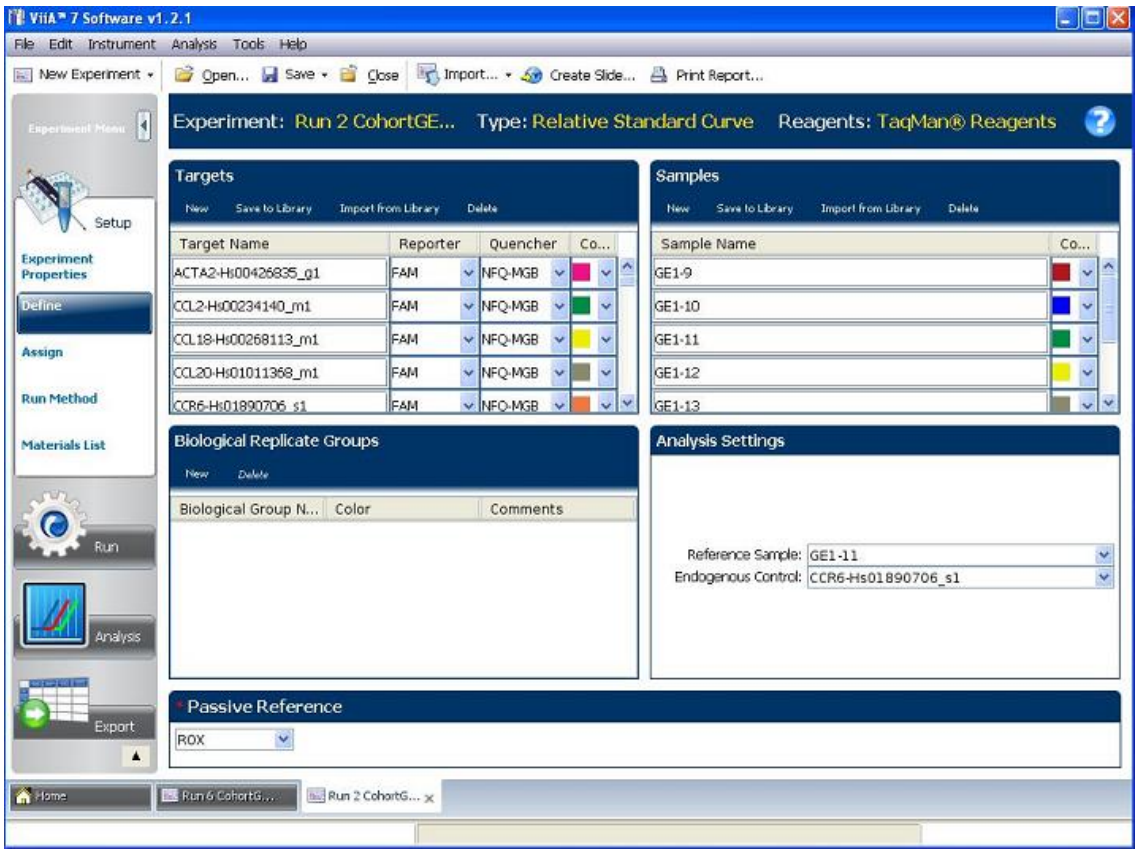


- Click close to close the tray

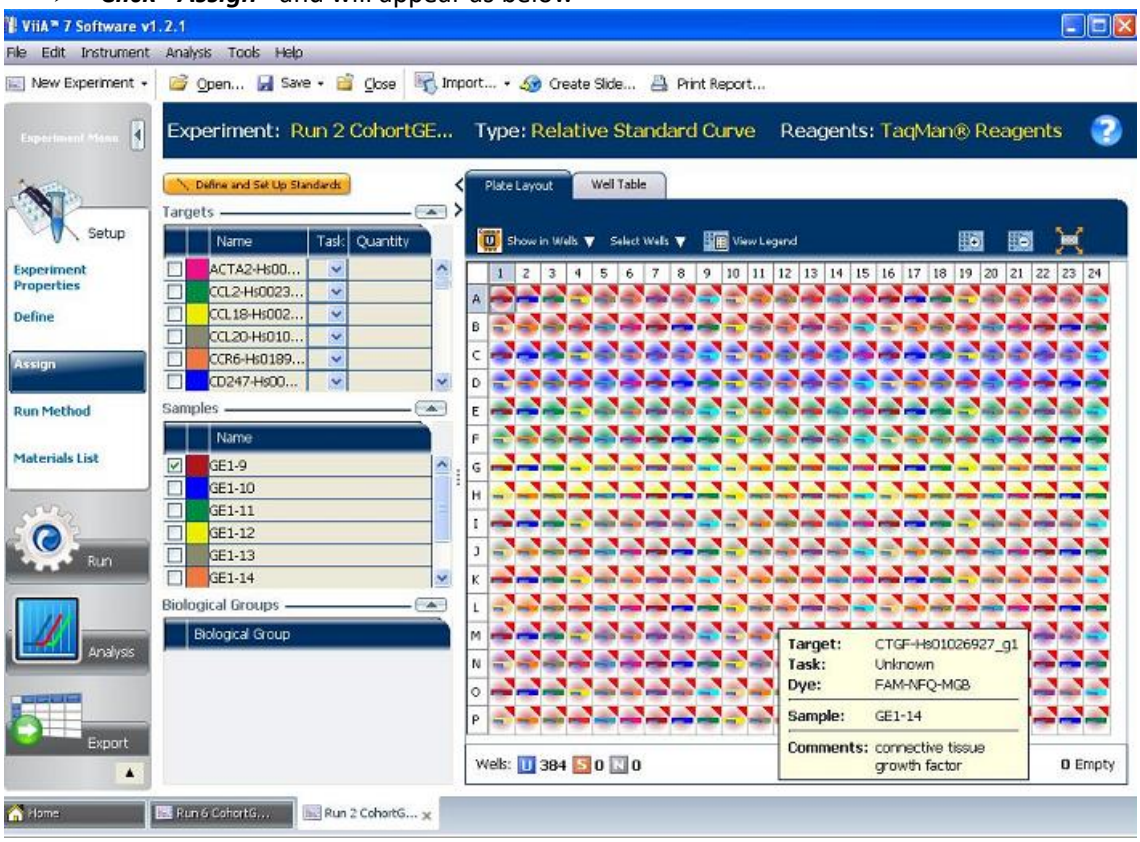
- ***New Experiment***
- ***Click "Setup"***
- ***Click "Experiment Properties"*** and fill the following;
  - ✓ Experiment Name_____
  - ✓ Barcode_____
  - ✓ User Name_____
  - ✓ Comments (if necessary)_____
- Tick the following as indicated below
  - ✓ Array Card Block
  - ✓ Relative Standard Curve
  - ✓ TaqMan@ Reagents
  - ✓ Standard



- ***Click "Define"***
- Import the targets names stored in the computer or CD
- Write samples names
- Flourophore: FAM (ROX as reference)

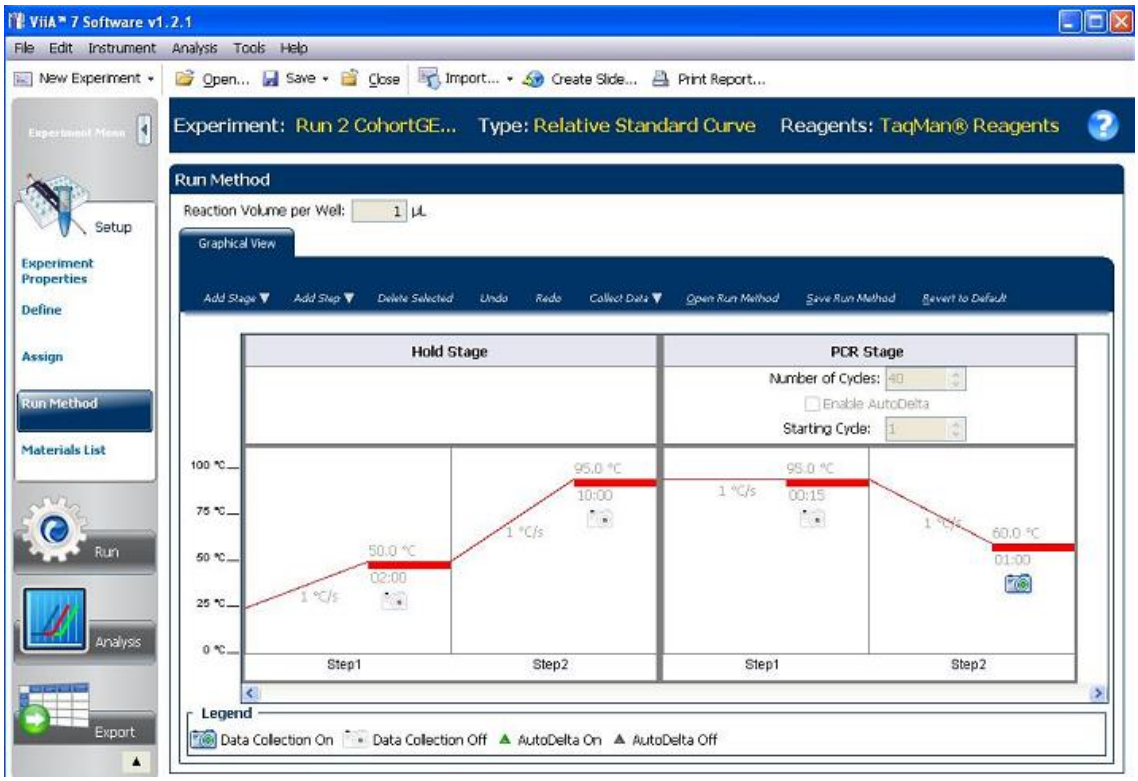


- ***Click "Assign"*** and will appear as below

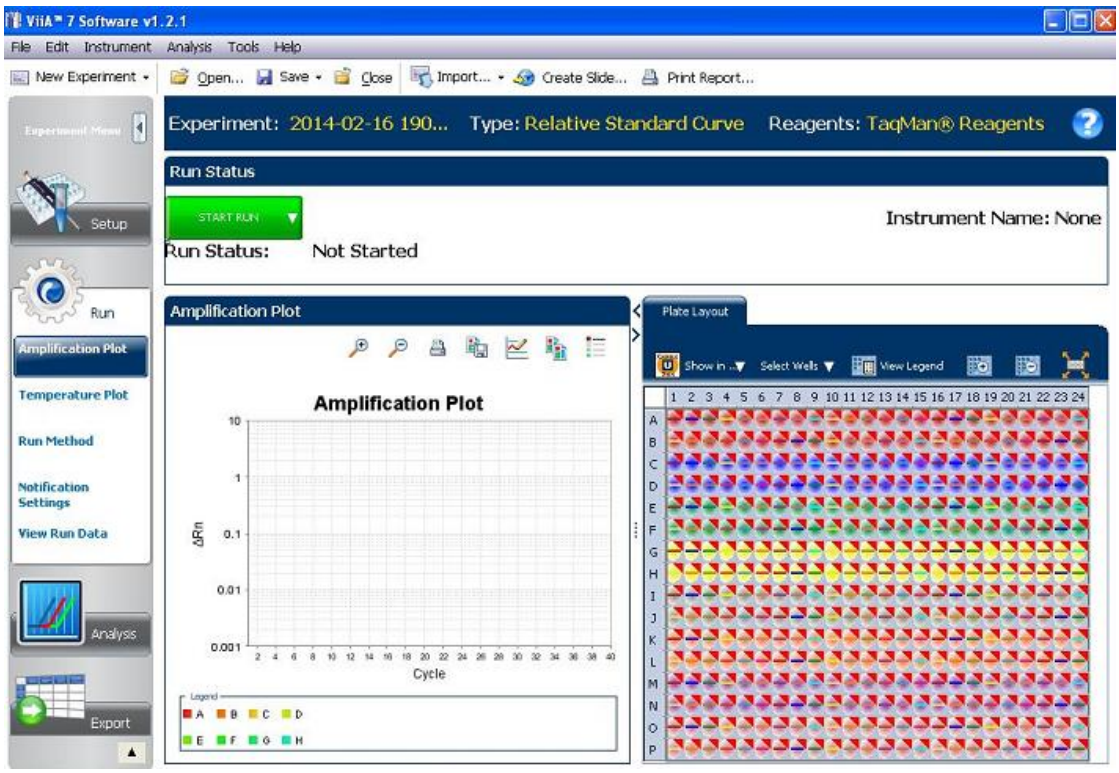


- ***Click "Run Methods",*** set cycling conditions of RT-PCR as below
- Cycling conditions of RT-PCR

Step	Temperature	Time
	✓ *Hold stage*	
1	50^0C	2min
2	95^0C	10min
	✓ *PCR Stage (Number of cycles 40)*	
1	95^0C	15sec
2	60^0C	1min (data detection)



- ***Click Run -------Start Run***
- Save the experiment in a designated folder


ViiA™ 7 Software v1.2.1
File Edit Instrument Analysis Tools Help
New Experiment
Open...
Save
Close
Import...
Create Slide...
Print Report...
Experiment: 2014-02-16 190...
Type: Relative Standard Curve
Reagents: TaqMan® Reagents
Setup
Run
Amplification Plot
Temperature Plot
Run Method
Notification Settings
View Run Data
Analysis
Export
Run Status
START RUN
Instrument Name: None
Run Status: Not Started
Amplification Plot
Amplification Plot
ΔRn
Cycle
Plate Layout
Show in ..
Select Wells
View Legend

Appendix 7: DNA Extraction Protocol for Microbiome

PowerSoil®DNA Isolation Kit

Aim
To extract DNA from Eye swabs

Kit storage
Both reagents and components are stored at room temperature (15-30^{0}C)

Precautions
Wear gloves all the time and avoid all skin contact with kit reagents (wash thoroughly with water in case there is contact or see material safety data sheet at www.mobio.com for details)

Equipment
Microcentrifuge (10,000 x *g*)
Pipettors (50 µl – 500 µl)
Bead beater
Vortex Adapter

Reagents preparation
Add 120ml of absolute ethanol into solution C5-D to make total of 240ml

Procedures

1. **Please wear gloves at all times**
2. **Sample/solution 1 preparation**
   a) Prepare a maximum of 16 swab samples (equal number with the bead beater available)
   b) Check **Solution C1**. If it is precipitated, heat solution to 60^{0}C until dissolved before use
   c) Add dry swab samples to the PowerBead Tubes.
3. **Cells lysis**
   a) Add 60 µl of **Solution C1**
   b) Beat on bead beater for 1 minute
   c) Repeat beating for another 1 minute
   d) Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature (RT).
   e) Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

   Note: Expect between 400 to 500 µl of supernatant.
4. **Inhibitor removal**
   a) Add 250 µl of **Solution C2** and vortex for 5 seconds.
   b) Incubate at 4^{0}C for 5 minutes.
   c) Centrifuge the tubes at RT for 1 minute at 10,000 x *g.*
   d) Avoiding the pellet, transfer up to, but no more than, 600µl of supernatant to a clean **2 ml**
   Collection Tube (provided).
   e) Add 200µl of **Solution C3** and vortex briefly.
   f) Incubate at 4^{0}C for 5 minutes.
   g) Centrifuge the tubes at RT for 1 minute at 10,000 x *g.*
   h) Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml**
   Collection Tube (provided).
5. **DNA binding**
   a) Shake to mix **Solution C4** before use.
   b) Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

c) Load approximately 600 µl of supernatant onto a **Spin Filter**
d) Centrifuge at 10,000 x *g* for 1minute at RT.
e) Discard the flow through
f) Repeat as in 5 (c-e) above
g) Again repeat as in 5(c-e) above

6. Washing

a) Add 500µl of **Solution C5**
b) Centrifuge at RT for 30 seconds at 10,000 x *g*.
c) Discard the flow through.
d) Centrifuge again at RT for 1 minute at 10,000 x *g*.
e) Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any
Solution C5 onto the **Spin Filter**.

7. **Elution**

a) Add 100µl of **Solution C6** to the centre of the white filter membrane. **Or** 100µl of sterile
DNA-Free PCR Grade Water
b) Centrifuge for **2 minutes** at **200 x *g,***
c) Centrifuge for 30 seconds at 10,000 x *g*.
d) Discard the **Spin Filter**.

8. Place extracted DNA samples in -80^{0}C freezer (for long storage) or -20^{0}C for short term storage

Appendix 8: Ocular Immune Responses, *Chlamydia trachomatis* infection and Clinical Signs of Trachoma Before and After Azithromycin Mass Drug Administration in a Treatment Naïve Trachoma-Endemic Tanzanian Community (Equivalent to Chapter 7)

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Ocular immune responses, *Chlamydia trachomatis* infection and clinical signs of trachoma before and after azithromycin mass drug administration in a treatment naïve trachoma-endemic Tanzanian community

Athumani M. Ramadhani[1,2]*, Tamsyn Derrick[1,2], David Macleod[3], Patrick Massae[2], Aiweda Malisa[2], Kelvin Mbuya[2], Tara Mtuy[1,2], William Makupa[2], Chrissy H. Roberts[1], Robin L. Bailey[1], David C. W. Mabey[1], Martin J. Holland[1], Matthew J. Burton[1]

1 Clinical Research Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, **2** Kilimanjaro Christian Medical Centre, Moshi, Tanzania, **3** Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, United Kingdom

* Athumani.Ramadhani@lshtm.ac.uk

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Abstract

Background

Trachoma, caused by *Chlamydia trachomatis*, remains the leading infectious cause of blindness worldwide. Persistence and progression of the resulting clinical disease appears to be an immunologically mediated process. Azithromycin, which is distributed at the community level for trachoma control, has immunomodulatory properties. We investigated the impact of one round of oral azithromycin on conjunctival immune responses, *C. trachomatis* infection and clinical signs three- and six- months post treatment relative to three pre-treatment time-points.

Methodology

A cohort of children aged 6 to 10 years were recruited from a trachoma endemic region of northern Tanzania and were visited five times in a 12-month period. They were examined for clinical signs of trachoma and conjunctival swabs were collected for laboratory analysis. *C. trachomatis* infection was detected and the expression of 46 host genes was quantified using quantitative PCR. All community members were offered azithromycin treatment immediately after the six-month timepoint according to international guidelines.

Findings

The prevalence of *C. trachomatis* infection and inflammatory disease signs were significantly reduced three- and six- months post-mass drug administration (MDA). *C. trachomatis* infection was strongly associated with clinical signs at all five time-points. A profound anti-inflammatory effect on conjunctival gene expression was observed 3 months post-MDA,

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however, gene expression had largely returned to pre-treatment levels of variation by 6 months. This effect was less marked, but still observed, after adjusting for *C. trachomatis* infection and when the analysis was restricted to individuals who were free from both infection and clinical disease at all five time-points. Interestingly, a modest effect was also observed in individuals who did not receive treatment.

Conclusion

Conjunctival inflammation is the major clinical risk factor for progressive scarring trachoma, therefore, the reduction in inflammation associated with azithromycin treatment may be beneficial in limiting the development of potentially blinding disease sequelae. Future work should seek to determine whether this effect is mediated directly through inhibition of pro-inflammatory intracellular signalling molecules, through reductions in concurrent, sub-clinical infections, and/or through reduction of infection exposure.

Author summary

Trachoma, caused by conjunctival infection with *Chlamydia trachomatis*, remains the leading infectious cause of blindness. Repeated infection during childhood can trigger prolonged inflammation, which is the main risk factor for conjunctival scarring. Azithromycin is distributed globally for trachoma control, however it is also widely reported to have immunomodulatory properties. This report investigated the impact of one round of oral azithromycin for trachoma control on conjunctival immune responses, clinical signs and *C. trachomatis* infection in Tanzanian children. A large anti-inflammatory effect of azithromycin on conjunctival gene expression was observed 3 months post-treatment, however, gene expression patterns had mostly resumed to pre-treatment levels by 6 months. The effect was evident after adjusting for *C. trachomatis* infection and when analysis was restricted to uninfected individuals, however it was also observed to a lesser extent in individuals that did not receive treatment. These findings suggest that azithromycin may have a direct immunomodulatory effect on conjunctival gene expression but that it may also reduce inflammation by reducing exposure to *C. trachomatis* and other infections. This anti-inflammatory effect could have therapeutic potential in limiting the development of disease sequelae, that goes beyond its effect on the clearance of ocular *C. trachomatis* infection.

Introduction

Trachoma remains the leading infectious cause of blindness worldwide, with the greatest burden in sub-Saharan Africa [1]. Trachomatous disease is initiated by repeated conjunctival infection with C*hlamydia trachomatis*, which triggers prolonged inflammatory episodes that contribute to the development of conjunctival scarring [2]. Infection and clinical signs of active trachoma (follicular and papillary inflammation) are most frequently found in younger children [3]. Conjunctival scarring gradually accumulates through childhood, adolescence and into adult life. Eventually this results in the in-turning of the eyelid (entropion) and eyelashes (trichiasis), abrasion of the eyelashes against the cornea, severe visual impairment and blindness in later life. According to recent World Health Organisation

(WHO) estimates, around 165.1 million people live in trachoma-endemic areas (of whom 89% are from WHO's African region) [4] and 2.8 million have trichiasis [5]. The WHO advocates the use of the **SAFE** Strategy for trachoma control: **S**urgery to correct trichiasis, **A**ntibiotics to treat *C. trachomatis* infection, **F**acial cleanliness and **E**nvironmental improvements to suppress transmission [6].

