KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLGY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF MOLECULAR MEDICINE

Ph.D. IMMUNOLOGY

MICRO RNAs, NEW PLAYERS IN THE IMMUNOPATHOGENESIS OF TUBERCULOSIS.

ANTHONY AFUM-ADJEI AWUAH

OCTOBER 2014

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLGY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF MOLECULAR MEDICINE

MICRO RNAs, NEW PLAYERS IN THE IMMUNOPATHOGENESIS OF TUBERCULOSIS.

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY (KNUST), IN FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF Ph.D. DEGREE IN IMMUNOLOGY

BY

ANTHONY AFUM-ADJEI AWUAH

BSc (HONS) BIOCHEMISTRY

DECLARATION

I, Anthony Afum-Adjei Awuah, author of this thesis, "micro RNAs, new players in the immunopathogenesis of tuberculosis" do hereby declare that, apart from references to past and current literature duly cited in thesis, the entire research work presented in this thesis was done by me as a student of the Department of Molecular Medicine, KNUST.

It has neither in whole nor in part been submitted for a degree elsewhere

ANTHONY AFUM-ADJEI AWUAH	……..	……..
Student	Signature	Date
PROF. MRS. MARGARET FREMPONG	……..	……..
Academic Supervisor	Signature	Date
DR. ELLIS OWUSU-DABO	……..	……..
Field Supervisor	Signature	Date
DR. R. A. NGALA	……..	……..
Head of Department	Signature	Date

DEDICATION

I dedicate this work to Rev David Seth Quansah who believed in me when I needed it the most, helped me every step of the way and for his exemplary leadership, which I have used as template for my Christian and professional development.

ACKNOWLEDGMENT

My strength, thy grace; my rule, your word; my end, the glory of the Lord. I thank God and give Him all the glory for this work. Without Him would nothing be done that has been done. I would also like to acknowledge the German Leprosy and TB relief association (DAHW) for funding this study. Again, I wish to acknowledge the potent help of my PIs, Dr. Ellis Owusu-Dabo and Prof PD. Marc Jacobsen for giving me the opportunity without which this would not be possible. Their support and training have been the solid rock on which I built skill and understanding to do this work. I wish to thank my supervisor Prof Mrs M.T. Frempong who's selfless tutoring and patience saw me through the difficult times of my study. I also want to show my appreciation to the AG Jacobsen in BNITM, Hamburg and the AG Jacobsen in Dusseldorf who without fail trained and helped me in various phases of my work. I wish to thank Malta and Nora from Dusseldorf who accepted me to stay in their home when I travelled to do my work.

The KCCR team has been phenomenal not only at giving me the opportunity to engage in high end advanced biomedical training but given me the support every step of the work. Especially to Dorothy Sarpong, Dr. Mrs Augustina Sylverken, Michael Frimpong, Richard Larbi, Kwadwo Sarfo Marfo, Sandra Baffour Awuah and Esimebia Amegashie who literally assisted me with my study when the going got tough.

Last but not the least, I wish to acknowledge my parents, Mr Thomas Williams Awuah and Mrs Florence Oppong for their love, encouragement, support and personal interest in my professional development. Also, thank you to family and friends especially Abena Serwaa Berchie who in diverse ways supported me throughout my study.

I thank you all and pray that the Lord bless you in all of your life's endeavours.

ABSTRACT

Whereas a third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), there is still a lot to learn about the specific role of cellular immune system in particular the role that is played by $CD4^+$ T cell derived IFN-γ and micro RNAs in immune modulation during active tuberculosis. Although IFN-γ remains a very important mediator of host immune response to *M. tuberculosis* infection, there has been documented speculations about IFN-γ independent mechanism of mtb control by $CD4^+$ T cells emphasizing on IFN-γ as a measure of inflammatory status other than an appropriate immune response. Although miRNAs had been documented to be implicated in autoimmunity and cancer, it was not until recently that investigators have suggested a role for them in host pathogen interaction especially in modulating several processes in humoral and cell mediated immunity. This study investigated the dynamics of IFN-γ production during acute pulmonary tuberculosis in a tuberculosis endemic region of Ghana. Again pathogen induced immune modulation in $CD4^+$ T cells via miRNAs was investigated in tuberculosis patients and their exposed but healthy household contacts.