Annual mass drug administration (MDA) with oral azithromycin for a minimum of 3 years is recommended for communities where the initial prevalence of the clinical sign *trachomatous inflammation-follicular* (TF) is $\geq$10% in children aged 1 to 9 years, with a recommended coverage of 80% of the whole community [7]. In low-prevalence settings this usually leads to a sustained reduction in *C. trachomatis* infection prevalence over time [8–11], however in highly endemic areas infection can re-emerge shortly after MDA [12].

Inflammatory disease signs are reported to persist longer than infection at both the individual and population levels, resulting in the observation of clinical signs in the absence of infection [13–16]. The correlation between clinical signs and *C. trachomatis* infection in communities prior to MDA is further reduced following treatment [14].

Previously we reported on the relationship between clinical signs, *C. trachomatis* infection and the expression of 91 immuno-fibrogenic and cell marker genes at the baseline time-point of a long-term cohort study of Tanzanian children [17]. We found an increase in transcripts related to Th1 and NK cell activity in individuals with *C. trachomatis* infection and an increase in matrix and fibrogenic factors in individuals with active disease in the absence of infection, supporting the findings of several earlier studies [16, 18–24]. However, the changes of these transcriptional responses in an untreated population and the changes that might occur following MDA with azithromycin have not previously been investigated.

Azithromycin is a macrolide antibiotic which has anti-inflammatory and immunomodulatory properties via inhibition of the transcription factor Nuclear Factor Kappa-B [25]. Azithromycin has been reported *in vitro* to suppress T-cell proliferation and activation and to reduce the expression of mucins and pro-inflammatory cytokines [26–28]. As a result azithromycin is found to be beneficial in the treatment of diseases characterised by pathological inflammation [29]. Azithromycin therefore has the potential to exert broad anti-inflammatory effects on conjunctival gene expression, independently of the clearance of *C. trachomatis*.

Here we investigate the changes in clinical signs of trachoma, *C. trachomatis* infection and host immune responses in a cohort of Tanzanian children three- and six-months post azithromycin MDA relative to three pre-treatment time-points. We also investigate the associations between clinical signs, infection and immune responses before and after MDA. This investigation uses data from the first five time-points of a four-year longitudinal study, the baseline findings of which have previously been reported [17].

Methods

Ethics statement

This study was reviewed and approved by the Ethics Committees of the Tanzania National Institute for Medical Research, Kilimanjaro Christian Medical University College and the London School of Hygiene & Tropical Medicine. The study adhered to the tenets of the Declaration of Helsinki. A field worker explained the nature of the study in detail in either Kiswahili or Maasai. Prior to enrolment of a child into this study, their parent or guardian provided written informed consent, on a consent form in Kiswahili, which was witnessed by a third person.

Study design and population

This study was conducted in three adjacent trachoma endemic communities in Kilimanjaro and Arusha regions, Northern Tanzania. In January 2012 we recruited a cohort of children aged 6–10 years from these communities to study the pathogenesis of trachomatous conjunctival scarring. The cohort has subsequently been followed-up every three months for four years. All children aged 6–10 years, who were normally resident in the three villages, were eligible for inclusion. We chose this restricted age group as we considered that they were more likely to show evidence of incident or progressive conjunctival scarring during the four years of the study. The investigation presented in this paper is nested within this overall longitudinal study and uses data from the first five time-points only. The objectives of this nested investigation were to examine changes in *C. trachomatis* infection, clinical signs of trachoma and host immune responses, and the associations between them, three- and six-months post treatment relative to three pre-treatment time-points.

The study population and participant recruitment process are described in more detail in the report of baseline (time-point 1) findings [17]. In brief, these villages are relatively remote, geographically neighbours and have similar patterns of life and traditions. This area is predominately inhabited by people of the Maasai ethnic group. Pastoral activities are the main occupation. The area is dry for much of the year, except for the rainy season (February to May). Water supply is therefore limited, and largely depends on a long-distance water pipe scheme from Mount Kilimanjaro. Family units are organised in *Boma*, with living huts arranged in a circle around a central animal enclosure, which is often characterised by a high density of flies.

Clinical assessments and sample collection

We visited the cohort of children every three months at their homes or schools. An experienced ophthalmic nurse examined their left eye for clinical signs of trachoma using x2.5 loupes and a bright torch. Signs were graded using the 1981 WHO 'FPC' detailed grading system [30]. This sub-divides the features into several four-point severity scales: follicles (F), papillary inflammation (P) and conjunctival scarring (C). This system corresponds to the WHO Simplified Trachoma Grading System in the following way: *Trachomatous inflammation-Follicular* (TF) is equivalent to F2/F3 and *Trachomatous inflammation-Intense* (TI) is equivalent to P3 [31]. Where we refer to "Active Trachoma", we follow the widely used definition of TF (F2/3) and/or TI (P3). However for the purpose of this study, we also consider that both P2 and P3 represent clinically significant papillary inflammation, and refer to this as "TP" [18]. High resolution photographs (Nikon D90 camera with 105mm Macro lens) were taken of the examined eye for independent grading.

The conjunctiva of the left eye was anaesthetised with a drop of preservative-free proxymetacaine hydrochloride 0.5% w/v (Minims, Chauvin Pharmaceuticals Ltd, Surrey, UK). Two conjunctival swab samples (Dacron polyester, Puritan Medical Products Company, Maine, USA) were collected for *C. trachomatis* detection and gene expression analysis. The swabs were passed across the upper tarsal conjunctiva four times, with a quarter turn between each pass. The first swab was placed directly into a tube containing RNAlater solution (Thermo Fisher Scientific, Massachusetts, USA) and the second into a dry tube. The samples were placed into a cool box. Later the same day the dry swab samples were stored directly at -80˚C and the RNAlater samples kept at 4–8˚C overnight and then stored at -80˚C.

Trachoma control measures

The SAFE Strategy is being implemented in this region of Tanzania. Community members who had trachomatous trichiasis were offered free surgery in the local health facility.

Azithromycin MDA was distributed to the members of the three villages by our field team under the auspices of the Tanzanian National NTD Control Programme in the Ministry of Health and with supervision by district eye coordinators. Azithromycin, donated to the National NTD Control Programme by Pfizer through the International Trachoma Initiative, was offered to all community members over the age of 6 months. Single-dose azithromycin MDA (1g for adults and 20mg/kg for children) was distributed annually for three years. For infants under six months, tetracycline eye ointment was provided to their primary carer to be applied twice a day to both eyes for six weeks. The project team provided repeated health education messages around hygiene and sanitation. The first round of MDA was administered to all individuals in the three cohort villages immediately after time-point 3. No adverse effects were reported.

C. trachomatis detection

Two protocols were used for both genomic DNA extraction and *C. trachomatis* detection. For time-point 1 samples, genomic DNA was extracted from dry swabs using the PowerSoil DNA isolation Kit (MO Bio Laboratories, California, USA) according to manufacturer's instructions. For time-points 2 to 5, genomic DNA was extracted from samples stored in RNAlater using the Norgen DNA/RNA Purification Kit (Norgen Biotek Corp, Canada) following the manufacturer's instructions.

C. trachomatis was detected in the time-point 1 samples using a droplet digital PCR assay (ddPCR) and at time-points 2 to 5 using multiplex quantitative real-time PCR (qPCR) previously evaluated against ddPCR [17, 32, 33]. Both assays detect chlamydial plasmid open reading frame 2 (*pORF2*), *C. trachomatis* outer membrane complex protein B (*omcB*) and human endogenous control gene ribonuclease P/MRP Subunit P30 (*RPP30*) [33], using the same primer and probe sequences. The ddPCR reaction contained 5μl of DNA template and primers/probes at a final concentration of 0.3nM using Taqman mastermix. PCR reaction conditions were as follows: 95˚C for 10 minutes, then 40 cycles of 95˚C for 10 seconds and 60˚C for 30 seconds and finally 98˚C for 12 minutes. Droplets were then examined for fluorescence on a QX200TM Droplet Reader (Bio-Rad, UK), providing a quantitative result. The qPCR assay was performed on a ViiA7 thermal cycler (Thermo Fisher Scientific, Massachusetts, USA) using TaqMan Multiplex Master mix in a final volume of 20 μl, containing 4μl of DNA template and primers and probes each at a final concentration of 0.3nM. Cycling conditions were as follows: 95˚C hold for 20 seconds followed by 40 cycles of 95˚C for 1 second and 60˚C for 20 seconds. Samples were tested in duplicate and were considered *C. trachomatis* positive if either replicate amplified *omcB* and/or *pORF2* with a cycle threshold (CT) value <40. In order to compare agreement between ddPCR and qPCR assays, Norgen-extracted DNA from time-point 2 samples (extracted from the first swab stored in RNAlater) were tested using both methods and the results are shown in S1A Table.

Analysis of human gene expression

Total RNA was extracted from samples stored in RNAlater using the Norgen DNA/RNA Purification Kit (Norgen Biotek Corp, Canada) and reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies) following the manufacturer's instructions. Relative abundance of host gene targets was quantified by real-time PCR using customized TaqMan Microfluidic 384-well Array Cards (Thermo Fisher Scientific, Massachusetts, USA) on a ViiA7 real-time PCR machine (Thermo Fisher Scientific, Massachusetts, USA), as previously described [17]. A total of 46 genes of interest were selected based on our previously reported time-point 1 findings, in which we selected genes that were significantly associated with

clinical signs and/or *C. trachomatis* infection status. *HPRT1* was included to each PCR run as an endogenous control gene.

Statistical analysis

Data were managed in Microsoft Access. The ΔCT method was used to adjust for the concentration of input RNA by subtracting the cycle threshold (CT) value of each gene from the CT value of *HPRT1* in the same sample [34]. The distribution of ΔCT values were plotted to assess normality. Host gene expression, *C. trachomatis* infection and clinical data were analysed in STATA v14.

For each time-point the prevalence of clinical signs and *C. trachomatis* infection was estimated and the association between infection and each of TF and TP was estimated using logistic regression. The effect of MDA on infection, TF, TP and AT (Active Trachoma) was estimated using a random effects logistic regression. Each of infection, TF, TP and AT were used as the outcome variable in four separate regressions, the observations from the first three time-points were compared with the observations from time-point 4 (the first observation after the MDA) and participant ID was included as a random effect to account for the fact that these were repeated observations within individuals. An identical analysis was repeated comparing the observations from time-point 5 to those from before MDA to assess whether the estimated effect persisted at six months post treatment.

The change in mean ΔCT value from time-point 1 was plotted for 46 genes at each of the four subsequent time-points and inspected to identify any clear differences between time-points. The change in mean ΔCT following MDA was formally tested by comparing the mean ΔCT in the first three time-points with that from the fourth time-point using a random effects linear regression, with the ΔCT value of each gene as the outcome variable, whether an observation was before or after MDA as the exposure. Participant ID was included as a random effect to again account for repeated observations of the same individuals. An interaction term was included between before/after MDA and whether an individual was actually treated or not to assess the evidence of whether gene expression response after MDA was different in the treated and the untreated groups. These analyses were also repeated comparing the fifth time-point with the three pre-MDA time-points to identify if the effect persisted. Gene expression was then compared at time-point 4 only between treated and untreated individuals. These analyses were initially performed unadjusted and then adjusted for infection status (clinical signs were not adjusted for as they were likely to be caused by both infection and gene expression, rather than the other way around so adjusting for these could bias our estimates). The Benjamini and Hochberg method was used to control for the false discovery rate of 5% [35].

Multivariable linear regression was used at each of the five time-points presented in this report, to test the association of each gene's expression with clinical signs and infection, adjusting for age and sex and assuming a false discovery rate (FDR) of 5% in multiple comparisons [35].

A Preferred Reporting Item (**STROBE_checklist_cohort 2-12-18**) is included in the supporting information. Accession numbers for each gene included in this analysis are included at the end of this manuscript. The protocols used in our analyses are accessible on protocols.io website using the accession number https://dx.doi.org/10.17504/protocols.io.zyhf7t6

List of accession numbers for genes

HPRT1-Hs02800695, ALOX5-Hs01095330, CCL18-Hs00268113, CCL2-Hs00234140, CCL20-Hs01011368, CD247-Hs00609515, NCAM1-Hs00941830, CDH1-Hs01023894, CDH2-Hs00983056, CXCL13-Hs00757930, CXCL5-Hs01099660, DEFB4B;

DEFB4A-Hs00175474, DUOX2-Hs00204187, FGF2-Hs00266645, IFNG-Hs00989291, IL10-Hs00961622, IL12B-Hs01011518, IL17A-Hs00174383, IL19-Hs00604657, IL1B-Hs01555410, IL21-Hs00222327, IL22-Hs01574154, IL23A-Hs00900828, IL6-Hs00985639, IL8-Hs00174103, MMP12-Hs00899662, MMP7-Hs01042796, MMP9-Hs00234579, MUC1-Hs00159357, MUC4-Hs00366414, MUC5AC-Hs00873651, MUC7-Hs03047182, MZB1-Hs00414907, NCR1-Hs00183118, PDGFB-Hs00966522, CD274-Hs01125301, S100A4-Hs00243202, S100A7-Hs01923188, SERPINB4;SERPINB3-Hs00741313, SOCS1-Hs00705164, SOCS3-Hs02330328, SPARCL1-Hs00949881, TGFB1-Hs00998133, VIM-Hs00185584, CTGF-Hs00170014, PTGS2-Hs00153133,

Results

Study participants

At census we registered a total of 666 children aged between 6 and 10 years who were eligible for recruitment at the beginning of this study from three trachoma-endemic villages. At time-point one 506 participants were assessed; their demography, clinical signs and infection status have previously reported in detail [17]. In general participants were predominantly from the Maasai ethnic group (652/666, 97.9%) with a similar number of males (332, [49.9%]) and females (334 [50.1%]) and a mean age of 7.01 years (SD 2.0) at the time of commencing the study. At time-points 2, 3, 4, and 5 we assessed 537, 466, 467 and 477 children, respectively. At each time-point some children were not examined due to being absent in the village, having moved away or declining to participate. After time-point 1, the recruitment of new participants into the longitudinal study was permitted at the second time-point only. MDA was offered immediately after time-point 3 to all members of the three cohort villages. The reported community-wide coverage was 68.7%. All study participants examined in time-point 3 (466) were treated except one who refused. At time-point 4, 392/466 (84.1%) of the individuals seen had been treated.

Clinical signs of trachoma

At time-point 1 the clinical signs previously reported were based on grading of conjunctival photographs, to enable subsequent comparison with the final time-point for determination of scarring incidence and progression [17]. However, for consistency within this analysis of the first five time-points, the field grading data was used. The agreement between field and photograph grading for time-point 1 is shown in S1B Table. Kappa scores between field and photographs grading were 0.92 for TF and 0.68 for TP, with TP being slightly under-reported by field graders.

The prevalence of TF in the first three time-points prior to MDA was fairly consistent (171/506 [33.8%], 163/537 [30.2%] and 104/467 [22.3%]), dropping to 52/467 (11.1%) and 61/479 (12.6%) post-MDA at time-points 4 and 5 (Fig 1). The prevalence of TP was consistently lower than TF and also dropped substantially following MDA (Fig 1). There were no statistically significant differences between males and females in terms of the proportion showing signs of TF and/or TP at any time-point (S2 Table), with the exception of time-point 1 where there was possibly a weak association between TP and female sex.

C. trachomatis infection

The prevalence of infection was fairly consistent prior to MDA, dropping very slightly from 15.4% and 15.3% at time-points 1 and 2 to 11.6% at time-point 3. Three months after azithromycin MDA (time-point 4) infection prevalence dropped to 1.3% and then increased slightly

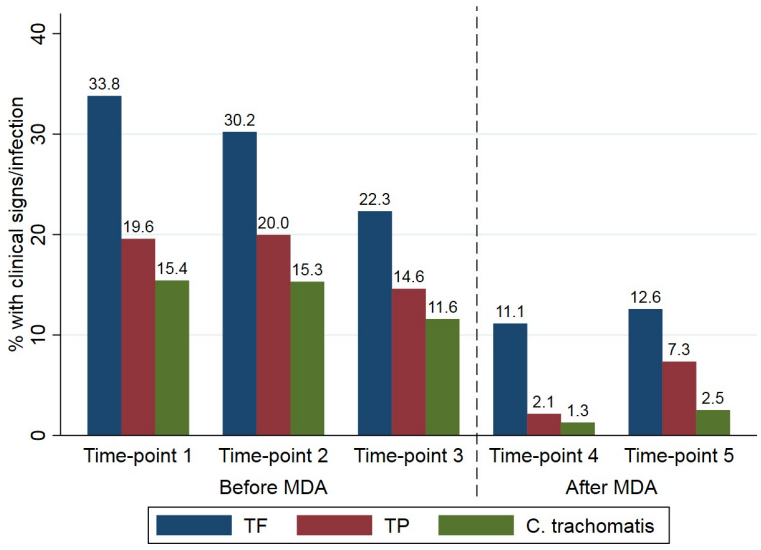

Fig 1. The prevalence of *C. trachomatis* infection and clinical signs before and after MDA. At time-points 1, 2, 3, 4, and 5 data are shown for 506, 537, 466, 467 and 477 children respectively.

https://doi.org/10.1371/journal.pntd.0007559.g001

to 2.5% at time-point 5 (Fig 1). There was strong evidence for an association between *C. trachomatis* infection and clinical signs (TF, TP) at all five time-points (Table 1), with the exception of TP at time-point 5. There was a significant reduction in TF, TP and *C. trachomatis* detection in post-MDA time-points 4 and 5 relative to the combined odds at pre-MDA time-points 1–3 (Table 2). The inflammatory disease (TF and/or TI (active trachoma)) and infection status for each individual at each time-point is shown in Fig 2; participants were grouped by infection and disease status at baseline. There were no statistically significant differences between males and females in terms of the proportion testing positive for *C. trachomatis* at any time-point (S2 Table).

Table 1. The association between *C. trachomatis* detection and clinical signs by time-point. Clinical signs are based on field grading from all five time-points.

Time-point		Follicular Inflammation (TF)		Papillary inflammation (TP)	
		No TF (%)	TF (%)	No TP (%)	TP (%)
1 (n = 506)	Ct infected (%)	18/335 (5.4)	60/171 (35.1)	38/407 (9.3)	40/99 (40.4)
	OR (95%CI)	-	9.52 (5.4–16.8)	-	6.58 (3.9–11.1)
	p-value	-	<0.0001	-	<0.0001
2 (n = 536)	Ct infected (%)	29/374 (7.8)	53/162 (32.7)	38/429 (8.9)	44/107 (41.1)
	OR (95%CI)	-	5.78 (3.5–9.5)	-	7.19 (4.3–12.0)
	p-value	-	<0.0001	-	<0.0001
3 (n = 466)	Ct infected (%)	19/362 (5.3)	35/104 (33.7)	30/398 (7.5)	24/68 (35.3)
	OR (95%CI)	-	9.16 (4.9–16.9)	-	6.69 (3.6–12.5)
	p-value	-	<0.0001	-	<0.0001
Azithromycin MDA					
4 (n = 467)	Ct infected (%)	3/415 (0.7)	3/52 (5.8)	4/457 (0.9)	2/10 (20.0)
	OR (95%CI)	-	8.41 (1.7–42.8)	-	28.31 (4.5–177.5)
	p-value	-	0.010	-	<0.0001
5 (n = 477)	Ct infected (%)	4/417 (1.0)	8/60 (13.1)	10/442 (2.3)	2/35 (5.7)
	OR (95%CI)	-	15.88 (4.6–54.5)	-	2.62 (0.6–12.4)
	p-value	-	<0.0001	-	0.226

https://doi.org/10.1371/journal.pntd.0007559.t001

Table 2. Comparison of *C. trachomatis* infection and disease signs between the pre-treatment (odds of time-points 1, 2 and 3) to the post-treatment time-points (4 and 5 analysed separately). Clinical signs are based on field grading from all five time-points.

		Infection	TF	TP	Active Trachoma
Combined odds of pre-MDA time-points vs. time-point 4 (3 months post-MDA)	OR	0.01	0.10	0.03	0.08
	(95%CI)	(0.004–0.04)	(0.06–0.17)	(0.01–0.07)	(0.05–0.13)
	p-value	<0.0001	<0.0001	<0.0001	<0.0001
Combined odds of pre-MDA time-points vs. time-point 5 (6 months post-MDA)	OR	0.04	0.15	0.18	0.14
	(95%CI)	(0.02–0.10)	(0.10–0.23)	(0.11–0.30)	(0.10–0.22)
	p-value	<0.0001	<0.0001	<0.0001	<0.0001

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Conjunctival gene expression

Forty-six genes of interest were quantified in all individuals who were sampled at each of the five time-points. All amplified targets were included in the analyses. For each time-point, multivariate linear regression models were constructed for expression of each gene to investigate associations with TF, TP and *C. trachomatis* infection, adjusting for age and sex (S3 Table). The associations between the expression of specific genes with clinical signs and *C. trachomatis* infection was similar at each time-point and was consistent with the baseline (time-point 1) report [17]. Briefly, in individuals with *C. trachomatis* infection, *IFNG*, *IL22*, *CCL2*, *IL12B*, *CD274*, *IL21*, *IL17A* and *SOCS1* genes were consistently the most upregulated and *S100A4*, *ALOX5*, *MMP7*, *MUC5AC*, *MUC7*, *MUC4*, *MUC1*, *CDH2* and *CDH1* genes were the most downregulated. In individuals with TF and TP, *S100A7*, *CCL18*, *MMP12*, *CXCL13*, *IL10*, *IL19*, *IL21* and *IL17A* were the most upregulated while *S100A4*, *SPARCL1*, *ALOX5* and *MUC5AC* were the most downregulated.

For each target, the difference in mean gene expression (across all individuals) was calculated at each time-point relative to the mean expression at time-point 1 (Fig 3). There was only modest variability between these time-points, with the exception of time-point 4, three months after MDA, which showed marked differences compared to time-point 1 (and the other time-points). The largest increases in expression at time-point 4 relative to time-point 1 were found in

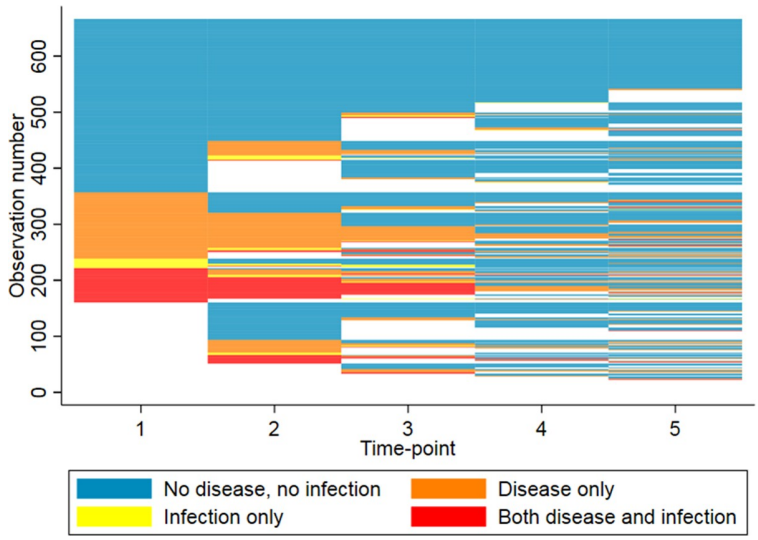

Fig 2. The clinical disease (Active Trachoma) and infection status of individuals at each time-point, ordered by status at baseline. Each row represents an individual and each column represents a time-point.

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SPARCL1, *MUC5AC*, *CDH2*, *CTGF*, *NCAM1*, *CDH1*, *MUC7*, *S100A4*, and *IL12B* (Fig 3). The largest decreases were in *S1007A*, *CCL18*, *CXCL5*, *DEFB4A*, *CXCL13*, *IL19*, *MMP12*, *IDO1*, *IL1B*, and *IL17A* (Fig 3). By time-point 5, six months after MDA, difference in mean gene expression had mostly returned to levels that were similar to those prior to treatment (Fig 3).

The change in mean ΔCT for the expression of each gene from the three time-points before MDA to the fourth time-point three months after MDA was estimated for all participants, and also separately for both individuals who received MDA and those who did not, adjusting for changes in *C. trachomatis* infection status (Table 3). This showed a significant change in mean ΔCT of most targets from before MDA compared with three months after MDA. Interestingly, this change was still observed in the untreated sub-group, albeit at a much reduced scale. The changes in mean ΔCT were larger when the analysis was not adjusted for *C. trachomatis* infection (S4 Table).

To investigate the differences between the treated and untreated groups further, mean ΔCT were compared between the groups at time-point 4 only (S5 Table). This revealed only subtle differences in mean ΔCT between treated and untreated individuals. The anti-inflammatory effect of MDA on gene expression was observed even in individuals without any detectable episodes of chlamydial infection or clinical disease (F0, P0) at any of these five time-points (S6 Table).

Discussion

Infection

The prevalence of *C. trachomatis* was similar across the three time-points before MDA, suggesting that the infection prevalence was relatively stable in this antibiotic-naïve community at around 11% - 16%. The drop in the prevalence of infection and clinical signs at time-point 3 may have been due to medium term natural variation in the prevalence, as strains of *Chlamydia trachomatis* come and go, due to the introduction of public health education to the communities by the field team or possibly due to the change in seasons. There was a

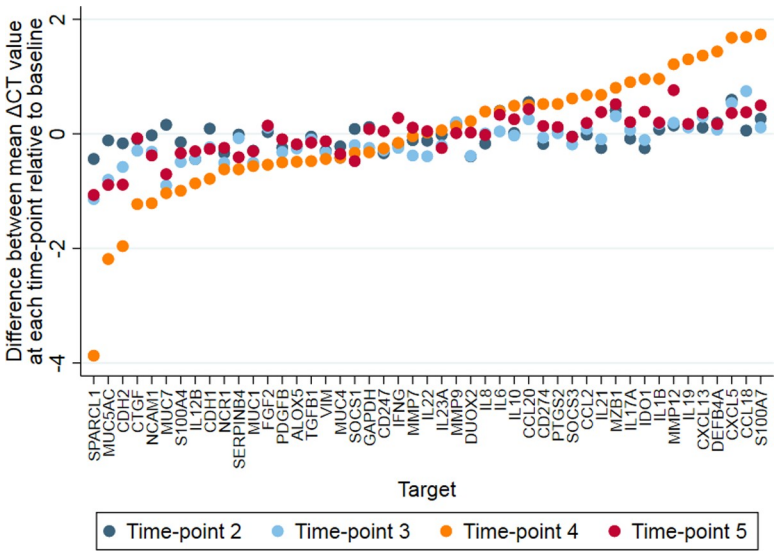

Fig 3. Variation in conjunctival gene expression. The difference in mean ΔCT value (across all individuals) for each gene at time-points 2, 3, 4 and 5 is shown relative to time-point 1. Values are adjusted for *C. trachomatis* infection and active trachoma and are ordered by difference in mean gene expression at Time-point 4.

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Table 3. Estimated Fold Changes (FC) with their respective p-values comparing the expression of each gene pre-MDA (time-points 1,2 and 3) and post-MDA (time-point 4). A FC of >1 indicates increased expression of the gene after MDA. Column 1 includes all participants, columns 2 and 3 show the results stratified by whether an individual actually received the treatment or not. The final column (p-value for interaction) assesses the evidence as to whether the fold change from before to after MDA is different in the treated and untreated groups. Results are ordered by FC of "**All**" individuals (column 1).

Target	All		Untreated Only		Treated Only		p-value for interaction
	FC	p-value	FC	p-value	FC	p-value	
SPARCL1	11.89	7.76 x10^-186	6.81	9.04 x10^-19	13.13	2.54 x10^-173	0.005
MUC5AC	4.02	3.95 x10^-145	2.89	1.20 x10^-14	4.26	5.35 x10^-136	0.009
CDH2	3.42	2.97 x10^-141	2.67	9.16 x10^-16	3.58	3.30 x10^-130	0.026
NCAM1	2.23	9.10 x10^-89	1.83	1.95 x10^-9	2.31	8.32 x10^-83	0.031
CTGF	2.20	3.57 x10^-96	2.25	1.27 x10^-17	2.20	4.10 x10^-81	0.818
MUC7	1.89	7.10 x10^-29	2.17	1.01 x10^-7	1.84	2.24 x10^-23	0.291
S100A4	1.82	7.83 x10^-79	1.75	1.56 x10^-12	1.83	5.50 x10^-69	0.612
CDH1	1.72	1.23 x10^-80	1.69	9.80 x10^-14	1.72	2.55 x10^-69	0.808
FGF2	1.50	2.86 x10^-9	1.48	0.019	1.50	4.19 x10^-8	0.934
SERPINB4	1.43	1.91 x10^-3	1.65	0.067	1.39	0.009	0.570
IL12B	1.42	5.47 x10^-15	1.49	4.10 x10^-4	1.41	1.97 x10^-12	0.657
TGFB1	1.34	2.53 x10^-45	1.43	4.80 x10^-12	1.33	6.55 x10^-36	0.178
ALOX5	1.29	7.49 x10^-39	1.37	7.71 x10^-11	1.27	1.24 x10^-30	0.133
MUC1	1.24	8.04 x10^-17	1.35	4.05 x10^-6	1.22	9.95 x10^-13	0.161
NCR1	1.23	2.51 x10^-10	1.25	0.007	1.23	7.76 x10^-9	0.835
PDGFB	1.22	7.96 x10^-14	1.35	1.26 x10^-5	1.20	2.59 x10^-10	0.130
GAPDH	1.20	5.05 x10^-11	1.49	1.68 x10^-8	1.16	1.33 x10^-6	0.001
SOCS1	1.20	2.64 x10^-8	1.49	5.69 x10^-7	1.15	6.66 x10^-5	0.003
MUC4	1.16	1.80 x10^-7	1.34	6.1 x10^-5	1.13	6.01 x10^-5	0.034
VIM	1.15	9.83 x10^-10	1.22	4.08 x10^-4	1.14	2.11 x10^-7	0.242
CD247	1.01	0.757	1.10	0.196	0.99	0.856	0.205
MMP9	0.95	0.242	0.98	0883	0.95	.0224	0.731
IFNG	0.95	0.219	0.93	0.521	0.95	0.293	0.860
MMP7	0.93	0.033	1.08	0.441	0.90	0.009	0.086
IL23A	0.88	5.88 x10^-4	1.00	0.989	0.86	1.73 x10^-4	0.127
IL22	0.83	0.020	1.26	0.232	0.76	0.002	0.016
IL6	0.83	5.24 x10^-4	0.83	0.150	0.83	0.001	0.959
CCL20	0.82	3.80 x10^-6	0.92	0.447	0.80	2.29 x10^-6	0.240
IL8	0.72	1.42 x10^-18	0.72	4.81 x10^-4	0.72	4.02 x10^-16	0.980
PTGS2	0.70	1.97 x10^-17	0.79	0.02.52	0.69	8.27 x10^-17	0.210
DUOX2	0.69	7.01 x10^-25	0.87	0.134	0.67	1.81 x10^-26	0.006
IL10	0.67	7.68 x10^-24	0.80	0.026	0.64	6.84 x10^-24	0.047
MZB1	0.65	3.20 x10^-17	0.71	0.008	0.64	6.23 x10^-16	0.466
CD274	0.63	6.37 x10^-37	0.77	0.004	0.61	1.28 x10^-36	0.018
CCL2	0.60	2.63 x10^-22	0.63	5.52 x10^-4	0.59	5.94 x10^-20	0.617
SOCS3	0.59	1.13 x10^-38	0.76	0.006	0.57	5.67 x10^-39	0.007
IL1B	0.52	1.78 x10^-43	0.61	2.81 x10^-5	0.50	1.55 x10^-40	0.140
IL21	0.51	3.18 x10^-24	0.72	0.044	0.48	1.03 x10^-24	0.022
IL17A	0.48	6.01 x10^-44	0.64	7.36 x10^-4	0.46	2.57 x10^-43	0.017
IDO1	0.44	6.25 x10^-90	0.68	1.69 x10^-4	0.41	4.74 x10^-93	9.55 x10^-6
MMP12	0.43	9.98 x10^-70	0.56	1.87 x10^-6	0.41	6.82 x10^-67	0.018
IL19	0.40	5.00 x10^-63	0.55	1.42 x10^-5	0.37	4.32 x10^-61	0.012
CXCL5	0.39	2.79 x10^-65	0.61	3.88 x10^-4	0.36	6.18 x10^-66	7.64 x10^-4
DEFB4A	0.38	1.32 x10^-64	0.52	1.16 x10^-5	0.36	1.94 x10^-62	0.015

(*Continued*)

Table 3. (Continued)

Target	All		Untreated Only		Treated Only		p-value for interaction
	FC	p-value	FC	p-value	FC	p-value	
CXCL13	0.38	1.19 x10^-47	0.50	3.58 x10^-5	0.36	3.28 x10^-45	0.067
CCL18	0.33	8.10 x10^-49	0.47	5.75 x10^-5	0.31	7.95 x10^-47	0.032
S100A7	0.28	4.90 x10^-55	0.53	0.002	0.25	3.61 x10^-56	7.03 x10^-4

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substantial reduction in infection prevalence at three months post-MDA, however, it rose slightly by six months post-MDA, suggesting some limited re-emergence of infection. This may be due to insufficient MDA coverage within the community, contact with individuals from surrounding untreated communities, or failure to complete the 6-week daily treatment course of tetracycline eye ointment for infants under 6 months. Members of these communities travel quite frequently to search for pastures and water for livestock, to visit markets and for social interactions with other communities. As a result, it was difficult to achieve high MDA coverage. Previous studies from Tanzania and The Gambia have also reported on the importance of contact between communities as a risk factor for reinfection following treatment [10, 11, 36].

Disease

Clinical signs of inflammation were strongly correlated with *C. trachomatis* infection at all five time-points. In our previous systematic review and meta-analysis we found a strong correlation between TF and *C. trachomatis* infection and a moderate correlation between intense papillary inflammation (TI) and infection prior to initiation of MDA, however after treatment the correlation was weaker for TF and no correlation was found for TI [14]. Most of these earlier studies included multiple rounds of MDA and reported data several years after initiating treatment, therefore it might be too early to see this trend in our cohort. There was no consistent difference in the prevalence of clinical signs of inflammation between males and females, with the exception of the first timepoint which showed a non-significant trend of more TP in females.

Gene expression

Our findings in this study of the associations between host gene expression, *C. trachomatis* infection and clinical signs of inflammation were consistent with previous reports from ourselves and others [16, 18, 21, 22, 24, 37, 38]. Targets that were consistently associated with clinical signs (TF/TP) at all five time-points included antimicrobial peptides (*S100A7)*, pro-inflammatory cytokines and chemokines *(CCL18*, *CXCL13*, *IL10*, *IL19*, *IL21*, *IL17A)*, matrix modifiers *(MMP12* and *SPARCL1)*, epithelial-mesenchymal transition markers *(S100A4)*, microbiota responses (*ALOX5)* and mucins (*MUC5AC)*. Likewise, *C. trachomatis* infection was consistently associated with pro-inflammatory cytokines and chemokines *(IFNG*, *IL22*, *CCL2*, *IL12B*, *IL21*, *IL17A)*, regulators/signalling pathways *(SOCS1*, *CD274)*, *S100A4*, *ALOX5*, matrix modifiers *(MMP7*, *SPARCL1)* and mucins (*MUC7)*. We discussed the functions of these genes and their potential roles in the clearance of *C. trachomatis* infection and immunopathology in detail in our baseline paper [17]. The results at each of the subsequent time-points support the data from baseline, suggesting that strong *IFNG/IL12* responses are important in the clearance of infection, whilst Th17 cell associated cytokines and matrix factors are associated with both infection and the clinical inflammation which persists after infection has been cleared.

Large changes in gene expression were detected at time-point 4, three months after MDA with azithromycin, relative to the three time-points prior to MDA. This variation in gene

expression largely returned to pre-MDA levels by time-point 5, six months post-MDA. Azithromycin appeared to have an anti-inflammatory effect on gene expression, reversing the direction of gene expression change usually associated with clinical signs and *C. trachomatis* infection. Genes normally downregulated in individuals with *C. trachomatis* infection and/or inflammation were upregulated post-MDA (*SPARCL1*, *MUC5AC*, *CDH2*, *CTGF*, *NCAM1*, *CDH1*, *S100A4*, *MUC7*, and *FGF2*), whilst genes normally upregulated (*S100A7*, *CCL18*, *CXCL5*, *CXCL13*, *IL19*, *IDO1*, *MMP12*, *IL17A*, *IL1B* and *IL21*) were strongly downregulated post-MDA. The effect was greatest when *C. trachomatis* infection was not adjusted for, however the effect was still large after adjustment for infection, suggesting that azithromycin has an immunomodulatory effect on gene expression that is independent of the concurrent reduction in infection. This effect was also seen in individuals without any episodes of *C. trachomatis* infection and clinical signs of inflammation across all 5 time-points, supporting this hypothesis. However, we cannot exclude the possibility that azithromycin treatment reduced ocular infections with other sub-clinical or mild inflammation-causing organisms in these individuals. Interestingly, a change in mean gene expression post-MDA was also observed in individuals who did not receive treatment. This could be due to a reduction in transmission and therefore exposure to *C. trachomatis* and/or other infectious organisms within the community as a whole.

Azithromycin has previously been reported to have anti-inflammatory effects in humans, animal and *in vitro* models, leading to improved clinical outcomes through a combined approach of clearing infection and reducing pathological host inflammatory responses [39]. One pre-surgical dose of azithromycin reduced the level of pro-inflammatory cytokines and chemokines detected in oral fluid 6 days following dental implant surgery relative to amoxicillin [40]. Relative to other non-macrolide antibiotics, azithromycin reduced levels of IL-6, IL-8, TNF-α and GM-CSF proteins in individuals with pneumonia and rhinovirus infections [41–43]. MMP9 expression was reduced in the airways of lung transplanted individuals treated with azithromycin between 3 and 6 months [44], and in an experimental laminectomy model in rats, azithromycin was associated with reduction of fibrosis and inflammatory cell density six weeks after administration [45]. Immunomodulatory effects of azithromycin are thought to be enhanced by its long half-life in tissue, lasting for several weeks [46, 47]. In addition to localised anti-inflammatory effects, one round of azithromycin, administered for trachoma control, was associated with a large reduction in infectious and all-cause childhood mortality [48]; a finding which was reinforced by a large multi-country placebo-controlled clinical trial [49]. Our findings of an immunomodulatory effect of azithromycin are therefore consistent with published evidence and suggest that MDA for trachoma control may have an additional protective effect through a systemic reduction in inflammation.

This study has several limitations. It was only feasible to sample one eye from study participants, thus only the left eye was examined and sampled throughout the longitudinal study. The age range of study participants was limited due to the study design of the overall longitudinal study, which this investigation was nested within. The method of *C. trachomatis* detection was changed after the first time-point, which could introduce inconsistencies between the infection results of the first relative to later time-points. Agreement between the two methods used was however deemed acceptable (S1A table). The infection loads of discrepant results were very low and at around the limit of detection. Given the large sample size and the use of three pre-MDA time-points, this variation is not expected to significantly alter the results or their interpretation.

Conclusions

We present evidence that one round of oral azithromycin treatment exerted a strong anti-inflammatory effect on conjunctival gene expression, detectable three months following

treatment but mostly returning to pre-MDA levels by six months. This effect was also observed in individuals without *C. trachomatis* infection and clinical signs of inflammation across all five time-points, indicating that the immunomodulatory effect was at least in part independent of the reduction of *C. trachomatis* infection. Interestingly, a reduced effect was also seen in individuals who did not receive treatment, which could reflect a community reduction in infection transmission and exposure. A limitation of this study is that we cannot determine whether this effect is mediated directly through inhibition of pro-inflammatory intracellular signalling molecules, through reductions in concurrent, sub-clinical infections, and/or through reduction of infection exposure, and future work should seek to understand these mechanisms. Conjunctival papillary inflammation is a significant risk factor for scarring progression [2], therefore the anti-inflammatory effect of azithromycin might have therapeutic potential in limiting the development of disease sequelae, that goes beyond its effect on the prevalence of ocular *C. trachomatis* infection.

Supporting information

S1 Checklist. STROBE checklist.
(DOC)

S1 Table. Table A) 2 x 2 table showing agreement between qPCR and ddPCR assays for C. trachomatis detection in DNA extracted from conjunctival swabs at time-point 2. Sensitivity = 82.6% (95% CI 72.8–89.9), Specificity = 96.7% (95% CI 96.7–99.4), NPV = 96.6% (95% CI 94.8–97.6%); PPV = 91.0% (95% CI 82.9–95.5%); Cohens Kappa = 0.84, Accuracy: overall probability that a sample will be correctly classified 95.8% (95% CI 93.7–97.4), for these samples at this prevalence (16.4%) with ddPCR as the reference standard. Table B) Agreement between field and photo grading at baseline (time-point 1) for follicular and papillary inflammation in the conjunctiva. Kappa scores between field and photographs grading were 0.92 for TF and 0.68 for TP.
(DOCX)

S2 Table. The relationship between sex and (i) clinical signs (from field grading) and (ii) C. trachomatis infection of each of the 5 time-points. The number of individuals with each clinical phenotype or infection is shown as a proportion of the total number of males and females at each time-point. Associations between sex and clinical phenotypes or infection were tested using logistic regression.
(DOCX)

S3 Table. Multivariable linear regression models for conjunctival gene expression associated with clinical signs, *C. trachomatis*, female sex and age. FC = fold change. Using the Benjamini and Hochberg approach to adjust for multiple comparisons, in order to control the false discovery rate <5% only tests with a p-value below 0.027 are considered statistically significant.
(XLSX)

S4 Table. Estimated Fold Changes (FC) with their respective p-values comparing the expression of each gene between the combined first three time-points (time-points 1, 2 and 3) before MDA and time-point 4 (three months after MDA), not adjusted for *C. trachomatis* infection. A FC of >1 indicates increased expression of the gene at time-point 4. Random effects multivariable linear regression of all individuals (first panel), untreated only (second panel) and treated only (third panel). The final column (p-value for interaction) provides evidence as to whether the fold change from before to after MDA is different in the

treated and untreated groups. Results are ordered by FC of "**All**" individuals. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control the false discovery rate <5%, only tests with a p-value <0.035 are considered statistically significant.
(DOCX)

S5 Table. Estimated Fold Change (FC) with their respective p-values for the expression of each gene at time-point 4 only (three months post MDA), comparing MDA treated (after time-point 3) to untreated individuals. Multivariable linear regression of all individuals adjusted (first panel) and not adjusted (second panel) for *C. trachomatis* infection. Results are ordered by FC of adjusted data with infection. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control false discovery rate <5%, only tests with a p-value <0.009 are considered statistically significant.
(DOCX)

S6 Table. Estimated fold changes (FC) with their respective p-values comparing the expression of each gene between the combined first three time-points (1, 2 and 3) before MDA and separately time-points 4 and 5 (three and six months following MDA treatment), in 122 individuals who were free from infection and disease (F0, P0) at all 5 time-points. Results are ordered by Fold Change (FC) in pre-MDA time-points vs. time-point 4. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control the false discovery rate <5%, only tests with a p-value <0.035 are considered statistically significant.
(DOCX)

Author Contributions

Conceptualization: Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

Data curation: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod.