This case control study nested with a cohort design recruited a total of 32 clinically confirmed Tuberculosis patients and 56 exposed but healthy household contacts otherwise referred to as Latent tuberculosis infected persons (LTBIs) consecutively from three hospitals in Kumasi, Ghana (Komfo Anokye Teaching Hospital, Kwame Nkrumah University of science and Technology Hospital and Kumasi South Hospital). PBMCs were isolated from whole blood for stimulation using PPD and ESAT-6 as Tuberculosis specific antigen and PMA/Io, PHA and SEB as positive control for 5 days. IFN-γ from culture supernatants were quantified using ELISA. $CD4^+$ T cells were isolated from PBMCs using the Imag system and its purity determined using FACS analysis. MiRNAs were extracted from $CD4^+$ T cells and the differential expression of candidate miRNAs were quantified using qRT-PCR.

It is shown in this study that IFN-γ response is higher in LTBIs than tuberculosis patients. IFN-γ response pattern does not change significantly during antituberculous chemotherapy. Again, concomitant diseases, age, sex and prior BCG vaccination does not influence IFN-γ response during acute pulmonary tuberculosis.

Moreover, study findings reveal that 7 different miRNAs may be involved in the T cell response during acute Pulmonary Tuberculosis in Ghanaian study population. Their differential expression was repressed prior to antituberculous chemotherapy but increased significantly during therapy. The miRNAs are miR-25, miR-26a, miR-29a, miR-99b, miR-101, miR-146a and miR-223. Candidate miRNAs' differential expressions were not significantly different from LTBIs.

Lastly miR-29a had a tendency to correlate positively with IFN-γ response in tuberculosis patients and LTBIs but this was not statistically significant except for tuberculosis specific ESAT-6 induced IFN-γ response in LTBIs that showed a significant positive correlation.

IFN-γ mediated Th1 response is critical for control of *Mycobacterium tuberculosis* infection. However there exists the possibility of a Th1- ($CD4^+$ T cell) dependent IFN-γ independent mechanism of *M. tuberculosis* control. IFN-γ repression may be a likely immune evasive mechanism employed by *M. tuberculosis* to overwhelm the host immunity. Consistent pattern of significantly altered differential expression of miR-25, miR-26a, miR-29a, miR-99b, miR-101, miR-146a and miR-223 suggests a role of these candidates in $CD4^+$ T cell immunity during tuberculosis disease and recovery. These candidates may represent future candidates for immunotherapies and vaccines against *M. tuberculosis* infections. Although miR-29a may be involved with CD4+ T cell response to *M. tuberculosis* infection it appears not to be a none redundant repressor of IFN-γ production in tuberculosis patients and LTBIs. Thus, IFN-γ and miRNAs may be important determinants of disease outcome during *M. tuberculosis*

infection. Future studies would consider possible targets for successful candidates with significantly altered differential expression and varying expression in $CD4^+$ T cell sub population. The current study is considered as an initial step to characterize the role of IFN-γ and microRNAs in tuberculosis.

TABLE OF CONTENT

Declaration ii
Dedication iii
Acknowledgment iv
Abstract v
List of Tables xiii
List of Figures xv
List of Abbreviations xix
Chapter One 1
1.0 Introduction 1
1.1 Rationale of the Study 5
1.2 Hypothesis 8
1.3 General Objectives 8
1.4 specific Objective 8
Chapter Two 10
2.0 Literature Review 10
2.1 Overview of Tuberculosis 10
2.2 History of Tuberculosis 11
2.3 Diagnosis 12
2.4 Current Treatment in Ghana 12
2.4.1 Standard Regime 13
2.4.2 Retreatment Regime 13
2.5 Etiology 14
2.6 Epidemiology 15
2.7 Overview of Immunopathogenesis 16
2.7.1 Humans generate Th1 immunity during tuberculosis 18
2.7.2 Route and Site of Infection 19
2.7.3 Events after *M. tuberculosis* infection 19
2.7.4 Antimycobacterial Functions 20
2.7.5 Binding of *M. tuberculosis* to Phagocytes 20
2.7.6 Phagolysosome fusion 20
2.7.7 Macrophage initiated mechanism of Mycobacterial growth inhibition or killing 21
2.7.8 Reactive Nitrogen Intermediates (RNI) 21
2.7.9 Reactive Oxygen Intermediate (ROI) 22