Formal analysis: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod.

Funding acquisition: Martin J. Holland, Matthew J. Burton.

Investigation: Athumani M. Ramadhani, Tamsyn Derrick, Martin J. Holland, Matthew J. Burton.

Methodology: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Patrick Massae, Aiweda Malisa, Kelvin Mbuya, Chrissy H. Roberts, Martin J. Holland, Matthew J. Burton.

Project administration: Athumani M. Ramadhani, Patrick Massae, Aiweda Malisa, Kelvin Mbuya, Tara Mtuy, William Makupa, Martin J. Holland, Matthew J. Burton.

Resources: Patrick Massae, Aiweda Malisa, Tara Mtuy, William Makupa, Martin J. Holland, Matthew J. Burton.

Supervision: Martin J. Holland, Matthew J. Burton.

Visualization: David Macleod.

Writing – original draft: Athumani M. Ramadhani.

Writing – review & editing: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Tara Mtuy, William Makupa, Chrissy H. Roberts, Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

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Supplementary Table 1a. 2 x 2 table showing agreement between qPCR and ddPCR assays for *C. trachomatis* detection in DNA extracted from conjunctival swabs at time-point 2.

		ddPCR		
		+	**-**	**Total**
qPCR	**+**	**71 (13.6%)**	**7 (1.3 %)**	**78**
	-	**15 (2.9%)**	**430 (82.2%)**	**445**
	Total	**86**	**437**	**523**

Sensitivity = 82.6% (95% CI 72.8 – 89.9), Specificity = 96.7% (95% CI 96.7 -99.4), NPV = 96.6% (95% CI 94.8 – 97.6%); PPV = 91.0% (95% CI 82.9 – 95.5%); Cohens Kappa = 0.84, Accuracy: overall probability that a sample will be correctly classified 95.8% (95% CI 93.7 – 97.4),for these samples at this prevalence (16.4%) with ddPCR as the reference standard.

Supplementary Table 1b. Agreement between field and photo grading at baseline (time-point 1) for follicular and papillary inflammation in the conjunctiva. Kappa scores between field and photographs grading were 0.92 for TF and 0.68 for TP.

Field Grading	**Photo Grading**			
	No TF (%)	***TF (%)***		
No TF	317 (94.63%)	18 (5.37%)		
TF	19 (11.11%)	152 (88.89%)		
	No TP (%)	***TP (%)***		
No TP	345 (84.77%)	62 (15.23%)		
TP	1 (1.01%)	98 (98.99%)		
	F0 (%)	***F1 (%)***	***F2 (%)***	***F3 (%)***
F0	140 (96.55%)	4 (2.76%)	1 (0.69)	0 (0.00%)
F1	61 (32.11%)	112 (58.95%)	17 (8.95%)	0 (0.00%)
F2	0 (0.00%)	16 (21.33%)	49 (65.33%)	10 (13.33%)
F3	0 (0.00%)	3 (3.13%)	14 (14.58%)	79 (82.29%)
	P0 (%)	***P1 (%)***	***P2 (%)***	***P3 (%)***
P0	173 (60.49%)	99 (34.62%)	13 (4.55%)	1 (0.35%)
P1	6 (4.96%)	67 (55.37%)	41 (33.88%)	7 (5.79%)
P2	1 (1.85%)	0 (0.00%)	38 (70.37%)	15 (27.78%)
P3	0 (0.00%)	0 (0.00%)	4 (8.89%)	41 (91.11%)

Supplementary Table 2. The relationship between sex and (i) clinical signs (from field grading) and (ii) *C. trachomatis* infection of each of the 5 time-points. The number of individuals with each clinical phenotype or infection is shown as a proportion of the total number of males and females at each time-point. Associations between sex and clinical phenotypes or infection were tested using logistic regression.

Time-point 1				Time-point 2				Time-point 3				MDA	Time-point 4				Time-point 5			
Male (%)	**Female (%)**	**OR (95%CI)**	**p-value**	**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**	**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**		**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**	**Male (%)**	**Female (%)**	**OR (95%CI)**	**p-value**
Follicular Trachoma (TF)																				
77/251 (30.7)	94/255 (36.9)	1.32 (0.9-1.9)	0.142	72/258 (27.9)	90/278 (32.4)	1.24 (0.9-1.8)	0.261	44/218 (20.2)	60/248 (24.2)	1.28 (0.8-2.0)	0.278		23/216 (10.7)	29/251 (11.6)	1.10 (0.6-2.0)	0.756	27/229 (11.8)	34/248 (13.7)	1.19 (0.7-2.0)	0.531
Papillary Inflammation (TP)																				
41/251 (16.3)	58/255 (26.8)	1.51 (1.0-2.4)	0.070	45/258 (17.4)	63/278 (22.7)	1.39 (0.9-2.1)	0.133	28/218 (12.8)	41/248 (16.5)	1.34 (0.8-2.3)	0.264		6/216 (2.8)	4/251 (1.6)	0.57 (0.2-2.0)	0.384	12/229 (5.2)	23/248 (9.3)	1.85 (0.9-3.8)	0.096
Chlamydia trachomatis																				
34/252 (13.5)	44/255 (17.3)	1.34 (0.8-2.2)	0.241	32/258 (12.4)	48/278 (17.3)	1.47 (0.9-2.4)	0.116	31/218 (14.2)	24/248 (9.7)	0.65 (0.4-1.1)	0.131		1/216 (0.5)	4/251 (1.60)	3.48 (0.4-31)	0.266	6/229 (2.6)	6/248 (2.4)	0.92 (0.3-2.9)	0.889

Supplementary Table 3: Multivariable linear regression models for conjunctival gene expression associated with clinical signs, *C. trachomatis* , female sex and age. FC = fold change. Using the Benjamini and Hochberg approach to adjust for multiple comparisons, in order to control the false discovery rate <5% only tests with a p-value below 0.027 are considered statistically significant.

	Time-point 1										Time-point 2									
	TF		TP		*C. trachomatis*		Sex		Age		TF		TP		*C. trachomatis*		Sex		Age	
Target	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value
Antimicrobial Peptides																				
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	1.21	0.314	2.2	0.000	1.67	0.024	1.51	0.005	0.89	0.002	1.28	0.089	2.29	0.000	1.89	0.001	1.37	0.010	0.86	0.000
Psoriasin-1 (*S100A7*)	2.41	0.001	3.68	0.000	2.01	0.024	1.6	0.021	0.86	0.004	2.03	0.000	5.25	0.000	2.12	0.002	1.52	0.009	0.79	0.000
Cytokines/Chemokines																				
Chemokine ligand 2 (*CCL2*)	1.49	0.019	2.46	0.000	3.52	0.000	1.31	0.045	0.86	0.000	1.5	0.002	2.52	0.000	3.85	0.000	1.17	0.151	0.86	0.000
Chemokine ligand 5 (*CXCL5*)	1.6	0.019	2.18	0.001	0.97	0.889	0.94	0.697	0.9	0.008	1.76	0.000	2.1	0.000	1.53	0.034	1.04	0.791	0.88	0.000
Chemokine ligand 13 (*CXCL13*)	2.13	0.000	2.7	0.000	2.5	0.000	1.4	0.039	0.86	0.000	2.3	0.000	3.33	0.000	2.79	0.000	1.35	0.037	0.83	0.000
Chemokine ligand 18 (*CCL18*)	1.79	0.004	2.95	0.000	2.25	0.001	1.11	0.521	0.86	0.000	2.2	0.000	4.28	0.000	2.26	0.000	1.07	0.660	0.81	0.000
Chemokine ligand 20 (*CCL20*)	1.76	0.000	1.61	0.004	1.47	0.028	1.12	0.338	0.94	0.048	1.66	0.000	1.52	0.001	1.86	0.000	1.11	0.272	0.9	0.000
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	1.5	0.013	1.73	0.003	2.33	0.000	1.54	0.001	0.88	0.000	1.41	0.014	1.53	0.008	2.58	0.000	1.5	0.001	0.83	0.000
Interferon gamma(*IFNG*)	1.67	0.000	1.36	0.055	7.95	0.000	1.5	0.000	0.9	0.001	1.77	0.000	1.73	0.000	7.19	0.000	1.28	0.011	0.88	0.000
Interleukin 1 beta(*IL1B*)	1.54	0.011	2.09	0.000	1.89	0.002	1.32	0.041	0.9	0.003	1.44	0.005	1.57	0.002	2.46	0.000	1.07	0.531	0.88	0.000
Interleukin 6 (*IL6*)	1.32	0.077	1.82	0.001	1.2	0.000	1.07	0.589	0.93	0.020	1.5	0.001	1.43	0.012	2.46	0.000	1	0.982	0.91	0.000
Interleukin 8 (*IL8*)	1.25	0.085	1.76	0.000	1.55	0.006	1.16	0.154	0.96	0.140	1.38	0.005	1.88	0.000	1.53	0.003	1.04	0.691	0.92	0.001
Interleukin 10 (*IL10*)	1.51	0.003	2.03	0.000	2.12	0.000	1.32	0.012	0.91	0.001	1.72	0.000	2.31	0.000	2.29	0.000	1.15	0.150	0.91	0.000
Interleukin 12 beta (*IL12B*)	1.69	0.000	1.43	0.005	3.71	0.000	1.37	0.001	0.97	0.154	1.36	0.000	1.32	0.019	4.99	0.000	1.11	0.214	0.94	0.007
Interleukin 17A (*IL17A*)	1.73	0.001	1.82	0.001	2.81	0.000	1.37	0.014	0.87	0.000	2.03	0.000	2.23	0.000	2.95	0.000	1.39	0.005	0.87	0.000
Interleukin 19 (*IL19*)	1.56	0.018	2.73	0.000	2.63	0.000	1.65	0.001	0.85	0.000	1.79	0.000	3.04	0.000	2.82	0.000	1.54	0.001	0.84	0.000
Interleukin 21 (*IL21*)	2.22	0.000	1.97	0.000	3.75	0.000	1.37	0.017	0.89	0.001	2.08	0.000	2.66	0.000	3.51	0.000	1.28	0.063	0.88	0.000
Interleukin 22 (*IL22*)	1.31	0.114	1.83	0.001	7.29	0.000	1.4	0.015	0.92	0.020	1.48	0.021	1.71	0.004	6.99	0.000	1.26	0.099	0.98	0.497
Interleukin 23A (*IL23A*)	1.49	0.005	1.98	0.000	2.15	0.000	1.15	0.219	0.92	0.002	1.45	0.000	1.7	0.000	2.47	0.000	1.19	0.040	0.9	0.000
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	1.4	0.025	1.78	0.001	1.47	0.033	1.06	0.534	0.96	0.137	1.34	0.006	1.62	0.000	1.68	0.000	0.97	0.748	0.94	0.007
EMT Markers																				
Epithelial cadherin (*CDH1*)	1.22	0.060	1.28	0.034	0.8	0.081	1.05	0.562	0.99	0.762	1.01	0.901	1.12	0.220	1.1	0.364	1.07	0.295	0.98	0.189
Neuronal cadherin (*CDH2*)	0.97	0.787	1.15	0.295	1.29	0.072	1.03	0.271	1.11	0.218	1.01	0.916	1.04	0.756	1.51	0.002	1.13	0.168	1.03	0.279
S100 calcium binding protein A4 (*S100A4*)	1.08	0.523	1.05	0.755	0.49	0.000	0.98	0.846	1.05	0.061	0.82	0.020	0.81	0.036	0.74	0.007	1.04	0.595	1.02	0.371
Vimentin (*VIM*)	1.52	0.002	1.42	0.019	1.65	0.002	1.02	0.845	1.01	0.836	1.31	0.001	1.43	0.000	2.05	0.000	1.12	0.095	0.93	0.000
Matrix Modifiers																				
Connective tissue growth factor (*CTGF-1*)	1.05	0.701	1.2	0.190	1.47	0.009	0.87	0.142	1.01	0.712	0.97	0.735	1.25	0.023	1.24	0.047	0.91	0.171	1	0.992
Fibroblast growth factor 2 (basic) (*FGF2*)	0.9	0.447	1.83	0.000	1.77	0.001	1.03	0.818	0.99	0.658	1.03	0.823	1.69	0.001	1.71	0.001	0.95	0.696	0.97	0.320
Matrix metallopeptidase 7 (*MMP7*)	1.46	0.009	1.43	0.029	0.46	0.000	1.02	0.849	0.94	0.042	1.17	0.225	1.36	0.032	0.61	0.002	1.06	0.576	0.93	0.006
Matrix metallopeptidase 9 (*MMP9*)	1.91	0.000	2.01	0.000	2.06	0.000	1.14	0.251	0.95	0.064	1.67	0.000	1.54	0.001	2.72	0.000	1.13	0.192	0.92	0.001
Matrix metallopeptidase 12 (*MMP12*)	1.78	0.000	2.16	0.000	2.27	0.000	1.28	0.052	0.92	0.013	1.92	0.000	2.59	0.000	2.67	0.000	1.17	0.165	0.88	0.000
Platelet-derived growth factor beta polypeptide (*PDGFB*)	1.3	0.012	1.5	0.001	1.7	0.000	1.2	0.011	0.9	0.010	1.29	0.002	1.56	0.000	1.93	0.000	1.15	0.029	0.93	0.000
SPARC-like 1 (hevin) (*SPARCL1*)	0.57	0.004	0.54	0.006	0.52	0.006	0.77	0.095	1.18	0.000	0.51	0.000	0.43	0.000	0.47	0.001	0.88	0.384	1.18	0.000
Transforming growth factor, beta 1 (*TGFβ1*)	1.3	0.012	1.25	0.067	1.51	0.001	1.14	0.109	0.98	0.274	1.21	0.013	1.19	0.048	1.76	0.000	1.06	0.371	0.94	0.000
Microbiota Response																				
Arachidonate 5-lipoxygenase (*ALOX5*)	1.17	0.157	1.21	0.125	0.85	0.217	1.08	0.351	0.1	0.893	1.04	0.628	1.1	0.301	1.02	0.848	1.04	0.551	0.95	0.004
Dual oxidase 2 (*DUOX2*)	1.2	0.206	1.71	0.001	1.58	0.010	1.36	0.007	0.91	0.001	1.28	0.028	1.56	0.001	1.58	0.001	1.39	0.000	0.88	0.000
Mucins																				
Mucin 1, cell surface associated (*MUC1*)	1.15	0.227	1.28	0.056	1.08	0.602	1.11	0.220	0.97	0.264	1.12	0.154	1.27	0.012	1.14	0.211	1.12	0.087	0.95	0.003
Mucin 4, cell surface associated (*MUC4*)	1.22	0.089	1.55	0.001	0.83	0.182	1	0.988	0.96	0.056	1.05	0.615	1.32	0.008	1.02	0.856	1.16	0.046	0.91	0.000
Mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*)	0.86	0.266	0.94	0.679	0.71	0.038	1.09	0.437	1.04	0.111	0.87	0.207	0.85	0.214	0.73	0.026	1.04	0.634	1.06	0.009
Mucin 7, secreted (*MUC7*)	0.98	0.902	0.9	0.555	0.52	0.000	0.66	0.001	1.08	0.014	0.79	0.065	0.76	0.053	0.64	0.005	0.77	0.012	1.06	0.021
NK Cell Markers																				
CD247 molecule (CD247)	1.41	0.003	1.32	0.031	2.15	0.000	1.2	0.049	1	0.212	1.32	0.001	1.51	0.000	2.14	0.000	1.12	0.106	0.93	0.000
Neural cell adhesion molecule 1 (*NCAM1*)	0.9	0.413	1.12	0.435	2.13	0.000	1.14	0.210	1.01	0.702	1.02	0.832	1.04	0.683	2.01	0.000	1.08	0.268	0.98	0.240
Natural cytotoxicity triggering receptor 1 (*NCR1*)	1.4	0.003	1.33	0.023	2.86	0.000	1.28	0.005	0.94	0.007	1.43	0.000	1.41	0.001	2.86	0.000	1.22	0.009	0.94	0.001
Regulators/Signalling Pathways																				
CD274 molecule (*CD274*)	1.37	0.029	1.64	0.003	3.36	0.000	1.31	0.020	0.9	0.019	1.46	0.000	1.81	0.000	3.42	0.000	1.18	0.053	0.89	0.000
Marginal zone B and B1 cell-specific protein (*MZB1*)	1.76	0.001	1.33	0.117	2.61	0.000	1.29	0.048	0.96	0.201	1.51	0.008	2.84	0.000	2.92	0.000	1.34	0.020	0.89	0.000
Serpin peptidase inhibitor clade B member 4, (*SERPINB4*)	1.08	0.739	3.72	0.000	5.1	0.000	1.05	0.811	0.97	0.564	1.03	0.878	2.94	0.000	5.77	0.000	1.12	0.514	0.9	0.018
Suppressor of cytokine signalling 1 (*SOCS1*)	1.55	0.000	1.48	0.001	2.26	0.000	1.18	0.057	0.94	0.004	1.36	0.000	1.5	0.000	2.76	0.000	1.1	0.191	0.92	0.000
Suppressor of cytokine signalling 3 (*SOCS3*)	1.56	0.002	1.89	0.000	1.35	0.079	1.17	0.169	0.93	0.015	1.39	0.003	1.6	0.000	1.85	0.000	1.08	0.422	0.88	0.000

	Time-point 3										Time-point 4									
	TF		TP		*C. trachomatis*		Sex		Age		TF		TP		*C. trachomatis*		Sex		Age	
Target	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value
Antimicrobial Peptides																				
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	1.12	0.567	1.84	0.005	2.23	0.001	1.38	0.018	0.87	0.000	3.01	0.000	1.64	0.400	3.04	0.135	1.23	0.224	0.91	0.026
Psoriasin-1 (*S100A7*)	1.58	0.080	4.18	0.000	3.34	0.000	1.38	0.080	0.78	0.000	3.01	0.000	2.08	0.154	10.81	0.000	1.13	0.391	0.87	0.000
Cytokines/Chemokines																				
Chemokine ligand 2 (*CCL2*)	1.42	0.039	2.28	0.000	4.1	0.000	1.2	0.124	0.84	0.000	2.43	0.000	1.17	0.646	5.45	0.000	0.94	0.525	0.91	0.000
Chemokine ligand 5 (*CXCL5*)	1.19	0.387	1.72	0.019	2.03	0.004	1.04	0.771	0.82	0.000	4.38	0.000	1.5	0.462	2.51	0.185	1.06	0.731	0.88	0.001
Chemokine ligand 13 (*CXCL13*)	1.68	0.024	2.69	0.000	2.94	0.000	1.43	0.027	0.78	0.000	3.59	0.000	1.41	0.477	10.61	0.000	1.15	0.297	0.84	0.000
Chemokine ligand 18 (*CCL18*)	1.63	0.033	2.92	0.000	3.61	0.000	1.18	0.292	0.83	0.000	2.41	0.000	1.22	0.659	11.77	0.000	0.94	0.635	0.94	0.033
Chemokine ligand 20 (*CCL20*)	1.36	0.059	1.34	0.108	2.05	0.000	1.11	0.367	0.88	0.000	3.12	0.000	1.09	0.844	2.79	0.058	1.13	0.323	0.89	0.000
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	1.39	0.063	1.86	0.002	3.53	0.000	1.61	0.000	0.83	0.000	4.84	0.000	1.49	0.443	6.8	0.004	1.46	0.011	0.88	0.000
Interferon gamma(*IFNG*)	1.43	0.019	1.95	0.000	9.71	0.000	1.26	0.032	0.88	0.000	2.79	0.000	1.08	0.836	13.43	0.000	1.28	0.022	0.88	0.000
Interleukin 1 beta(*IL1B*)	1.27	0.173	1.71	0.006	2.53	0.000	1.15	0.240	0.89	0.000	2.86	0.000	1.78	0.183	3.29	0.030	1.09	0.502	0.94	0.048
Interleukin 6 (*IL6*)	1.03	0.875	1.4	0.087	2.71	0.000	1.05	0.684	0.93	0.018	2.04	0.001	0.72	0.476	3.73	0.022	1.22	0.124	0.91	0.003
Interleukin 8 (*IL8*)	0.93	0.649	1.72	0.003	2.18	0.000	1.12	0.312	0.92	0.006	2.2	0.000	1.63	0.273	1.71	0.335	1.1	0.462	0.98	0.598
Interleukin 10 (*IL10*)	1.25	0.154	2.25	0.000	2.38	0.000	1.17	0.145	0.89	0.000	2.11	0.000	1.38	0.273	4.81	0.000	1.02	0.769	0.91	0.000
Interleukin 12 beta (*IL12B*)	1.44	0.007	1.43	0.018	5.47	0.000	1.16	0.121	0.92	0.000	1.88	0.000	1.06	0.844	10.34	0.000	1.11	0.265	0.92	0.000
Interleukin 17A (*IL17A*)	1.65	0.008	2.18	0.000	3.25	0.000	1.43	0.007	0.83	0.000	4.15	0.000	1.57	0.309	10.54	0.000	1.27	0.055	0.88	0.000
Interleukin 19 (*IL19*)	1.24	0.283	2.73	0.000	3.83	0.000	1.57	0.002	0.83	0.000	3	0.000	2.09	0.110	6.86	0.001	1.23	0.119	0.91	0.004
Interleukin 21 (*IL21*)	1.66	0.015	2.62	0.000	4.41	0.000	1.37	0.029	0.83	0.000	3.49	0.000	1.67	0.236	13.11	0.000	1.2	0.164	0.87	0.000
Interleukin 22 (*IL22*)	1.7	0.014	1.54	0.066	8.75	0.000	1.19	0.252	0.96	0.282	1.41	0.148	1.63	0.332	13.13	0.000	1.17	0.321	0.99	0.736
Interleukin 23A (*IL23A*)	1.11	0.440	1.8	0.000	2.65	0.000	1.11	0.291	0.89	0.000	1.93	0.000	1.02	0.957	5.04	0.000	0.97	0.778	0.91	0.000
Prostaglandin-endoperoxide synthase 2	1.02	0.515	1.42	0.041	1.84	0.001	1.16	0.172	0.9	0.000	2.4	0.000	1.85	0.109	1.83	0.211	0.97	0.778	0.94	0.022
EMT Markers																				
Epithelial cadherin (*CDH1*)	1.02	0.797	1.14	0.212	1.22	0.079	1.06	0.359	0.94	0.000	1.55	0.020	0.88	0.763	1.49	0.441	1.05	0.663	1	0.873
Neuronal cadherin (*CDH2*)	1.03	0.808	0.99	0.947	1.24	0.181	1.13	0.189	1.03	0.317	1.02	0.892	0.59	0.158	1.55	0.344	1.05	0.645	0.99	0.644
S100 calcium binding protein A4 (*S100A4*)	0.96	0.738	0.9	0.447	0.72	0.028	1	0.987	1	0.912	1.52	0.060	0.86	0.746	0.84	0.777	0.87	0.318	1.02	0.483
Vimentin (*VIM*)	1.18	0.102	1.3	0.024	2.17	0.000	1.07	0.360	0.92	0.000	2.17	0.000	1.01	0.978	3.4	0.017	1.03	0.794	0.97	0.216
Matrix Modifiers																				
Connective tissue growth factor (*CTGF-1*)	0.99	0.915	0.98	0.872	1.4	0.012	0.9	0.173	0.97	0.089	1.44	0.045	0.59	0.174	2.22	0.105	0.91	0.397	1.02	0.492
Fibroblast growth factor 2 (basic) (*FGF2*)	1.2	0.182	1.34	0.044	1.85	0.000	0.97	0.725	0.95	0.041	1.46	0.035	1.04	0.922	1.96	0.179	0.96	0.756	0.97	0.263
Matrix metallopeptidase 7 (*MMP7*)	1.36	0.052	1.17	0.366	0.68	0.040	0.97	0.754	0.89	0.000	2.3	0.000	1.14	0.760	1.06	0.917	0.97	0.777	0.94	0.056
Matrix metallopeptidase 9 (*MMP9*)	1.78	0.000	1.18	0.332	2.51	0.000	1.06	0.580	0.9	0.000	2.75	0.000	1.55	0.268	3.44	0.014	1.16	0.187	0.9	0.000
Matrix metallopeptidase 12 (*MMP12*)	1.37	0.097	2.16	0.000	2.89	0.000	1.36	0.020	0.83	0.000	3.5	0.000	1.65	0.332	6.38	0.005	1.18	0.256	0.89	0.002
Platelet-derived growth factor beta polypeptide	1.12	0.247	1.48	0.001	2.08	0.000	1.14	0.066	0.92	0.000	1.8	0.000	1.15	0.690	2.98	0.014	1.1	0.319	0.97	0.270
SPARC-like 1 (hevin) (*SPARCL1*)	0.7	0.142	0.58	0.042	0.42	0.003	0.73	0.066	1.12	0.010	0.36	0.000	0.32	0.025	0.32	0.077	0.85	0.252	1.05	0.178
Transforming growth factor, beta 1 (*TGFβ1*)	1.1	0.338	1.15	0.179	1.81	0.000	1.02	0.741	0.93	0.000	2	0.001	0.94	0.878	3.55	0.020	1.04	0.728	0.99	0.621
Microbiota Response																				
Arachidonate 5-lipoxygenase (*ALOX5*)	0.95	0.633	1.17	0.193	1.1	0.430	1.01	0.845	0.94	0.002	1.75	0.008	1.17	0.726	1.28	0.663	1.03	0.837	0.99	0.729
Dual oxidase 2 (*DUOX2*)	1.16	0.315	1.55	0.008	2.13	0.000	1.5	0.000	0.84	0.000	2.74	0.000	1.95	0.186	4.26	0.023	1.36	0.031	0.91	0.010
Mucins																				
Mucin 1, cell surface associated (*MUC1*)	1.08	0.496	1.26	0.054	1.35	0.021	1.14	0.074	0.9	0.000	2.04	0.001	1.09	0.857	1.69	0.366	1.1	0.453	0.98	0.497
Mucin 4, cell surface associated (*MUC4*)	0.96	0.766	1.19	0.243	1.47	0.016	1.04	0.686	0.89	0.000	1.46	0.056	1.64	0.254	1.65	0.362	1.15	0.257	0.95	0.075
Mucin 5AC, oligomeric mucus/gel-forming	0.84	0.185	0.84	0.229	0.77	0.083	1.09	0.315	1.05	0.036	0.79	0.317	0.6	0.314	0.3	0.059	1.2	0.200	1.08	0.032
Mucin 7, secreted (*MUC7*)	1.02	0.913	0.71	0.064	0.78	0.199	0.7	0.002	0.99	0.819	1.52	0.055	0.66	0.375	0.76	0.653	0.76	0.039	0.95	0.111
NK Cell Markers																				
CD247 molecule (CD247)	1.24	0.061	1.43	0.006	2.8	0.000	1.12	0.178	0.92	0.000	2.07	0.000	0.92	0.812	5.16	0.000	1.07	0.469	0.95	0.019
Neural cell adhesion molecule 1 (*NCAM1*)	0.96	0.719	1.09	0.468	1.83	0.000	0.92	0.271	0.96	0.050	1.57	0.007	0.59	0.150	2.2	0.085	0.9	0.312	0.98	0.539
Natural cytotoxicity triggering receptor 1	1.35	0.011	1.41	0.008	3.01	0.000	1.09	0.291	0.92	0.000	2.46	0.000	1.14	0.704	5.33	0.000	1.17	0.097	0.9	0.000
Regulators/Signalling Pathways																				
CD274 molecule (*CD274*)	1.27	0.090	1.88	0.000	3.56	0.000	1.22	0.047	0.87	0.000	2.64	0.000	1.16	0.683	9.87	0.000	1.14	0.223	0.9	0.000
Marginal zone B and B1 cell-specific protein	1.58	0.015	1.68	0.013	2.3	0.000	1.03	0.811	0.86	0.000	2.7	0.000	1.09	0.867	10.7	0.000	1.16	0.307	0.88	0.000
Serpin peptidase inhibitor clade B member 4,	1.36	0.205	2.46	0.001	3.69	0.000	0.93	0.672	0.87	0.003	0.88	0.736	1.11	0.892	7.78	0.021	0.75	0.220	0.93	0.211
Suppressor of cytokine signalling 1 (*SOCS1*)	1.26	0.032	1.43	0.003	2.97	0.000	1.13	0.105	0.89	0.000	2.06	0.000	1.32	0.379	4.65	0.000	1.02	0.793	0.96	0.040
Suppressor of cytokine signalling 3 (*SOCS3*)	1.21	0.180	1.6	0.003	2.08	0.000	1.15	0.170	0.87	0.000	2.68	0.000	1.54	0.244	3.27	0.012	1.04	0.678	0.94	0.012

		Time-point 5									
		TF		TP		*C. trachomatis*		Sex		Age	
Target	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value
Antimicrobial Peptides											
Defensin, beta 4B,defensin, beta 4A (*DEFB4A*)	0.026	1.98	0.009	3.44	0.000	4.51	0.018	1.52	0.009	0.82	0.000
Psoriasin-1 (*S100A7*)	0.000	3.31	0.000	4.39	0.000	7.39	0.000	2.06	0.000	0.78	0.000
Cytokines/Chemokines											
Chemokine ligand 2 (*CCL2*)	0.000	1.69	0.008	1.71	0.022	8.74	0.000	1.29	0.035	0.92	0.006
Chemokine ligand 5 (*CXCL5*)	0.001	2.5	0.001	2.99	0.001	2	0.215	1.47	0.024	0.82	0.000
Chemokine ligand 13 (*CXCL13*)	0.000	3.26	0.000	2.41	0.003	4.07	0.005	1.59	0.002	0.79	0.000
Chemokine ligand 18 (*CCL18*)	0.033	2.88	0.000	4.07	0.000	7.05	0.000	1.13	0.388	0.84	0.000
Chemokine ligand 20 (*CCL20*)	0.000	2.23	0.000	1.45	0.133	2.61	0.023	1.41	0.007	0.92	0.007
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	0.000	2.49	0.000	3.16	0.000	3.77	0.008	1.46	0.013	0.85	0.000
Interferon gamma(*IFNG*)	0.000	1.44	0.032	1.68	0.011	16.98	0.000	1.41	0.001	0.94	0.013
Interleukin 1 beta(*IL1B*)	0.048	2.1	0.000	2.09	0.003	3.24	0.006	1.19	0.180	0.87	0.000
Interleukin 6 (*IL6*)	0.003	1.44	0.073	0.96	0.866	4.67	0.000	1.16	0.242	0.94	0.048
Interleukin 8 (*IL8*)	0.598	1.61	0.016	1.58	0.051	3.27	0.003	1.08	0.512	0.94	0.035
Interleukin 10 (*IL10*)	0.000	1.96	0.000	1.66	0.007	3.9	0.000	1.2	0.057	0.88	0.000
Interleukin 12 beta (*IL12B*)	0.000	1.36	0.044	0.98	0.909	6.43	0.000	1.2	0.044	1	0.876
Interleukin 17A (*IL17A*)	0.000	2.31	0.000	2.4	0.000	5.98	0.000	1.59	0.000	0.86	0.000
Interleukin 19 (*IL19*)	0.004	2.53	0.000	3.49	0.000	7.06	0.000	1.6	0.001	0.83	0.000
Interleukin 21 (*IL21*)	0.000	2.39	0.000	2.1	0.001	7.25	0.000	1.66	0.000	0.88	0.000
Interleukin 22 (*IL22*)	0.736	1.37	0.109	1.85	0.010	13.77	0.000	1.17	0.209	0.98	0.461
Interleukin 23A (*IL23A*)	0.000	1.77	0.001	1.71	0.011	3.67	0.000	1.12	0.285	0.91	0.000
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	0.022	1.7	0.003	1.66	0.018	2.22	0.029	1.25	0.049	0.87	0.000
EMT Markers											
Epithelial cadherin (*CDH1*)	0.873	1.19	0.207	1.2	0.272	1.24	0.444	1.12	0.188	0.95	0.032
Neuronal cadherin (*CDH2*)	0.644	0.89	0.461	0.8	0.236	1.63	0.126	1.23	0.038	1.06	0.030
S100 calcium binding protein A4 (*S100A4*)	0.483	0.88	0.499	0.87	0.523	0.84	0.641	1.03	0.780	1.02	0.587
Vimentin (*VIM*)	0.216	1.49	0.002	1.38	0.034	2.58	0.000	1.08	0.330	0.95	0.008
Matrix Modifiers											
Connective tissue growth factor (*CTGF-1*)	0.492	1.08	0.661	0.93	0.720	1.93	0.056	1	0.985	0.99	0.690
Fibroblast growth factor 2 (basic) (*FGF2*)	0.263	1.37	0.065	1.04	0.836	2.32	0.010	1.09	0.448	1.02	0.417
Matrix metallopeptidase 7 (*MMP7*)	0.056	1.49	0.043	1.34	0.211	0.91	0.810	1.01	0.923	0.89	0.000
Matrix metallopeptidase 9 (*MMP9*)	0.000	1.87	0.001	1.56	0.036	3.42	0.001	1.11	0.330	0.91	0.001
Matrix metallopeptidase 12 (*MMP12*)	0.002	1.89	0.004	1.63	0.065	5.91	0.000	1.23	0.125	0.87	0.000
Platelet-derived growth factor beta polypeptide (*PDGFB*)	0.270	1.37	0.017	1.55	0.005	2.77	0.000	1.28	0.002	0.95	0.007
SPARC-like 1 (hevin) (*SPARCL1*)	0.178	0.37	0.000	0.21	0.000	0.27	0.017	0.95	0.736	1.25	0.000
Transforming growth factor, beta 1 (*TGFβ1*)	0.621	1.31	0.054	1.31	0.100	1.89	0.025	1.13	0.166	0.96	0.040
Microbiota Response											
Arachidonate 5-lipoxygenase (*ALOX5*)	0.729	1.06	0.719	1.03	0.881	1.43	0.263	1.06	0.576	0.94	0.018
Dual oxidase 2 (*DUOX2*)	0.010	1.96	0.002	2.42	0.001	2.79	0.022	1.32	0.039	0.86	0.000
Mucins											
Mucin 1, cell surface associated (*MUC1*)	0.497	1.12	0.504	1.09	0.673	1.91	0.064	1.08	0.449	0.94	0.013
Mucin 4, cell surface associated (*MUC4*)	0.075	1.26	0.183	1.17	0.442	1.61	0.174	1.17	0.142	0.93	0.010
Mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*)	0.032	0.83	0.293	0.99	0.960	0.76	0.442	1.23	0.062	1.11	0.000
Mucin 7, secreted (*MUC7*)	0.111	0.88	0.533	1.15	0.555	0.7	0.380	0.85	0.174	0.94	0.063
NK Cell Markers											
CD247 molecule (CD247)	0.019	1.32	0.097	1.53	0.033	3.21	0.001	1.22	0.056	0.92	0.002
Neural cell adhesion molecule 1 (*NCAM1*)	0.539	0.89	0.427	1.15	0.418	1.72	0.071	1	0.996	1.02	0.363
Natural cytotoxicity triggering receptor 1 (*NCR1*)	0.000	1.43	0.022	1.53	0.021	2.97	0.001	1.28	0.011	0.91	0.000
Regulators/Signalling Pathways											
CD274 molecule (*CD274*)	0.000	1.74	0.002	2.02	0.001	5.56	0.000	1.29	0.024	0.87	0.000
Marginal zone B and B1 cell-specific protein (*MZB1*)	0.000	1.83	0.005	1.53	0.102	4.2	0.001	1.46	0.005	0.87	0.000
Serpin peptidase inhibitor clade B member 4, (*SERPINB4*)	0.211	1.18	0.600	2.15	0.044	21.14	0.000	0.98	0.940	1	0.965
Suppressor of cytokine signalling 1 (*SOCS1*)	0.040	1.67	0.001	1.52	0.021	2.89	0.001	1.1	0.311	0.9	0.000
Suppressor of cytokine signalling 3 (*SOCS3*)	0.012	1.76	0.003	1.86	0.006	2.47	0.020	1.12	0.345	0.85	0.000

Supplementary Table 4. Estimated Fold Changes (FC) with their respective p-values comparing the expression of each gene between the combined first three time-points (time-points 1, 2 and 3) before MDA and time-point 4 (three months after MDA), not adjusted for *C. trachomatis* infection. A FC of >1 indicates increased expression of the gene at time-point 4. Random effects multivariable linear regression of all individuals (first panel), untreated only (second panel) and treated only (third panel). The final column (p-value for interaction) provides evidence as to whether the fold change from before to after MDA is different in the treated and untreated groups. Results are ordered by FC of ''**All**'' individuals. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control the false discovery rate <5%, only tests with a p-value <0.035 are considered statistically significant.

Target	All		Untreated Only		Treated Only		p-value for interaction
	FC	p-value	FC	p-value	FC	p-value	
SPARCL1	18.65	2.75x10^-250	8.21	3.65x10^-23	21.77	1.32x10^-235	2.69X10^-5
MUC5AC	5.33	7.6x10^-215	3.14	3.06x10^-18	5.88	7.21x10^-206	1.19X10^-5
CDH2	3.92	2.22x10^-169	2.72	3.72x10^-17	4.24	1.9x10^-158	6.84X10^-4
MUC7	2.71	2.52x10^-72	2.45	9.19x10^-11	2.74	5.15x10^-62	0.458
CTGF	2.40	2.04x10^-118	2.29	1.12x10^-19	2.43	1.68x10^-101	0.553
NCAM1	2.34	5.3x10^-90	1.79	8.89x10^-9	2.49	8.21x10^-86	0.003
S100A4	2.30	1.87x10^-143	1.95	5.02x10^-17	2.37	4.66x10^-129	0.026
CDH1	1.94	1.28x10^-122	1.78	1.38x10^-17	1.97	3.13x10^-107	0.173
ALOX5	1.42	1.61x10^-65	1.45	1.37x10^-13	1.41	2.70x10^-53	0.606
MUC1	1.39	7.87x10^-39	1.40	4.01x10^-8	1.40	2.12x10^-33	0.975
FGF2	1.39	6.72X10^-6	1.41	0.046	1.42	1.77x10^-5	0.994
TGFB1	1.34	9.68x10^-46	1.41	5.79x10^-12	1.32	2.41x10^-35	0.272
MUC4	1.30	5.69x10^-19	1.40	2.54x10^-6	1.29	4.82x10^-15	0.273
GAPDH	1.29	6.09x10^-18	1.50	1.65x10^-8	1.25	1.06x10^-11	0.020
IL12B	1.24	7.69x10^-6	1.28	0.035	1.24	5.35x10^-5	0.794
PDGFB	1.22	1.49x10^-12	1.29	1.54x10^-4	1.20	1.01x10^-9	0.351
NCR1	1.21	2.44x10^-8	1.15	0.086	1.22	6.90x10^-8	0.530
VIM	1.13	7.56x10^-8	1.17	0.005	1.13	3.59x10^-6	0.515
SOCS1	1.13	2.42x10^-4	1.36	1.35x10^-4	1.09	0.021	0.012
SX10RPINB4	1.13	0.341	1.34	0.320	1.09	0.565	0.520
MMP7	1.11	0.006	1.17	0.086	1.09	0.028	0.487
CD247	0.96	0.122	1.02	0.770	0.94	0.059	0.297
MMP9	0.84	9.28x10^-5	0.91	0.371	0.83	1.37x10^-4	0.457
IL23A	0.83	2.24x10^-6	0.92	0.369	0.81	2.43x10^-6	0.250
IL6	0.80	2.75x10^-5	0.77	0.050	0.81	3.65x10^-4	0.758
CCL20	0.78	5.98x10^-8	0.88	0.263	0.76	8.80x10^-8	0.232
IFNG	0.76	7.28x10^-8	0.76	0.030	0.77	1.07x10^-6	0.985
DUOX2	0.71	1.51x10^-22	0.87	0.116	0.69	1.05x10^-22	0.011
PTGS2	0.68	1.96x10^-19	0.77	0.011	0.67	3.94x10^-18	0.203
IL8	0.68	1.75x10^-24	0.69	7.73x10^-5	0.68	2.06x10^-20	0.822
IL22	0.65	1.91x10^-6	0.95	0.801	0.60	2.79x10^-7	0.042
IL10	0.60	7.40x10^-35	0.74	0.002	0.58	2.33x10^-33	0.029
SOCS3	0.58	8.67x10^-40	0.73	0.001	0.56	3.03x10^-38	0.015
MZB1	0.56	6.51x10^-26	0.65	0.001	0.55	7.23x10^-24	0.260
CD274	0.54	1.82x10^-57	0.68	3.92x10^-5	0.51	4.37x10^-55	0.008
CCL2	0.48	3.48x10^-37	0.55	1.23x10^-5	0.47	1.01x10^-32	0.341
IL1B	0.46	5.42x10^-59	0.56	7.91x10^-07	0.44	4.31x10^-54	0.054
IDO1	0.41	7.77x10^-102	0.65	2.11x10^-5	0.38	3.28x10^-104	1.56X10^-6
IL17A	0.40	4.45x10^-62	0.57	2.46x10^-5	0.38	2.21x10^-59	0.005
IL21	0.40	1.52x10^-39	0.61	0.003	0.37	6.53x10^-39	0.007
MMP12	0.37	1.06x10^-87	0.51	4.56x10^-8	0.35	3.18x10^-82	0.005
CXCL5	0.36	3.84x10^-72	0.59	2.11x10^-4	0.32	1.17x10^-72	1.04X10^-4
DX10FB4A	0.35	1.46x10^-64	0.50	4.51x10^-6	0.33	5.23x10^-61	0.013
IL19	0.33	1.01x10^-79	0.48	2.57x10^-7	0.31	1.71x10^-75	0.005
CXCL13	0.30	4.71x10^-66	0.44	9.38x10^-7	0.28	3.44x10^-62	0.015
S100A7	0.24	2.33x10^-60	0.47	3.39x10^-4	0.21	3.46x10^-61	4.20X10^-4
CCL18	0.23	1.32x10^-77	0.41	1.17x10^-6	0.21	9.46x10^-76	8.98X10^-4

Supplementary Table 5. Estimated Fold Change (FC) with their respective p-values for the expression of each gene at time-point 4 only (three months post MDA), comparing MDA treated (after time-point 3) to untreated individuals. Multivariable linear regression of all individuals adjusted (first panel) and not adjusted (second panel) for *C. trachomatis* infection. Results are ordered by FC of adjusted data with infection. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control false discovery rate <5%, only tests with a p-value <0.009 are considered statistically significant.