2.7.10 Evasion of Host immune mechanisms by *M. tuberculosis* 23
2.8 Innate immunity 25
2.8.1 Nramp 25
2.8.2 Neutrophils 25
2.8.3 Natural Killer (NK) Cells 27
2.8.4 CD1d-restricted Natural killer T (NKT) cells 28
2.8.5 Gamma/lambda (γ/δ) T-cells 28
2.8.6 Macrophages (MAC) 29
2.8.7 Dendritic Cell (DC) 32
2.9 Adaptive immunity 34
2.9.1 T cells 34
2.9.1.1 $CD4^+$ T Cells 34
2.9.1.2 $CD8^+$ T Cells 35
2.10 Cytokines 36
2.10.1 Interleukin 12 (IL-12) 36
2.10.2 Interferon gamma (IFN-γ) 37
2.10.3 Tumour Necrosis Factor α (TNF- α) 38
2.10.4 Interleukin 10 (IL-10) 39
2.11 MICRO RNAs (miRNAs) 41
2.11.1 Biogenesis 41
2.11.2 Role of miRNAs in immune system development and function 44
2.11.3 MiRNAs in the regulation of innate immunity 44
2.11.4 MiRNAs in the regulation of adaptive immunity 46
2.11.5 MiRNAs and bacterial infection 47
2.11.6 MiRNAs and Mycobacterial infection 48
Chapter three 50
3.0 Methodology 50
3.1 Study population 50
3.2 Study area 50
3.3 Study design 50
3.4 Diagnosis at the hospital 51
3.5 Inclusion criteria 51
3.6 Exclusion criteria 51

3.7 Field work 51
3.8 Sample collection 52
3.9 Laboratory assay 53
3.9.1 PBMC isolation 53
3.9.1.1 Preparing samples 53
3.9.1.2 Blood Layering 53
3.9.1.3 PBMC separation 54
3.9.1.4 1st washing 54
3.9.1.5 2nd washing 54
3.9.2 Cell Counting 55
3.9.3 *In vitro* stimulation of PBMC with *M. tuberculosis* specific antigens 56
3.9.6 Enzyme Linked Immunosorbent Assay (ELISA) 57
3.9.6.1 Antibody Coating 58
3.9.6.2 Blocking of unspecific binding sites 58
3.9.6.3 Incubation of Culture supernatant 58
3.9.6.4 Detection 58
3.9.6.5 Elisa Reading 59
3.9.7 MiRNA Isolation 60
3.9.8 RNA Quantification 61
3.9.9 TaqMan Reverse Transcription for Plate assay 61
3.9.10 TaqMan Reverse Transcription for Single assay 62
3.9.11 qRT-PCR for Plate Array 63
3.9.12 qRT-PCR for Single Assay 65
3.10 Statistical Analysis 66
3.11 Data handling 67
3.12 Ethical approval 67
Chapter Four 68
4.0 Results 68
4.1 Dynamics of Interferon-gamma response 68
4.1.1 Interferon-gamma response over treatment period 68
4.1.2 Interferon-gamma response between patients and their contacts 70
4.1.3 Interferon-gamma response between vaccinated and non-vaccinated patients 71
4.1.4 Influence of age, sex and concomitant diseases on interferon-gamma response among patients. 72

4.2 Candidate miRNA screening 75
4.2.1 miR-25 76
4.2.2 miR-26a 78
4.2.3 miR-29a 79
4.2.4 miR-99b 80
4.2.5 miR-101 82
4.2.6 miR-146a 84
4.2.7 miR-223 86
4.2.8 MiRNA expression between patients and contacts 87
4.3 Modulation of Interferon gamma response by miR-29a 90
Chapter Five 93
5.0 Discussion 93
5.1 Interferon-gamma response over treatment period 93
5.2 Interferon-gamma response between patients and their contacts 94
5.3 Interferon-gamma response between vaccinated and non-vaccinated patients. 96
5.4 Influence of age, sex and concomitant diseases on interferon-gamma response among patients. 97
5.5 Candidate miRNA Screening 98
5.6 Limitations of the study 104
Chapter six 105
6.0 Conclusions and Recommendations 105
6.1 Conclusions 105
6.2 Recommendations 105
6.3 Novelty and Lessons learnt 106
6.4 Contribution to knowledge 107
References 108
Appendices 154
Appendix A 154
Patient demographics and laboratory coding 154
Appendix B 157
Cell counts of patients at various time points 157
Appendix C 158
Patient Interferon-gamma response for first time point 158
Appendix D 161

Patient Interferon-gamma responses for second time point....161
Appendix E....164
Patient Interferon-gamma responses for third time point....164
Appendix F....167
LTBIs Interferon-gamma responses....167
Appendix G....171
Compiled Patient Ct values for single assay....171
Appendix H....173
Compiled LTBI Ct values for Assay....173
Appendix i....175
First part of compiled patient Ct values for plate assay....175
Appendix J....179
Second part of compiled patient Ct values for plate assay....179
Appendix K....183
Raw Data display from the Applied Biosystem's real time PCR....183
Appendix L....195
Converted RT-PCR results from raw data....195
Appendix M....197
Data Entry Form for controls....197
Appendix N....198
Data Entry form for TB patients....198
Appendix O....200
Ethics Approval Letter....200
Appendix P....201
Data Entry form template for TB patients....201
Appendix Q....204
Pictures of study from field to the laboratory....204

LIST OF TABLES

Table 1 ANTIGENS AND THEIR VOLUMES USED PER WELL 57

Table 2 SCHEME OF MASTERMIX FOR PRIMER POOL A 61

Table 3 SCHEME OF MASTERMIX FOR PRIMER POOL B 62

Table 4 PCR PROGRAMME 62

Table 5 SCHEME OF MASTERMIX FOR MIR-29A 63

Table 6 SCHEME OF MASTERMIX FOR RNU48 63

Table 7 SCHEME FOR RT-PCR MASTERMIX 64

Table 8 SCHEME FOR PIPPETING UNTO AN OPTICAL 96 WELL PLATE 64

Table 9 PLATE ARRAY PCR PROGRAMME 65

Table 10 SCHEME FOR PREPARING MASTERMIX 66

Table 11 DEMOGRAPHIC CHARACTERISTICS OF TUBERCULOSIS PATIENTS AND LTBIS 68

Table 12 MEDIAN DIFFERENTIAL EXPRESSION OF NONE ALTERED MIRNAs IN LTBI AND TB PATIENTS 89

Table 13 PATIENT DEMOGRAPHICS AND LABORATORY CODING 154

Table 14 CELL COUNTS OF PATIENTS AT VARIOUS TIME POINTS 157

Table 15 PATIENT INTERFERON-GAMMA RESPONSES FOR FIRST TIME POINT . 158

Table 16 PATIENT INTERFERON-GAMMA RESPONSES FOR SECOND TIME POINT 161

Table 17 PATIENT INTERFERON-GAMMA RESPONSES FOR THIRD TIME POINT 164

Table 18 LTBIs INTERFERON-GAMMA RESPONSES 167

Table 19 COMPILED PATIENT Ct VALUES FOR SINGLE ASSAY 171

Table 20 COMPILED LTBI Ct VALUES FOR PLATE ASSAY 173

Table 21 FIRST PART OF COMPILED PATIENT Ct VALUES FOR PLATE ASSAY 175

Table 22 SECOND PART OF COMPILED PATIENT Ct VALUES FOR PLATE ASSAY 179

Table 23 RAW DATA DISPLAY FROM THE APPLIED BIOSYSTEM'S REAL TIME PCR.....183

Table 24 CONVERTED RT-PCR RESULTS FROM RAW DATA 195

LIST OF FIGURES

Figure 1 estimated Global tuberculosis incidence rate in 2010 15

Figure 2 Neutrophils ingest *M. tuberculosis* (Palomino et al., 2007) 27

Figure 3 Coloured scanning electron micrograph of a macrophage cell engulfing a bacterium 31

Figure 4 The intracellular lifestyle of Mycobacterium tuberculosis 31

Figure 5 Innate immunity to tuberculosis infection 33

Figure 6 miRNA biogenesis and action in animal cells 44

Figure 7 miRNAs in the regulation of innate immunity (Dai and Ahmed, 2011) 45

Figure 8 miRNAs in the regulation of adaptive immunity 47

Figure 9 The first 2 tubes on the left contains blood diluted with PBS and the right tube contains biocoll layered with blood. 53

Figure 10 Layering biocoll with blood. 54

Figure 11 cells pelleted at the base of the falcon tube after 2nd washing. 55

Figure 12 Neubauer hemacytometer loaded with samples for counting on the Zeiss Axiovert 25 inverted microscope 56

Figure 13 Colour of positive wells changes from blue to yellow upon addition of sulphuric acid just before reading with SUNRISE ELISA machine 60

Figure 14 monitor displaying Amplification plot from a ran sample using the 7500 software 65

Figure 15 shows PPD induced IFN-γ response of Tuberculosis patients at 3 time points (Time points 0(n=19), 1 (n=19), and 6 (n=31)). 69

Figure 16 shows SEB induced IFN-γ response of TB patients at 3 time points (time point 1(n=18), 2 (n=18), 3 (n=31)) 70