Target	Adjusted for infection		Not adjusted for infection	
	FC	p-value	FC	p-value
SPARCL1	2.06	3.10x10^-04	2.34	2.55 x10^-5
MUC5AC	1.44	0.011	1.62	9.45 x10^-4
MUC7	1.28	0.081	1.35	0.030
CDH2	1.27	0.062	1.30	0.037
NCAM1	1.19	0.055	1.19	0.048
IFNG	1.16	0.225	1.03	0.802
S100A4	1.08	0.238	1.14	0.052
MZB1	1.07	0.660	0.97	0.820
NCR1	1.05	0.568	0.99	0.904
CDH1	1.03	0.701	1.05	0.464
CD247	1.02	0.748	0.97	0.668
IL12B	1.01	0.890	0.92	0.468
VIM	1.00	0.950	0.97	0.599
TGFB1	0.98	0.738	0.96	0.429
SERPINB4	0.97	0.913	0.89	0.700
ALOX5	0.95	0.389	0.98	0.725
CCL2	0.95	0.602	0.89	0.247
PDGFB	0.95	0.440	0.93	0.300
IL6	0.94	0.627	0.91	0.479
MMP9	0.94	0.552	0.90	0.331
CTGF	0.93	0.462	0.94	0.529
IL23A	0.93	0.340	0.88	0.110
GAPDH	0.92	0.330	0.91	0.275
MMP7	0.91	0.370	0.93	0.520
SOCS1	0.90	0.183	0.86	0.057
MUC1	0.88	0.046	0.89	0.071
IL8	0.88	0.208	0.88	0.203
CD274	0.87	0.088	0.80	0.006
CCL20	0.86	0.199	0.84	0.123
PTGS2	0.85	0.100	0.84	0.083
IL10	0.85	0.028	0.80	0.004
IL1B	0.84	0.083	0.81	0.034
CXCL13	0.82	0.199	0.74	0.051
MUC4	0.82	0.015	0.84	0.024
FGF2	0.78	0.134	0.79	0.132
SOCS3	0.78	0.008	0.76	0.003
DUOX2	0.76	0.017	0.73	0.005
IL19	0.74	0.018	0.69	0.003
IL21	0.74	0.041	0.64	0.004
CCL18	0.73	0.042	0.67	0.008
IL17A	0.73	0.013	0.66	0.001
IL22	0.72	0.110	0.64	0.030
MMP12	0.71	0.009	0.66	0.001
DEFB4A	0.71	0.068	0.69	0.042
S100A7	0.70	0.030	0.63	0.005
IDO1	0.64	8.99 x10^-4	0.59	1.03 x10^-4
CXCL5	0.57	4.02E-04	0.56	1.98E-04

Supplementary Table 6. Estimated fold changes (FC) with their respective p-values comparing the expression of each gene between the combined first three time-points (1, 2 and 3) before MDA and separately time-points 4 and 5 (three and six months following MDA treatment), in 122 individuals who were free from infection and disease (F0, P0) at all 5 time-points. Results are ordered by Fold Change (FC) in pre-MDA time-points vs. time-point 4. Benjamini and Hochberg approach was used to adjust for multiple comparisons, in order to control the false discovery rate <5%, only tests with a p-value <0.035 are considered statistically significant.

	Pre-MDA vs time-point 4		Pre-MDA vs time-point 5	
Target	**FC**	**p-value**	**FC**	**p-value**
SPARCL1	7.84	$8.25x10^{-23}$	1.44	0.130
CDH2	3.34	$7.15x10^{-21}$	1.61	$1.78x10^{-04}$
MUC5AC	3.07	$1.75x10^{-16}$	1.38	0.033
CTGF	1.99	$2.71x10^{-10}$	0.96	0.684
NCAM1	1.93	$4.07x10^{-15}$	1.42	$4.88x10^{-05}$
CDH1	1.88	$3.74x10^{-19}$	1.17	0.019
S100A4	1.74	$3.57x10^{-14}$	0.97	0.724
MUC7	1.40	$9.60x10^{-03}$	1.03	0.846
IL12B	1.36	0.0147	1.01	0.927
TGFB1	1.35	$4.53x10^{-07}$	1.10	0.111
MUC1	1.29	$8.18x10^{-05}$	0.99	0.872
ALOX5	1.29	$8.31x10^{-09}$	1.00	0.998
PDGFB	1.27	$8.69x10^{-04}$	0.94	0.346
GAPDH	1.25	$2.23x10^{-04}$	0.94	0.351
SOCS1	1.23	0.0193	1.07	0.544
IL23A	1.16	0.13	1.04	0.694
MUC4	1.12	0.139	0.91	0.359
NCR1	1.09	0.355	0.92	0.416
FGF2	1.08	0.683	0.97	0.890
VIM	1.07	0.26	0.90	0.102
CD247	1.07	0.387	0.91	0.254
MMP7	1.04	0.698	0.73	$4.32x10^{-04}$
IFNG	0.93	0.534	0.82	0.125
IL22	0.89	0.636	0.94	0.769
CCL2	0.87	0.253	0.76	0.058
SERPINB4	0.86	0.661	1.72	0.166
MMP9	0.83	0.146	0.90	0.453
DUOX2	0.83	0.049	0.61	$5.06x10^{-04}$
IL8	0.78	0.015	0.86	0.118
CCL20	0.78	0.073	0.89	0.442
PTGS2	0.75	0.022	0.82	0.123
IL10	0.74	$3.28x10^{-03}$	0.83	0.061
IL6	0.72	0.0384	0.77	0.079
CD274	0.70	$7.10x10^{-04}$	0.83	0.092
IL21	0.65	0.0166	0.71	0.064
SOCS3	0.64	$6.95x10^{-04}$	0.76	0.057
MZB1	0.61	$3.29x10^{-04}$	0.82	0.142
IL17A	0.60	$8.87x10^{-04}$	0.82	0.234
IL1B	0.58	$5.77x10^{-05}$	0.79	0.101
IL19	0.56	$4.06x10^{-05}$	0.78	0.120
IDO1	0.55	$1.23x10^{-08}$	0.69	$2.28x10^{-03}$
CCL18	0.53	$3.07x10^{-04}$	0.74	0.103
CXCL13	0.48	$1.64x10^{-05}$	0.70	0.049
CXCL5	0.46	$4.97x10^{-07}$	0.83	0.242
MMP12	0.44	$5.06x10^{-10}$	0.54	$7.17x10^{-05}$
DEFB4A	0.42	$4.16x10^{-08}$	0.64	0.012
S100A7	0.41	$6.23x10^{-08}$	0.68	0.029

Appendix 9: Progression of Scarring Trachoma in Tanzanian Children: a Four-year Cohort Study (Equivalent to Chapter 8)

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Progression of scarring trachoma in Tanzanian children: A four-year cohort study

Athumani M. Ramadhani[1,2]*, Tamsyn Derrick[1,2], David Macleod[1], Patrick Massae[2], Elias Mafuru[2], Aiweda Malisa[2], Kelvin Mbuya[2], Chrissy h. Roberts[1], William Makupa[2], Tara Mtuy[1,2], Robin L. Bailey[1], David C. W. Mabey[1], Martin J. Holland[1], Matthew J. Burton[1]

1 London School of Hygiene and Tropical Medicine, London, United Kingdom, **2** Kilimanjaro Christian Medical Centre, Moshi, Tanzania

* Athumani.Ramadhani@lshtm.ac.uk

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Abstract

Background

Trachoma is a progressive blinding disease initiated by infection of the conjunctiva with *Chlamydia trachomatis*. Repeated infections are thought to cause chronic inflammation, which drives scarring, leading to in-turning of the eyelids. The relationship between *C. trachomatis*, clinical inflammation and scarring development in children is not fully understood due to a paucity of longitudinal studies with infection data at frequent follow-up.

Methods and findings

This longitudinal cohort study took place in northern Tanzania. Children aged 6–10 years at baseline were eligible for inclusion. Participants were visited every three months for four years. Clinical signs and conjunctival swabs for *C. trachomatis* detection by qPCR were collected at each time-point. Conjunctival photographs from baseline and final time-points were graded and compared side-by-side to determine scarring incidence and progression.

Of the 666 children enrolled in the study, outcome data were obtained for 448. Scarring progression was detected in 103/448 (23%) children; 48 (11%) of which had incident scarring and 55 (12%) had progression of existing scarring. Scarring was strongly associated with increasing episodes of trachomatous papillary inflammation (TP). Weaker associations were found between episodes of *C. trachomatis* infection and follicular trachoma (TF) with scarring progression in unadjusted models, which were absent in multivariable analysis after adjusting for inflammation (multivariable results: *C. trachomatis* p = 0.44, TF p = 0.25, TP p = <0.0001, age p = 0.13, female sex p = 0.05). Individuals having TP at 30% or more of the time-points they were seen had an odds ratio of 7.5 (95%CI = 2.7–20.8) for scarring progression relative to individuals without any TP detected during the study period.

Conclusions

These data suggest that the effect of infection on scarring progression is mediated through papillary inflammation, and that other factors contributing to the development of

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inflammation, in addition to *C. trachomatis* infection, may be important in driving conjunctival scarring progression in children. The addition of TP as a measure in trachoma control programs would provide an indication of the future risk of developing scarring sequelae.

Author summary

Trachoma is the leading cause of preventable blindness worldwide and is targeted for elimination as a public health problem by 2020. The natural history of trachoma is not completely understood however. We conducted a four-year longitudinal study in a trachoma-endemic area of northern Tanzania with detailed follow up every three months. In the four-year study period, nearly one quarter of children developed progression of conjunctival scarring, despite three rounds of annual mass drug administration (MDA) for trachoma control. Disease progression was strongly associated with increasing proportion of episodes with conjunctival papillary inflammation (TP), and only weakly associated with *Chlamydia trachomatis* infection and *trachomatous inflammation–follicular* (TF). Analysis revealed that associations between infection and TF with scarring progression were mediated through TP, and that other factors causing individual differences in TP were also contributing to scarring progression. These data have significant implications for trachoma control. We hypothesise that in individuals who have previously experienced ocular *C. trachomatis* infection, TP is the primary driver of scarring progression. The addition of TP to trachoma surveillance programs would provide an indicator for active disease progression in the community and a more accurate guide to the need for future trichiasis interventions.

Introduction

Sight loss from trachoma, the leading infectious cause of blindness, is the end result of an inflammatory-scarring process. Starting from early childhood, people growing-up in a trachoma endemic community may be repeatedly exposed to ocular challenge with *Chlamydia trachomatis*, the causative organism. This is thought to trigger inflammatory responses that lead to conjunctival scarring in some individuals[1]. As a result of conjunctival scarring the eyelids (entropion) and eyelashes (trichiasis) turn in, scratching the ocular surface and resulting in corneal opacification[1]. These complications of scarring usually develop during adulthood.

Trachoma control rests on the **SAFE** Strategy: **S**urgery for trichiasis, **A**ntibiotic treatment to treat *C. trachomatis* infection, **F**acial cleanliness and **E**nvironmental improvements to reduce transmission. Endemic countries and the international community have set the ambitious target of 2020 for the elimination of trachoma as a public health problem[2]. There is no specific treatment to halt the progression of scarring, beyond controlling the infection.

Around 3.2 million people are estimated to have trichiasis and 1.9 million of these are blind or have severe visual impairment[3]. Currently WHO estimates that 158 million people live in districts that require A, F and E interventions[4]. Nearly 90% of these people live in Sub-Saharan Africa.

Longitudinal data sets documenting the incidence or progression of conjunctival scarring trachoma are limited[1]. Such studies are complex and can take many years to complete. In this paper we report the clinical signs and infection results of children who were followed up every three months for four years. The aim of the study was to investigate the risk factors associated

with scarring incidence and progression in Tanzanian children. We investigated the association between scarring progression and clinical signs of inflammation, *C. trachomatis* infection, age and sex, in order to strengthen the evidence base that supports trachoma control programs.

Methods

Ethical statement

This study was reviewed and approved by Ethics Committees of the Tanzania National Institute for Medical Research, Kilimanjaro Christian Medical University College and the London School of Hygiene & Tropical Medicine. It adhered to the tenets of the Declaration of Helsinki. The study was explained in detail in Kiswahili or Maasai; written informed consent from a parent or legal guardian was necessary for enrollment.

Study design and population

We recruited a cohort of children from three neighboring villages in northern Tanzania. Two villages were in Siha district located in Kilimanjaro region and one was in Longido district located in Arusha region. They were assessed every three months for four years, totaling 17 time-points. The communities and recruitment have been previously described in detail[5]. These communities are predominantly comprised of Maasai people. Children aged between 6 and 10 years at baseline (February 2012), who were normally resident in the villages, were eligible for inclusion. This restricted age group was chosen as we anticipated that younger children may not have manifest incident / progressive conjunctival scarring during the four years of follow-up. A census was conducted and eligible children enrolled.

Clinical assessment and sample collection

At each time-point all available children were examined by an experienced ophthalmic nurse. The eye was first anaesthetized with preservative-free proxymetacaine hydrochloride 0.5% eyedrops. The left upper eyelid was everted and tarsal conjunctiva examined (using x2.5 loupes and torch) for signs of trachoma and graded using the 1981 WHO 'FPC' detailed grading system[6]. This grading system corresponds to the WHO Simplified Trachoma Grading System: F2/F3 equates to *Trachomatous Inflammation-Follicular* (TF), and P3 to *Trachomatous Inflammation-Intense* (TI)[7]. "Clinically Active Trachoma" was defined as presence of TF and/or TI. We also consider that both P2 and P3 represent clinically significant papillary inflammation, and refer to this as "TP"[8]. High resolution photographs (Nikon D90 camera with 105mm Macro lens) were taken of the conjunctiva for independent grading.

Two conjunctival swab samples were collected (Dacron polyester, Puritan Medical Products Company, Maine) at each time-point. The first was placed in RNAlater (Thermo Fisher, UK) and the second was stored dry. Clinical swabs and air control swabs were collected and stored as described previously[5]. Samples were stored on ice in the field and were transferred to a -80˚C freezer upon return to the laboratory later the same day.

Trachoma control

Following approval from the Ministry of Health (MoH) and in collaboration with the district eye coordinators the SAFE strategy was implemented in study villages by the study field team. Education was provided regarding facial cleanliness and environmental improvements and free trichiasis surgery was offered. Azithromycin mass drug administration (MDA) was administered by the study team, according to WHO guidelines, in August 2012, August 2013 and August 2014. In mid-2015, one of the three villages from Longido district, which had a

persistently elevated TF prevalence, received a further round of MDA. This was delivered by the local MoH team as part of the district-wide distribution. The other two villages, which are in the neighboring district (Siha), were not re-treated as the district-wide prevalence was below the treatment indication threshold, and these two villages had shown a good response to the three rounds of MDA.

Chlamydia trachomatis detection

At the first time-point, DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) from swab samples stored in dry tubes and *C. trachomatis* was detected by droplet digital PCR, as previously described[5, 9]. At all subsequent time-points, DNA was extracted from samples stored in RNAlater using the Norgen RNA/DNA purification kit (Norgen Biotek) and *C. trachomatis* was detected by triplex quantitative PCR (qPCR) for chlamydial chromosomal (*omcB*) and plasmid (*pORF2*) genes and a human endogenous control gene (*RPP30*), as described previously[10]. Time-point 2 Norgen-extracted samples were tested by both detection methods and the kappa score for agreement was 0.84. Samples were tested in duplicate and were defined as *C. trachomatis* positive if *RPP30* and *pORF2* and/or *omcB* amplified in <40 cycles in one or both replicates.

Analysis

We used photographic grading to determine whether there was either development of incident scarring in previously un-scarred conjunctiva, or increase in pre-existing scarring. Conjunctival photographs from baseline (time-point 1) were compared to the final time-point (time-point 17). For individuals not seen at time-point 1, the image from time-point 2 was used for their baseline. Similarly, if an individual was not seen at time-point 17, the image from time-point 16 was used as their final time-point. The images were assessed by an ophthalmologist experienced in using a detailed scarring grading system[11]. Baseline and final photographs were compared side-by-side to produce the main binary outcome variable of overall "scarring progression", defined as evidence of either incident scarring or worsening of pre-existing scarring. For further sub-analyses, we subdivided individuals with "no scarring progression" into (1) no scarring at either baseline and final; (2) scarring unchanged between baseline and final. We subdivided individuals with "scarring progression" into (3) incident scarring (no scarring at baseline and new scarring at final); (4) increasing scarring (some scarring at baseline and more at final).

All field data were managed in Access (Microsoft). Data were merged and analyzed in STATA v14. The total number of time-points at which participants were seen varied due to absence or refusal. We excluded from the analysis individuals who were seen on fewer than four occasions or did not have outcome data (time-point 16 or 17 assessments). A proportion variable was generated for each of TF, TP and *C. trachomatis* infection: number of time-points with each factor as a proportion of the total number of time-points that individual was seen. Proportions were subsequently categorized.

Separate mixed effects logistic regression models were used to determine the association between (1) TP, (2) TF, and (3) *C. trachomatis* infection with sex and baseline age, using data from all time-points in the longitudinal dataset. Mixed effects regression was also performed to assess the relationship between (1) TF and *C. trachomatis* infection, and (2) TP and *C. trachomatis*, again using data from all time-points in the longitudinal dataset (adjusting for age at baseline and sex). These analyses were limited to the 448 individuals with outcome data.

To identify risk factors for scarring progression, analysis was initially performed using logistic regression to assess the association between categorized proportions of TF, TP or *C. trachomatis* infection and overall scarring progression (either incident scarring in those

without scarring at baseline or progression of pre-existing scarring). Each of these were initially included as exposures separately in a logistic regression using scarring progression as the outcome variable and adjusting for age at baseline and sex. Following this, all three were included in a final multivariable model (adjusting for baseline age and sex), and likelihood ratio tests were performed between models including versus excluding each exposure to determine its overall *P* value. The analyses were subsequently repeated to identify risk factors for incident scarring and progression of pre-existing scarring separately. In the first set of univariable and multivariable analyses (using the same exposures as above) the analysis was restricted to individuals without scarring at baseline (incident scarring versus no scarring). In the second set, analysis was restricted to individuals with scarring at baseline (progression of existing scarring versus no progression of existing scarring).

Chlamydial load was calculated by extrapolating from a standard curve. *OmcB* in copies/μl was log10 transformed to normalize the distribution. In the longitudinal dataset random effects linear regressions were performed to look for associations between a) chlamydial load and scarring progression (adjusting for MDA-period, age at baseline and sex), and b) chlamydial load and age at time-point (in years) in *C. trachomatis* positive individuals (adjusting for sex and MDA-period). Age at time-point was split into four groups; <7.5 years, ≥ 7.5 - <10 years, ≥ 10 - <12.5 years, and ≥ 12.5 years. A random effects linear regression was then performed to assess for association between chlamydial load, age group and progression, including an interaction term between age group and progression in order to determine whether the association between chlamydial load and progression was modified by age. MDA-period (pre-MDA, post first MDA, post second MDA, post third MDA) was included in the model to adjust for confounding.

Results

Study participants

The participant flow is shown in Fig 1. There were 666 potentially eligible children and 616 enrolled. Fifty either refused or were absent. We excluded 57 who were examined on less than four occasions, and 111 without scarring progression outcome data (no time-point 16 or 17 assessment). This left 448 in this analysis, who were seen at a median of 15 time-points (1^{st}–3^{rd} quartiles = 13–16, S1 Fig).

The demographic characteristics of the entire cohort were described in the baseline report [5]. Of the 448 children included in this analysis, 242 (54.0%) were female, mean age at baseline was 6.8 years, and 438 (97.8%) were Maasai. Of the 218 children not included, 92 (42.2%) were female (OR = 1.61, 95%CI = 1.16–2.23, p = 0.004), mean age at baseline was 7.4 years (OR = 0.86, 95% CI = 0.79–0.93, $p<0.0002$) and 214 (98.2%) were Maasai. Younger children and females were therefore more likely to be included in this study analysis.

Antibiotic coverage of the 448 children included in scarring progression analysis was 355 (79.2%) in 2012, 374 (83.5%) in 2013 and 338 (75.4%) in 2014. The estimated community-wide MDA coverage in 2012, 2013 and 2014 were 68.7%, 42.9% and 72.9%, respectively.