Figure 17 shows PPD induced IFN-γ response between Tuberculosis (TB) patients (n= 18) and Latent Tuberculosis Infected contacts (LTBIs) (n= 38).....70
Figure 18 shows SEB induced IFN-γ response between Tuberculosis (TB) patients (n= 18) and Latent Tuberculosis Infected contacts (LTBIs) (n= 40).....71
Figure 19 shows PPD induced IFN-γ response between BCG vaccinated Tuberculosis patients (n= 27) and non-vaccinated Tuberculosis patients (n= 43).....71
Figure 20 shows SEB induced IFN-γ response between BCG vaccinated Tuberculosis patients (n= 26) and non-vaccinated Tuberculosis patients (n= 42).....72
Figure 21 Correlations between age of patients and PPD induced IFN-γ responses are shown on the left for time point 0 (a) (n=18), time point 1(b) (n=16) and time point 6(c) (n=29). On the right of the graph are Correlations between age of patients and SEB induced IFN-γ responses for time point 0 (e) (n=18), time point 1(f) (n=18) and time point 6(g) (n=31).....74
Figure 22 (a) shows PPD induced IFN-γ responses between male (n=51) and female (n=17) patients (b) shows SEB induced IFN-γ responses between male (n=50) and female (n=16) patients.....74
Figure 23 (a) shows PPD induced IFN-γ responses between Tuberculosis patients (TB) (n=18) and Tuberculosis patients with concomitant disease (TB+ CD) (n=3) (b) shows SEB induced IFN-γ responses between Tuberculosis patients (TB) (n=18) and Tuberculosis patients with concomitant disease (TB+ CD) (n=3).....74
Figure 24 shows preselected candidate miRNAs and successful candidates. Blue tabs on the left shows all 29 candidates, yellow tab shows the endogenous housekeeping gene used, middle red tabs shows detectable miRNAs and orange right tabs show significantly altered candidates.....76
Figure 25 Shows differential expression of miR-25 during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).....77

Figure 26 shows differential expression pattern of miR-25 for 5 different patients during antituberculous chemotherapy indicated by letters A-E. 77
Figure 27 Shows differential expression of miR-26a during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).....78
Figure 28 shows differential expression pattern of miR-26a for 5 different patients during antituberculous chemotherapy indicated by letters A-E. 79
Figure 29 Shows differential expression of miR-29a during antituberculous chemotherapy. Time point 0 (n=14), time point 1 (n=13) and time point 6 (n=25).....80
Figure 30 shows differential expression pattern of miR-29a for 5 different patients during antituberculous chemotherapy indicated by letters A-E. 80
Figure 31 Shows differential expression of miR-99b during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).....81
Figure 32 shows differential expression pattern of miR-99b for 5 different patients during antituberculous chemotherapy indicated by letters A-E. 82
Figure 33 Shows differential expression of miR-101 during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).....83
Figure 34 shows differential expression pattern of miR-101 for 5 different patients during antituberculous chemotherapy indicated by letters A-E. 84
Figure 35 Shows differential expression of miR-146a during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).....85
Figure 36 shows differential expression pattern of miR-146a for 5 different patients during antituberculous chemotherapy indicated by letters A-E. 85
Figure 37 Shows differential expression of miR-223 during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).....86

Figure 38 shows differential expression pattern of miR-146a for 5 different patients during antituberculous chemotherapy indicated by letters A-E. 87
Figure 39 shows relative expressions of preselected candidate miRNAs (that were significantly altered during treatment) of patients (n=13) and LTBIs (n=11). 88
Figure 40 shows correlation between miR-29a and IFN-γ responses induced by 5 different antigens for Tuberculosis patients (n=45). 91
Figure 41 shows correlation between miR-29a and IFN-γ responses induced by 5 different antigens for LTBIs (n=18). 92
Figure 42 A typical entered data sheet for household contacts of tuberculosis patients enrolled for this study 197
Figure 43 A typical entered data sheet for tuberculosis patients enrolled for this study 198
Figure 44 Ethics Approval letter from Local Ethics committee 200
Figure 45 Data entry form for tuberculosis patients 201
Figure 46 Data entry form for household contacts of tuberculosis patients 203
Figure 47 Study team arrives at the house of the patients to take samples from patients and their contacts 204
Figure 48 Taking blood samples from study participants. 204
Figure 49 Laboratory processing of samples 205

LIST OF ABBREVIATIONS

AFB	Acid Fast Bacillus
AIDS	Acquired Immune Deficiency Syndrome
AMC-1	Acute Myeloid Leukemia-1
APC	Antigen Presenting Cells
BCG	Bacillus Calmette Guerin
BSA	Bovine Serum Albumin
CC	Chemokine
CCR-7	Chemokine receptor 7
CD4	Cluster of Differentiation 4
cDNA	Complementary Deoxy Ribonucleic Acid
CHRPE	Committee on Human Research, Publication and Ethics
CMI	Cell Mediated Immune Response
CR1	Complement Receptor 1
CR2	Complement Receptor 2
CR3	Complement Receptor 3
DC	Dendritic Cells
DC-SIGN	Dendritic cell-specific intracellular-adhesion-molecule- grabbing non-integrin
DG-CR8	DG-CR8
DMSO	Dimethyl sulfoxide
DOTS	Directly Observed Treatment Short course
DPBS	Dulbecco's Phosphate Buffered Saline
DTH	Delayed Type Hypersensitivity
E	Ethanbutol
ELISA	Enzyme Linked Immunosorbent Assay
ESAT-6	Early Signs of Antigenic Target 6 kDa

FACS	Fluorescent Activated Cell Sorting
GKO	Gene Knockout
GM-CSF	Granulocyte-Macrophage-Colony Stimulating Factor
GNDP	Ghana National Drug Programme
GPI	Glycosylphosphatidylinositol
H	Isoniazid
H_2SO_4	Tetra Oxo Sulphate (VI) Acid
HIV	Human Immunodeficiency Virus
HKG	House Keeping Gene
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-12	Interleukin 12
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-18	Interleukin 18
IL-2	Interleukin 2
IL-23	Interleukin 23
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
iNOS2	Inducible nitric oxide synthase
Io	Ionomycin
KATH	Komfo Anokye Teaching Hospital
kDa	Kilo Dalton
KNUST	Kwame Nkrumah University of Science and Technology
KSH	Kumasi South Hospital

LAM	Lipoarabinomannan
LTBI	Latent Tuberculosis Infection
mAb	Monoclonal Antibody
MAC	Macrophages
M-CSFR	Macrophage colony-stimulating factor receptor
MDR-TB	Multi Drug Resistant Tuberculosis
MHC	Major Histocompatibility Complex
MiRNA	micro Ribonucleic Acid
MR	Mannose Receptor
NK cells	Natural Killer Cells
NKT cells	Natural Killer T cells
NO	Nitric oxide
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PGE2	Prostaglandine E2
PHA	Phytohaemagglutinin
PMA	Phorbol 12-Myrstate 13-Acetate
PMN	Polymorphonuclear Neutrophils
PPD	Purified Protein Derivative
PPDtub	Purified protein Derivative tuberculosis
Pre-miRNA	Precursor micro Ribonucleic Acid
qRT-PCR	Quantitative Real Time Reverse Transcription Polymerase Chain Reaction
R	Rifampicin
RISC	RNA-induced Silencing complex
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediate

ROI	Reactive Oxygen Intermediate
ROS	Reactive Oxygen specie
RT-PCR	Reverse transcription Polymerase Chain Reaction
SEB	Staphylococcal Enterotoxin B
SEM	Scanning Electron Microscope
SR	Spearman's Coefficient of correlation
TAP	Transporter Associated with Antigen Processing
TB	Tuberculosis
TGF-β	Transforming Growth Factor-beta
Th1	T helper type 1
Th2	T helper type 2
TLR-2	Toll Like Receptor 2
TLR-4	Toll Like Receptor 4
TMB	TetraMethylBenzidin
TNF-α	Tumour Necrosis Factor-alpha
USA	United States of America
WHO	World Health Organization
XDR-TB	Extreme Drug Resistance Tuberculosis
Z	Pyrazinamide
α-β cells	Alpha-beta cells
γ-δ T cells	Gamma-lambda T cells

CHAPTER ONE

1.0 INTRODUCTION

Tuberculosis commonly referred to as TB is a common and deadly infectious disease in the world caused by several strains of mycobacteria mostly *Mycobacterium tuberculosis* (*M. tuberculosis*) in humans (Flynn and Chan, 2001a). Approximately one third of humans are infected with *M. tuberculosis* (Kleinsteuber et al., 2013). It is the second most deadly infectious disease next to Human Immunodeficiency virus causing Acquired Immunodeficiency Syndrome (HIV/AIDS) (WHO, 2011). Tuberculosis mostly infects the lungs and is known as acute pulmonary tuberculosis. It can however spread to other parts of the body and infect other organs leading to miliary or disseminated tuberculosis. Classical symptoms of the disease include coughs, fever, night sweats and weight loss. The drastic weight loss experienced by patients particularly won it its name consumption.