Clinical disease

Of the 448 participants, 240 (53.6%) had TF (F2/F3), 185 (41.3%) had TP (P2/P3)), and 248 (55.4%) had clinically active trachoma (F2/3 and/or P3) at one or more time-point. The prevalence of TF and TP is shown for each time-point in Fig 2.

There was a significant reduction in inflammatory disease following MDA, although TP prevalence was particularly high at time-point 6. Examination of inflammation and infection prevalence by village revealed that this peak in TP was found in only two of the three villages

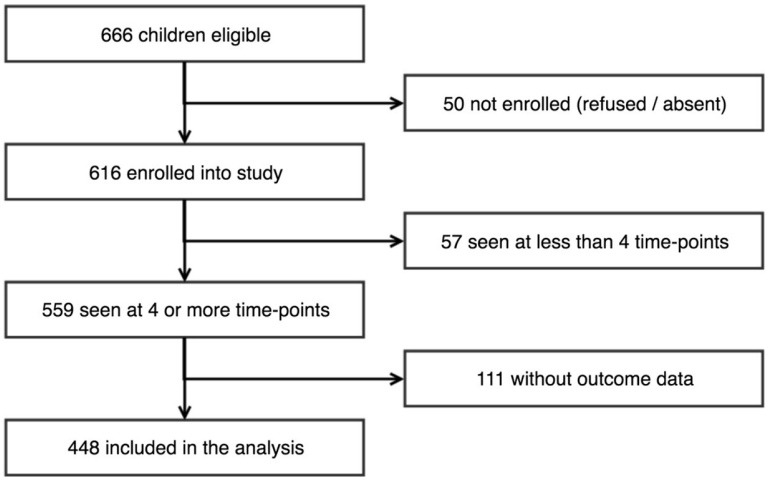

Fig 1. Flow chart for eligible study participants. The chart shows the number of individuals enrolled, excluded and included in the analysis of scarring progression.

https://doi.org/10.1371/journal.pntd.0007638.g001

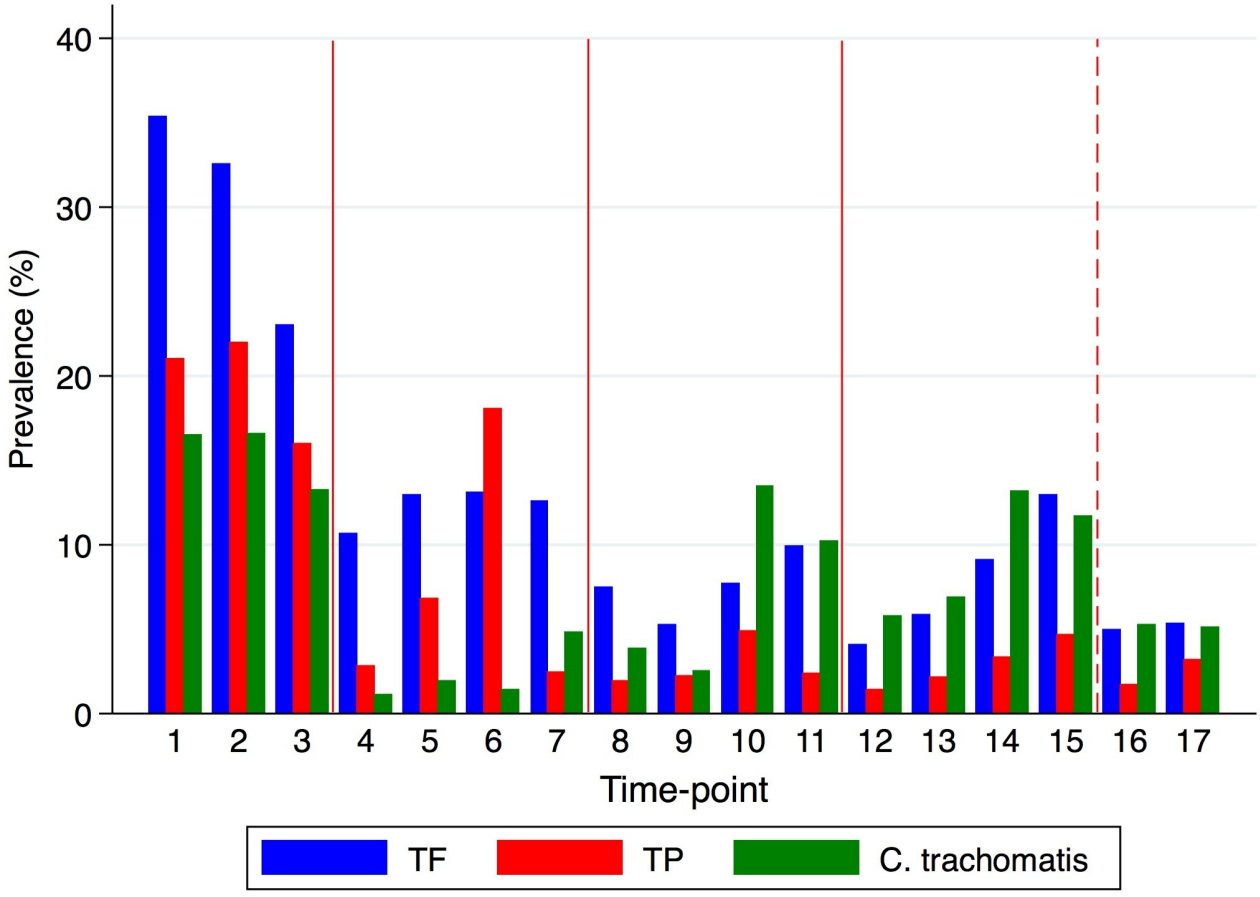

Fig 2. The prevalence of *C. trachomatis* infection and clinical signs at each time-point. Data are shown for the 448 individuals with outcome data. There were three-month intervals between time-points. Red vertical lines indicate annual MDA treatment given to all three study villages. The red dashed vertical line indicates treatment given to one village with residual disease.

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Table 1. Number of individuals with *C. trachomatis* infection, TF and TP, categorized by proportion of time-points. *C. trachomatis* infection, TF and TP were detected as a percentage of the total number of time-points that individual was seen.

Proportion of time-points	*C. trachomatis*		TF		TP	
	n	(%)	n	(%)	n	(%)
None	229	(51.1%)	208	(46.4%)	263	(58.7%)
<10%	78	(17.5%)	52	(11.6%)	71	(15.8%)
10–19%	79	(17.6%)	71	(15.9%)	48	(10.7%)
20–29%	43	(9.6%)	56	(12.5%)	41	(9.2%)
30% +	19	(4.2%)	61	(13.6%)	25	(5.6%)

https://doi.org/10.1371/journal.pntd.0007638.t001

("A" and "C"), and did not appear to correlate with infection (S2 Fig), indicating that it may have been driven by non-chlamydial infection.

The number of individuals with categorized proportions of time-points (none, <10%, 10–19%, 20–29%, 30%+) that they were found to have (1) TF, (2) TP and (3) *C. trachomatis* infection is shown in Table 1. At baseline, 93 (20.8%) had some degree of conjunctival scarring.

The odds of TF were estimated to be higher in females (OR = 1.49, 95%CI = 1.05–2.11, P = 0.025) and lower with each additional year of age (OR = 0.65, 95%CI = 0.59–0.71, p<0.0001) in the longitudinal dataset. The odds of TP were also estimated to reduce with age (OR = 0.79, 95%CI = 0.71–0.88, p<0.0001) but the evidence of an association with sex was much weaker (OR = 1.39, 95%CI = 0.92–2.09, p = 0.119). There was little difference between children included and those excluded in the analysis in terms of sex and baseline TF/TP/infection although those excluded tended to be slightly older on average than those included (mean baseline age 7.4 vs 6.8, p<0.001).

C. trachomatis infection

C. trachomatis was detected in 219/448 (48.9%) at one or more time-points. The prevalence of infection is shown for each time-point in Fig 2. The proportion of time-points that each individual had infection is shown in Table 1. The median proportion of time-points infected among the 219 individuals who had *C. trachomatis* detected on at least one occasion was 12.5%, which was equivalent to ~2 time-points if someone had been seen on all 17 visits.

Infection prevalence declined following each round of MDA, however at time point 10 (9 months after second MDA) it had increased and at time-point 14 (9 months after third MDA) infection prevalence had returned to pre-MDA levels (10–15%). Infection prevalence dropped again by time-points 16 and 17. Further examination of infection and clinical sign prevalence in each of the three villages showed that the majority of infection and TF at later time-points were found in only village "C" (S2 Fig). Village "C" is located in a different administrative district, which (unlike the other two villages) was eligible for and received MoH administered MDA treatment in July-2015. This was subsequently followed by a further drop in infection, TF and TP prevalence in village "C" at time-points 16 and 17 (S2 Fig).

In a mixed effects logistic regression of infection at any time-point, female sex (OR = 1.7, 95%CI = 1.25–2.34, p = 0.001) and younger baseline age (OR = 0.82, 95%CI = 0.75–0.99, p<0.0001) were significantly associated with *C. trachomatis* infection.

Clinical disease and infection

Overall, there was a strong association between *C. trachomatis* infection and TF (OR = 11.6, 95%CI = 8.9–15.0, P<0.0001) and TP (OR = 9.6, 95%CI = 7.1–12.8, P<0.0001) in the longitudinal dataset (adjusted for baseline age and sex). The odds ratios for TP and TF as predictors

of *C. trachomatis* infection (adjusted for TP/TF, age at baseline and sex) at each time-point were generally similar to or slightly higher after the initiation of MDA, however confidence intervals were much wider (S3 Fig).

Scarring progression

Overall, scarring progression was observed in 103/448 (23.0%) participants (Table 2). There were 307 (68.5%) who had no scarring; 38 (8.5%) with unchanged scarring; 48 (10.7%) with incident scarring; and 55 (12.3%) with increasing scarring.

The relationships between scarring progression and proportion of time-points when *C. trachomatis* was detected or signs of inflammation (TF or TP) were seen, adjusting only for age at baseline and sex, are shown in Table 3. In these models strong evidence was found of an association between progression and both TP and TF, but the association between infection and progression was weaker. There was also evidence of a greater risk of progression in females compared to males.

In a multivariable model (Table 4) for scarring progression (retaining infection, TF, TP, age at baseline and sex), the strong relationship between increasing proportion of time-points with TP and scarring progression remained. Female sex was marginally associated. There was no association with either TF or infection, suggesting that the associations between TF and infection with scarring were mediated through TP.

The analysis was repeated, restricted to individuals with (a) no scarring at baseline, and (b) some scarring at baseline, in order to differentiate between factors associated with incident scarring and progression of pre-existing scarring, respectively. In the unadjusted models there was evidence for associations between episodes of *C. trachomatis* infection, TF and TP and incident scarring (S1 Table). In the multivariable model however, only TP was significantly associated with incident scarring, again suggesting that the effect of infection and TF was mediated through TP (S2 Table). Neither infection, TF nor TP were significantly associated with progression of pre-existing scarring in either the unadjusted or adjusted models. There seemed to be a trend for increasing risk of progressive scarring with increasing episodes of TP, however the evidence for this effect was weak, it should be noted that the sample size for these sub-analyses was small. These data from children with pre-existing scarring did not demonstrate that additional episodes of *C. trachomatis* infection were associated with further progression of scarring. Female sex was associated with an increase in pre-existing scarring but not with incident scarring. There were no associations with age.

C. trachomatis infection load

Bacterial load in *C. trachomatis* positive individuals was equivalent between people with and without scarring progression, using data from all time-points (adjusting for age at baseline, sex

Table 2. Scarring progression category by presence of *C. trachomatis* infection and clinical features. Both infection and clinical features were detected at one or more time-points.

		Overall scarring		Scarring subgroups			
Clinical phenotype	**Total**	**Progression**	**No progression**	**No scarring**	**Unchanged scarring**	**Incident scarring**	**Increasing scarring**
Total	448	103 (23%)	345 (77%)	291 (65%)	54 (12%)	48 (10.7%)	55 (12.3%)
No infection/TP/TF	122	17 (13.9%)	105 (86.1%)	95 (77.9%)	10 (8.2%)	11 (9%)	6 (4.9%)
Any TF	240	67 (27.9%)	173 (72.1%)	136 (56.7%)	37 (15.4%)	31 (12.9%)	36 (15%)
Any TP	185	67 (36.2%)	118 (63.8%)	89 (48.1%)	29 (15.7%)	28 (15.1%)	39 (21.1%)
Any *C. trachomatis*	219	55 (25.1%)	164 (74.9%)	137 (62.6%)	27 (12.3%)	29 (13.2%)	26 (11.9%)

https://doi.org/10.1371/journal.pntd.0007638.t002

Table 3. Univariable logistic regression models of the associations between overall scarring progression and *C. trachomatis* infection and clinical features. *C. trachomatis* infection, TF and TP were categorized by proportions of time-points present, and were adjusted only for age at baseline and sex. The overall *P* value for each model is shown in line with the variable name.

	n/N	(%)	OR	95% CI	P value
C. trachomatis					0.041
None	48/229	(20.9%)			
<10%	12/78	(15.4%)	0.64	0.3–1.3	
10–19%	21/79	(26.6%)	1.29	0.7–2.4	
20–29%	15/43	(34.9%)	1.91	0.9–3.9	
30% +	7/19	(36.8%)	2.20	0.8–6.0	
TF					0.0004
None	36/208	(17.3%)			
<10%	8/52	(15.4%)	0.93	0.4–2.2	
10–19%	18/71	(25.4%)	1.96	1.0–3.9	
20–29%	15/56	(26.8%)	1.92	0.9–4.0	
30% +	26/61	(42.6%)	4.41	2.2–8.8	
TP					<0.0001
None	36/263	(13.7%)			
<10%	17/71	(23.9%)	2.14	1.1–4.2	
10–19%	15/48	(31.3%)	3.08	1.5–6.4	
20–29%	21/41	(51.2%)	7.26	3.5–15.0	
30% +	14/25	(56.0%)	8.41	3.5–20.2	
Age at baseline			1.01	0.9–1.1	0.853
Sex					
Male	38/206	(18.5%)	1.62	1.0–2.6	0.036
Female	65/242	(26.9%)			

https://doi.org/10.1371/journal.pntd.0007638.t003

and pre/post-MDA period) (OR = 1.1, 95% CI = 0.86–1.42, p = 0.45). There was evidence of an association between age at time-point (in years) and infection load among *C. trachomatis* positive individuals, with lower loads in older individuals (OR = 0.91, 95%CI = 0.86–0.97, p = 0.004), adjusting for sex and MDA period. Bacterial load in *C. trachomatis* positive progressors and non-progressors was plotted across different age groups (derived from age in years at that time-point) to determine whether the association between scarring progression and load varied by age. In the oldest age group, progressors had a slightly higher infection load relative to non-progressors, Fig 3, which was supported by evidence for an interaction between age group and progression in their association with bacterial load (p = 0.012). The model including the interaction explained the data better than the model without the interaction (p = 0.016).

Discussion

The development of trachomatous scarring is probably the result of a complex interaction between *C. trachomatis* infection, variation in the host immune response and possibly other pro-inflammatory stimuli. However, long-term data exploring the relative contributions of these factors to the development of scarring in children are limited.

In this cohort of Tanzanian children aged 6–10 years old at baseline we found that 23% had trachomatous scarring progression over the course of four years. Roughly half of this was new scarring whilst the other half involved progression of pre-existing scarring. Scarring progression was strongly associated with increasing frequency of conjunctival papillary inflammation (TP). This suggests that controlling TP could potentially limit progressive scarring trachoma.

Table 4. Multivariable logistic regression model for the association between overall scarring progression and *C. trachomatis* infection and clinical features. Categorized proportions of time-points with *C. trachomatis* infection, TF and TP were included, adjusting for age at baseline and sex. The overall P value for each variable is shown in line with the variable name, derived from a likelihood ratio test of the model including versus excluding that variable.

	OR	95% CI	P value
C. trachomatis			0.4396
None			
<10%	0.49	0.2–1.1	
10–19%	0.90	0.5–1.8	
20–29%	0.80	0.3–1.9	
30% +	0.72	0.2–2.5	
TF			0.2535
None			
<10%	0.85	0.3–2.1	
10–19%	1.07	0.5–2.3	
20–29%	0.84	0.3–2.0	
30% +	2.09	0.9–5.0	
TP			<0.0001
None			
<10%	2.19	1.1–4.4	
10–19%	2.94	1.3–6.7	
20–29%	6.67	3.0–14.9	
30% +	7.48	2.7–20.8	
Age at baseline	1.11	1.0–1.3	0.128
Sex (Female)	1.65	1.0–2.7	0.051

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We found only weak evidence of an association between *C. trachomatis* infection and scarring progression. Increasing proportions of infection episodes were associated with incident scarring, however, multivariable analysis showed that this effect was mediated through TP. These data suggest that other factors, in addition to *C. trachomatis* infection, are important determinants of the development of TP and progression of scarring. The data also indicate that the clinical sign of TF has no association with scarring progression after adjusting for TP, suggesting that TF is not a direct cause of, nor the best prognostic marker for scarring progression. For this purpose, TP prevalence might be a more informative clinical marker, and control programs could consider using TF in combination with TP prevalence to predict future risk of scarring.

Our finding that increasing frequency of papillary inflammation is strongly associated with scarring progression has consistently been reported by other studies[1], however the relationship between chlamydial infection and scarring progression is less definitive.

The first longitudinal study to try to investigate scarring progression was conducted by Dawson et al in Tunisia, starting in the late 1960's. They examined a group of children and younger adults (n = 213) on two occasions about 14 years apart; no tests for *C. trachomatis* infection were performed. TI (P3) was the strongest risk factor for developing severe scarring (RR = 18), whilst TF had a weaker association (RR = 2.8)[12]. Interestingly, there also appeared to be increased scarring risk associated with moderate papillary inflammation (P2).

West et al investigated the relationship between "constant severe inflammatory trachoma" (TI diagnosed at more than half of the examinations) in a group of children (n = 190) examined on four occasions during the baseline year and once again at 7 years[13]. TI was

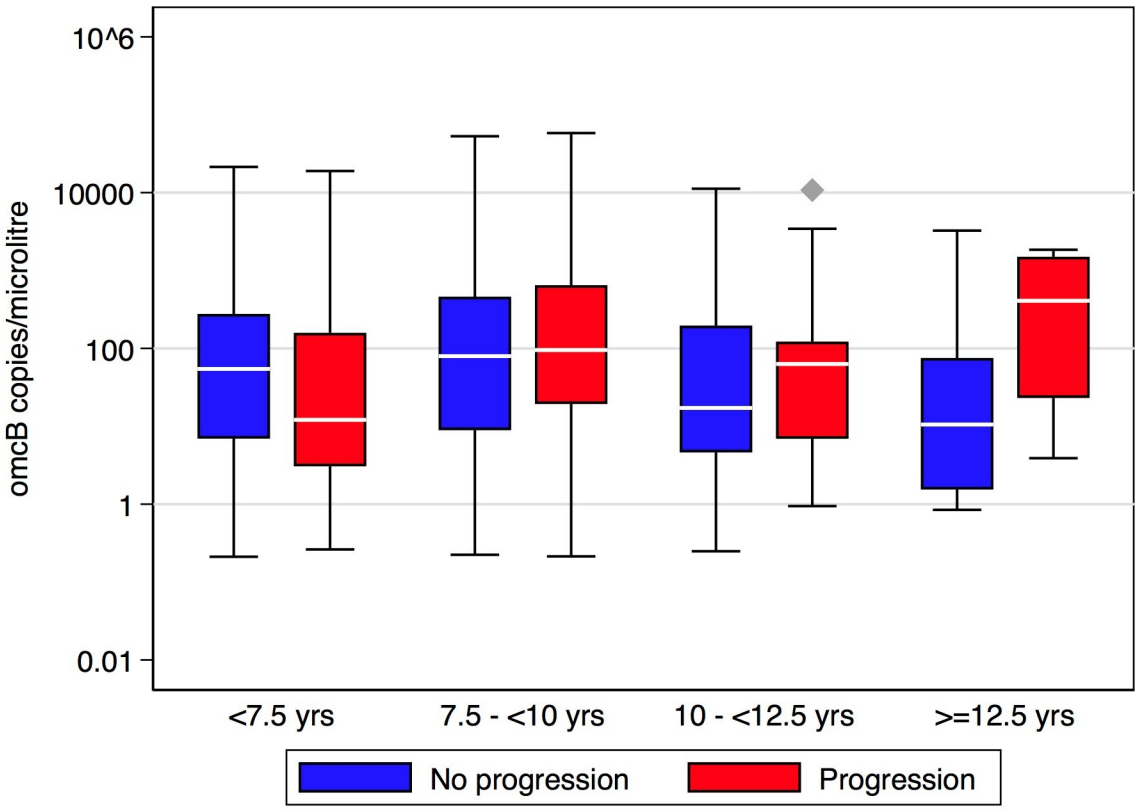

Fig 3. Distribution of *omcB* load in scarring progressors and non-progressors, split by age group at time-point.

https://doi.org/10.1371/journal.pntd.0007638.g003

associated with increased risk of developing scarring by 7 years. TF alone was not associated with development of scarring.