Tuberculosis is spread when *M. tuberculosis* is transferred via aerosols generated by an infected person by talking, sneezing or coughing. The extent of infection depends on a number of factors that include the host's immune strength, duration and frequency of exposure and the virulence of the mycobacteria species (Dheda et al., 2010).

There could be several outcomes to a person encountering *M. tuberculosis*. The innate immune response though not characteristic can eliminate the bacteria. The induction of acquired cell mediated immunity can in some cases revert the infection or contain it. Such individuals are considered to have Latent Tuberculosis Infection (LTBI). In this state the individual is asymptomatic and is incapable of spreading the infection. About 5-10% of people with LTBI would develop into acute tuberculosis from 1-3 years from the time of infection (Flynn and Chan, 2001a, Styblo, 1980). Others have also shown that development

of active tuberculosis can be from 2-5 years following the point of infection (Dheda et al., 2010, Cooper, 2009).

Development of active tuberculosis is not associated with immunodeficiency. In most cases, other factors such as HIV infection, treatment with corticosteroids, ageing, alcohol or drug abuse can predispose individuals to reactivation (Flynn and Chan, 2001a).

The outcome of *M. tuberculosis* infection depends on a number of factors prominent among which includes the pivotal containment by the cell mediated immune response to the aetiological agent by the host. This has been the focus for immunologists for decades. Although we know more about the immune interaction between *M. tuberculosis* and host immune system with its consequent infection outcome (Raja, 2004), exact mechanisms and key immunopathogenic pathways remains hypothetical at best. The help of modern day advanced tools for immunological studies have improved our understanding of this prehistoric infection (Flynn and Chan, 2001a).

Although bacterial virulence and host resistance are two independent variables, the pathogenesis of tuberculosis is largely attributed to the interactions between these variables. The last decade has seen a profound illumination into the specific role of bacterial and host components. These efforts are focused on identifying virulence factors, drug targets within the bacterium, resistance factors, mechanisms of immune modulation and components of the host's immune system that can be augmented and altered by vaccination (Cooper, 2009). Several factors have been identified which are crucial for protection against development of active tuberculosis. It is largely accepted that interactions between T cells and infected macrophages play a major role in protection from tuberculosis (Raja, 2004). Interferon gamma (IFN-γ), Tumour Necrosis Factor alpha (TNF-α) and Interleukin-12 (IL-12) are crucial mediators in this process. The most profound advantage of the acquired cellular

immune response which is crucial in resistance against *M. tuberculosis* is the rapidity with which it is expressed. A delay in the response would lead to high bacteraemia such that a potentially protective immune response would not contain it.

In the same regard, dose plays a role in the ability of the host to mount a protective immune response to contain the bacteria. If there is too high bacteraemia, the local bacterial burden may go beyond the threshold of immune containment and this will interfere with efficient expression of protective immunity (Cooper, 2009).

Interferon gamma has been studied extensively as a mediator of cellular immune response against intracellular bacteria like *M. tuberculosis* (Feng et al., 1999, Flynn et al., 1993). Increased bacterial load and decreased survival time was observed in interferon gamma (IFN-γ) receptor deficient mice (Flynn et al., 1993, Cooper et al., 1993). Nevertheless, it has been observed that quantitative assessment of single markers i.e. IFN-γ or tumour necrosis factor-alpha (TNF-α) is insufficient as a correlate of protection against tuberculosis (Boussiotis et al., 2000). The influence of other T helper cell subtypes in the immune response against *M. tuberculosis* is a matter of intense studies and has become increasingly complicated because of 'novel' T cell subtypes identified during recent years. E.g. interleukin 10 (IL-10) and Transforming growth factor beta (TGF-β) expressing regulatory T cells have been shown to play a role in a stage of hypo responsiveness of *M. tuberculosis*-specific T cells during active tuberculosis disease (Delgado et al., 2002, Boussiotis et al., 2000). Whereas there is a pool of data that suggests the importance of IFN-γ in the immune response to *M. tuberculosis* both in murine and human studies (Cooper et al., 2002, Dalton et al., 1993, Feng et al., 1999, Fenton et al., 1997, Flynn et al., 1993, Jo et al., 2003), there is new information that seeks to challenge the relevance of IFN-γ in IFN-γ dependent T cell mediated control of *M. tuberculosis* infection (Gallegos et al., 2011). In Gallegos et al.'s work, they provided compelling evidence that $CD4^+$ T cells can induce *M. tuberculosis* growth arrest, even when

unable to secrete IFN-γ, TNF or both cytokines. It however contradicts the work by Silva et al. who showed that mean IFN-γ response was higher in treated tuberculosis patients than in acute tuberculosis patients (Silva et al., 2012).