Only one study, by Wolle et al, has previously examined the relationship between *C. trachomatis* infection and the subsequent development of scarring in children[14]. They reported a five-year cohort of Tanzanian children (n = 189) that were examined on five occasions during the first 18 months and once again at 5 years. They found that incident scarring over the five years was associated with constant inflammation and/or constant *C. trachomatis* infection during the first 18 months (OR 5.74, 95%CI 2.39–13.8). However, the effects of infection and inflammation were not modelled independently of each other, possibly due to sample size limitations, therefore, the independent contribution from infection remained unclear.

Burton et al found a strong association between progression of pre-existing scarring in adults and increasing episodes of papillary inflammation (P2/P3) in Ethiopia (n = 585; OR 5.93, 95%CI 3.31–10.6, $p<0.0001$) and Tanzania (n = 577; OR 5.76, 95%CI 2.60–12.7, $p<0.0001$)[8]. The study examined and sampled participants on a 6-monthly basis for two years. Episodes of *C. trachomatis* infection were very infrequent, and they were not associated with disease progression.

Our finding that *C. trachomatis* infection was marginally associated with incident scarring (before adjustment) but not with progressive scarring is consistent with chlamydial infection being important for initiating the scarring disease process. However, once scarring is established, other factors driving TP are perhaps increasingly important for scarring progression. However, an alternative but perhaps less likely explanation for this finding might be that people who are more innately predisposed to developing scarring clear infection episodes rapidly

(which are therefore not readily detected), but also experience more severe and prolonged inflammation (TP) in the aftermath[15, 16]. Our analysis of progression of pre-existing scarring was also limited by a smaller sample size.

Conjunctival inflammation may be associated with other bacterial or viral infections, or with allergic conjunctivitis[17, 18]. Several cross-sectional studies have found associations between non-chlamydial ocular bacterial infections and active trachoma (TF/TP), conjunctival scarring, trichiasis/recurrent trichiasis and corneal scarring[11, 19–24]. A recent longitudinal study in 452 Tanzanian adults found that ocular commensal and pathogenic non-chlamydial bacterial infections were more common in scarring progressors relative to non-progressors and that, after adjusting for other factors, ocular infections were marginally associated with scarring progression at two years[25].

C. trachomatis infection might damage the barrier function or homeostasis of the conjunctival epithelium, such that external stimuli cause inflammation where they would not have done otherwise. The expression of mucins has consistently been found to be dysregulated in active and scarring trachoma, supporting this hypothesis[5, 26]. Long-term exposure to cooking smoke has also been linked to conjunctival inflammation[27], particularly affecting women, however a Tanzanian longitudinal study did not find any association between exposure to cooking fires and incident scarring[28]. Use of traditional medicines might also have a role, as could differences in diet or coinfections that lead to variation in host immune responses.

Genetic or epigenetic factors might also contribute to differences in host inflammatory responses. Several studies have reported associations between genetic differences and risk of trachoma, including one genome-wide association study[29–35]. Further detailed investigations of ocular microbial infections and host genetics are required to establish their roles in this disease.

There was a marginal association between female sex and overall scarring progression. Further analysis revealed that female sex was associated with progression of pre-existing scarring but not with incident scarring (S1 Table). Female sex was also associated with *C. trachomatis* infection, TF and only very marginally with TP. These findings are difficult to reconcile, as there were associations between infection and incident scarring, and between infection and female sex. Whereas, there was no association between infection and progressive scarring and only a marginal association between TP and female sex. Our findings also contrast those of previous longitudinal studies in which female sex was associated with scarring incidence but not progression[1]. The numbers in our analysis of pre-existing scarring were relatively small, therefore sample size limitations might explain these findings. Nevertheless, overall females were at greater risk of overall scarring progression, after adjustment for *C. trachomatis* infection and TP, suggesting that this effect was mediated by another mechanism.

We found no association between age and scarring progression, which may be due to the limited four year age range of our study participants. Age was strongly associated with *C. trachomatis* infection, TF and TP, all of which were more common in younger participants, perhaps suggesting that some level of acquired protective immunity develops. There was some evidence that in the oldest age group, scarring progressors had a higher chlamydial load relative to non-progressors, perhaps suggesting that their immunological control of the infection was less effective, although this evidence was very weak.

A strong association was seen between infection and TF or TP. In contrast to previous reports the association did not diminish after MDA[36], however *C. trachomatis* infection was still relatively common and the effect estimates had very wide confidence intervals. Infection prevalence recovered after MDA to near pre-MDA levels, whereas TP and TF prevalence remained low post-MDA. Most of the residual disease and infection was concentrated in

village "C", which lies within a district with historically higher levels of endemic trachoma. A study from central Tanzania has shown a significant reduction of both *C. trachomatis* infection and TF after each of three annual rounds of MDA in children aged 1 year and above, where MDA coverage was >80%[37]. The overall TF prevalence was reduced from 27.3% at baseline to 9.4% one year after the third MDA round, and infection prevalence was reduced from >20% in 1-9-year olds to <10% in the fourth year. The resurgence of infection prevalence during our study may have been due to a combination of insufficient MDA coverage and interaction with untreated neighboring communities. Community members are mainly pastoralists and often away from their homes during the day. These results highlight the need for high MDA coverage to effectively bring *C. trachomatis* prevalence under control. However, the sustained reduction in TP prevalence following MDA may be promising in terms of reducing scarring risk.

This study has several limitations. Only the left eye was examined and sampled throughout the longitudinal study as it was not feasible to process samples from both eyes at all time-points. There were changes in the detection method for *C. trachomatis* between baseline and all other time-points, which could have introduced some variability, however the agreement between methods was good and due to the large sample size and high number of time-points the interpretation of the results is not expected to be affected. As with any longitudinal study there was some loss to follow-up. In this study we found that the children for whom we were unable to collect outcome data were slightly older and more likely to be male. Although this moderate difference between those seen and not seen could potentially introduce some bias, the cohort size remained substantial to the end, and despite the loss to follow-up we were able draw clear conclusions. It is possible that MDA treatment could have had a beneficial effect against scarring progression through the known anti-inflammatory properties of azithromycin, however further investigation of this was outside the scope of the current study. The three-monthly spacing of observations are too far apart to be able to estimate the duration of disease and infection episodes. The study was designed to investigate individual level risk of progression in scarring and was of sufficient size to demonstrate this. However, it was not designed to provide community level estimates of the risk of progression, which would require a much larger number of communities and many more to children.

Conclusion

Progressive scarring trachoma was strongly associated with papillary inflammation in this longitudinal study. *C. trachomatis* infection was no longer associated with scarring progression after adjustment for TP, suggesting that the effect of infection is mediated through TP, and that other factors contributing to TP in addition to *C. trachomatis* infection are important determinants of disease progression. Further research is required to understand what these factors are; they might include other ocular or non-ocular infections, genetic variation in host immune responses or environmental factors. Females were at greater risk of *C. trachomatis* infection, clinical inflammation and scarring progression. The addition of TP as an indicator for trachoma control programs might provide a more accurate marker for the risk of disease progression and of the need for future trichiasis interventions, which are likely to be needed for many years to come in this community.

Supporting information

S1 Checklist. STROBE checklist.
(DOC)

S1 Fig. Histogram showing the number of time-points at which participants were seen. Data are shown for the 448 participants with outcome data.
(TIF)

S2 Fig. The prevalence of *C. trachomatis* infection and clinical signs at each time-point. Data are shown for the 448 individuals with outcome data, split by village. Red vertical lines indicate MDA treatment. The red dashed vertical line in village C indicates treatment given with residual disease
(TIF)

S3 Fig. The association between *C. trachomatis* infection and clinical signs at each time-point. TF is shown in blue and TP in red. Odds ratios with 95% confidence intervals are plotted. The grey vertical lines indicate MDA treatment given to all three study villages. The grey dashed vertical line indicates treatment given to one village with residual disease. The OR for TP at time-point 5 is missing as there was insufficient data to generate a result.
(TIF)

S1 Table. Univariable logistic regression models for scarring progression. These include; a) incident scarring and b) increase in pre-existing scarring, in individuals with (a) no scarring at baseline or (b) some scarring at baseline. Univariate associations between scarring and infection, TF and TP were adjusted for age at baseline and sex.
(DOCX)

S2 Table. Multivariable logistic regression models for scarring progression. These include; a) incident scarring and b) increase in pre-existing scarring, in individuals with (a) no scarring at baseline or (b) some scarring at baseline.
(DOCX)

Author Contributions

Conceptualization: Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

Data curation: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Patrick Massae, Elias Mafuru, Aiweda Malisa, Kelvin Mbuya, Chrissy h. Roberts, Tara Mtuy, Matthew J. Burton.

Formal analysis: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Martin J. Holland, Matthew J. Burton.

Funding acquisition: Martin J. Holland, Matthew J. Burton.

Investigation: Athumani M. Ramadhani, Tamsyn Derrick, Patrick Massae, Elias Mafuru, Aiweda Malisa, Kelvin Mbuya, Chrissy h. Roberts, William Makupa, Tara Mtuy, Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

Methodology: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Patrick Massae, Elias Mafuru, Aiweda Malisa, Kelvin Mbuya, Chrissy h. Roberts, Tara Mtuy, Martin J. Holland, Matthew J. Burton.

Project administration: Athumani M. Ramadhani, Patrick Massae, Aiweda Malisa, William Makupa, Tara Mtuy, Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

Resources: William Makupa, Tara Mtuy, Robin L. Bailey, David C. W. Mabey, Matthew J. Burton.

Supervision: Athumani M. Ramadhani, Tamsyn Derrick, William Makupa, Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

Validation: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Chrissy h. Roberts, Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

Visualization: Athumani M. Ramadhani, Tamsyn Derrick, Martin J. Holland, Matthew J. Burton.

Writing – original draft: Athumani M. Ramadhani.

Writing – review & editing: Athumani M. Ramadhani, Tamsyn Derrick, David Macleod, Patrick Massae, Elias Mafuru, Aiweda Malisa, Kelvin Mbuya, Chrissy h. Roberts, William Makupa, Tara Mtuy, Robin L. Bailey, David C. W. Mabey, Martin J. Holland, Matthew J. Burton.

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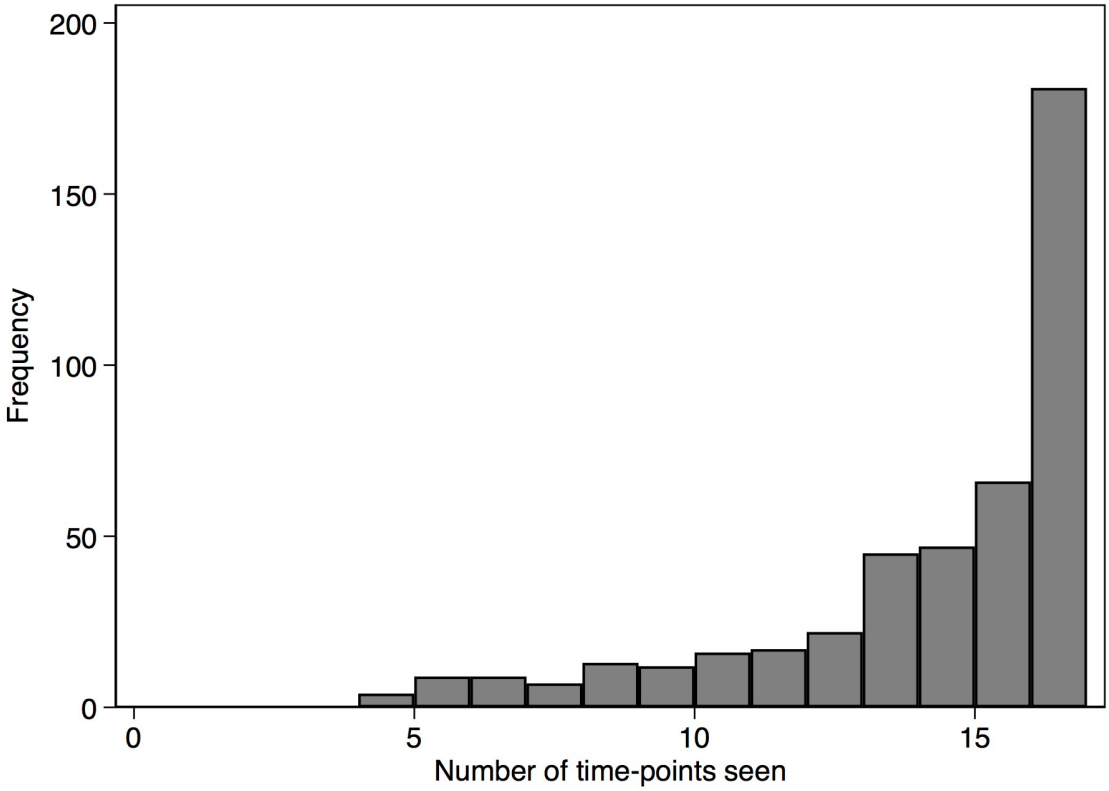
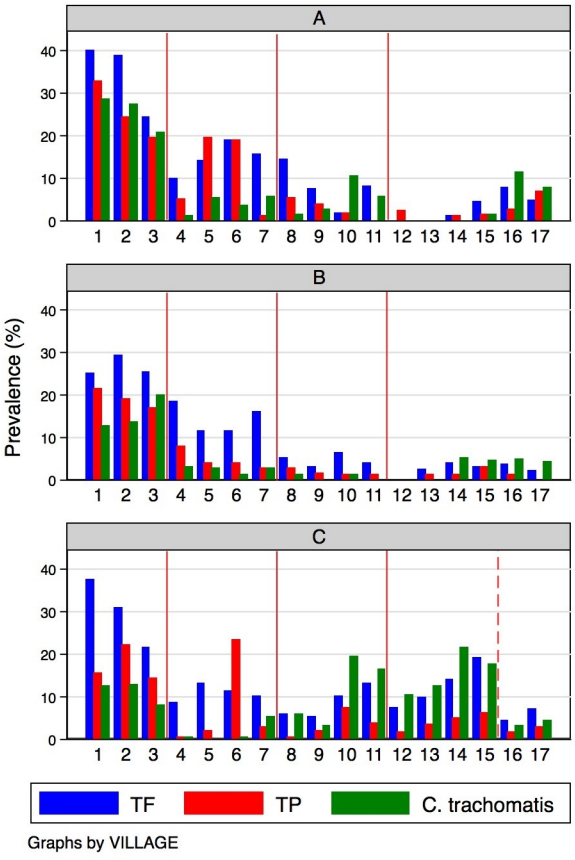
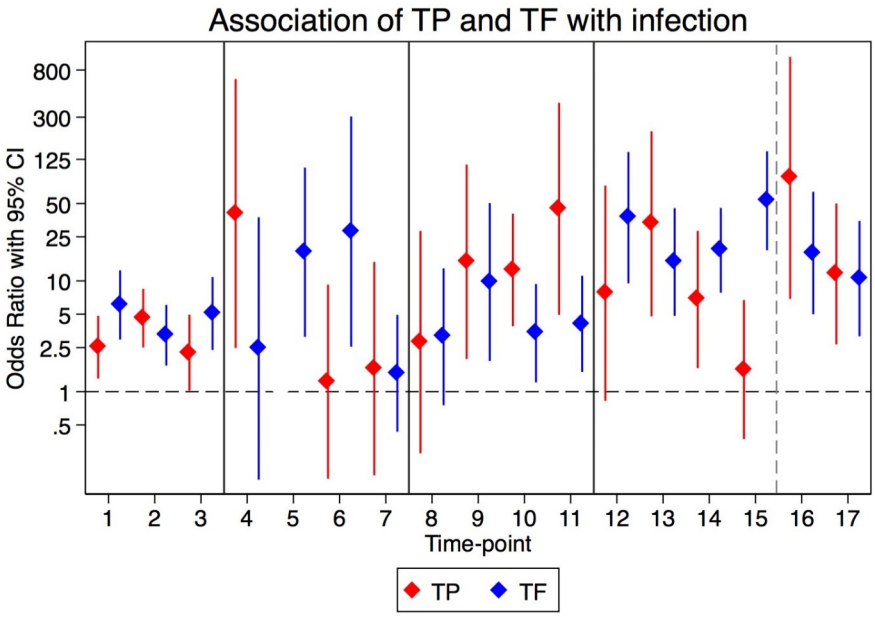

200
150
100
50
0
Frequency
0
5
10
15
Number of time-points seen


A
B
C
Prevalence (%)
0
10
20
30
40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
TF
TP
C. trachomatis
Graphs by VILLAGE


Association of TP and TF with infection
Odds Ratio with 95% CI
800
300
125
50
25
10
5
2.5
1
.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time-point
TP
TF

S1 Table. Univariate logistic regression models for scarring progression. That includes; a) incident scarring and b) increase in pre-existing scarring, in individuals with (a) no scarring at baseline or (b) some scarring at baseline. Univariate associations between scarring and infection, TF and TP were adjusted for age at baseline and sex

A) INCIDENT SCARRING	**n/N**	**%**	**OR**	**95% CI**	**P value**
	48/339	14.16			
C. trachomatis					0.0307
None	19/173	(11.0%)			
<10%	5/67	(7.5%)	0.68	0.24 - 1.91	
10-19%	10/53	(18.9%)	1.98	0.84 - 4.64	
20-29%	9/32	(28.1%)	3.40	1.33 - 8.68	
30% +	5/14	(35.7%)	4.75	1.42 - 15.90	
TF					0.0013
None	17/172	(9.9%)			
<10%	2/37	(5.4%)	0.62	0.13 - 2.86	
10-19%	6/47	(12.8%)	1.63	0.58 - 4.58	
20-29%	8/43	(18.6%)	2.54	0.96 - 6.71	
30% +	15/40	(37.5%)	7.35	2.91 - 18.54	
TP					<0.0000
None	20/222	(9.0%)			
<10%	4/49	(8.2%)	0.99	0.32 - 3.09	
10-19%	5/29	(17.2%)	2.40	0.80 - 7.19	
20-29%	15/28	(53.6%)	13.42	5.34 - 33.73	
30% +	4/11	(36.4%)	6.03	1.61 - 22.63	
Age at baseline			0.99	0.84 - 1.15	0.859
Sex					
Male	24/168	(14.3%)			
Female	24/171	(14.0%)	0.98	0.53 - 1.80	0.947

B) PROGRESSIVE SCARRING	n/N	%	OR	95% CI	P value
	55/109	(50.5%)			
C. trachomatis					0.3973
None	29/56	(51.8%)			
<10%	7/11	(63.6%)	0.93	0.21 - 4.18	
10-19%	11/26	(42.3%)	0.51	0.18 - 1.46	
20-29%	6/11	(54.6%)	0.80	0.20 - 3.18	
30% +	2/5	(40.0%)	0.62	0.09 - 4.36	
TF					0.5382
None	19/36	(52.8%)			
<10%	6/15	(40.0%)	0.68	0.19 - 2.40	
10-19%	12/24	(50.0%)	1.01	0.33 - 3.09	
20-29%	7/13	(53.9%)	1.19	0.31 - 4.60	
30% +	11/21	(52.4%)	0.87	0.26 - 2.91	
TP					0.1783
None	16/41	(39.0%)			
<10%	13/22	(59.1%)	2.17	0.73 - 6.46	
10-19%	10/19	(52.6%)	1.79	0.57 - 5.63	
20-29%	6/13	(46.2%)	1.44	0.40 - 5.23	
30% +	10/14	(71.4%)	3.63	0.94 - 14.04	
Age at baseline			1.02	0.84 - 1.24	0.839
Sex					
Male	14/38	(36.8%)			
Female	41/71	(57.8%)	2.34	1.04 - 5.27	0.039

S2 Table. Multivariable logistic regression models for scarring progression. That includes; a) incident scarring and b) increase in pre-existing scarring, in individuals with (a) no scarring at baseline or (b) some scarring at baseline. Multivariable associations between scarring and infection, TF and TP were adjusted for age at baseline and sex.

A) INCIDENT SCARRING	**OR**	**95% CI**	**P value**
C. trachomatis			0.3477
None			
<10%	0.46	0.14 - 1.48	
10-19%	1.52	0.57 - 4.04	
20-29%	1.19	0.36 - 3.93	
30% +	1.90	0.38 - 9.46	
TF			0.1472
None			
<10%	0.70	0.14 - 3.41	
10-19%	0.71	0.20 - 2.49	
20-29%	0.75	0.20 - 2.75	
30% +	2.77	0.79 - 9.65	
TP			0.0003
None			
<10%	1.04	0.30 - 3.56	
10-19%	2.00	0.57 - 7.08	
20-29%	11.97	3.74 - 38.31	
30% +	3.81	0.76 - 19.22	
Age at baseline	1.16	0.95 - 1.42	0.149
Sex (female)	0.95	0.46 - 1.94	0.885
B) PROGRESSIVE SCARRING			
C. trachomatis			0.5287
None			
<10%	0.89	0.16 - 4.87	
10-19%	0.42	0.13 - 1.38	
20-29%	0.41	0.07 - 2.35	
30% +	0.31	0.03 - 3.26	
TF			0.9483
None			

<10%	0.76	0.19 - 3.07	
10-19%	1.01	0.28 - 3.62	
20-29%	0.84	0.16 - 4.46	
30% +	0.59	0.13 - 2.63	
TP			0.1777
None			
<10%	2.26	0.73 - 6.98	
10-19%	2.95	0.76 - 11.48	
20-29%	1.51	0.40 - 5.79	
30% +	6.30	1.23 - 32.26	
Age at baseline	0.95	0.74 - 1.22	0.678
Sex (female)	2.54	0.91 - 7.03	0.074