Protective immunity by Bacillus Calmette Guerin (BCG) is documented to be dependent upon the induction of $CD4^+$ T cells that produce IFN-γ which subsequently induce bactericiding via macrophage activation (Hanekom, 2005, Hussey et al., 2007). In the case of tuberculosis, Abebe documents that IFN-γ produced by $CD4^+$ T cells has been used as a yardstick against which BCG or other tuberculosis vaccine induced immunity to tuberculosis is assessed (Abebe, 2012).

Though the general school of thought is that BCG induced immunity is lost in adults, some studies do report a lack of correlation between BCG induced immune protection and IFN-γ secreted by $CD4^+$ T cells (Connor et al., 2010, Hussey et al., 2007, McShane, 2011). Thus we investigated the disparity of IFN-γ expression between BCG vaccinated and none BCG vaccinated acute pulmonary tuberculosis patients in an attempt to define the effect of prior BCG vaccination on immune response during active disease state.

M. tuberculosis has been documented to employ several immune modulatory molecules to evade host immune response (Flynn and Chan, 2001b). One of such immune modulatory molecules of interest to immunologists is Micro-RNAs (miRNAs). MiRNAs are a promising novel group of molecules that specifically modulate gene expression post transcriptionally. These non-coding miRNAs have a strong influence on the development and function of the innate and adaptive immune response. MiR-155, for example, enhances TNF-α translation in cells of the innate immune response (Tili et al., 2007). TNF-α is crucial for the protective immune response against tuberculosis since monoclonal antibodies or soluble TNF-α receptor agonists dramatically increase the risk for reactivation of *M. tuberculosis* in LTBI.

MiR-181 expression influences the T cell sensitivity directly by targeting phosphatases involved in the T-cell receptor signalling (Li et al., 2007). MiR-146 has been identified as a marker for T helper type 1 cells, the crucial subset T cells for protection against *M. tuberculosis* but the role of miR-146 remains elusive (Monticelli et al., 2005). Pre-selection of 29 immune related miRNAs due to their reported dysregulation of immune response to infection particularly bacterial infection for this study was done.

Ma and colleagues (2011) have shown that miR-29a down regulates interferon gamma production in $CD4^+$ T cells post transcriptionally by targeting messenger RNA (mRNAs)(Ma et al., 2011b). Steiner et al. in 2011 proved that miR-29a mediates its effects through specific regulation of the IFN-γ production pathway rather than through general effects on T cell activation, cytokine production, or cell fitness (Steiner et al., 2011). Finally, Kleinsteuber et al. showed for the first time decreased expression of miR-21, miR-26a, miR-29a, and miR-142-3p in $CD4^+$ T Cells and peripheral blood from tuberculosis patients (Kleinsteuber et al., 2013). This could be suggestive of the role of miRNAs as immune modulatory molecule in T cell response during acute pulmonary tuberculosis infection. Over 150 human diseases have been documented to have a relationship with miRNA dysregulation (Jiang et al., 2009).

This study investigated the dynamics of interferon-gamma production during acute pulmonary tuberculosis infection and the potential role that 29 preselected, immune related miRNAs may play as immune modulatory molecule during disease in a tuberculosis endemic region, Ghana.

1.1 RATIONALE OF THE STUDY

Though strides have been made to understand immune evasive mechanisms employed during tuberculosis infection there is still much to do to better understand *M. tuberculosis* related immune evasive mechanism and host related immune evasive mechanism.

In 2010, there were an estimated 12.0 million prevalent cases of TB out of which 650,000 cases were multidrug resistance Tuberculosis (MDR-TB) (WHO, 2011, WHO, 2012). This is compounded by extreme drug resistance tuberculosis (XDR-TB) worldwide. In Ghana 50-90 per 100,000 new cases of tuberculosis were reported as at 2010. This number increased to 50-149 per 100,000 new cases in 2011. Forson et al. reports 38% MDR-TB from a tuberculosis tertiary referral centre in a teaching hospital in Ghana. This is higher than a reported 5% national average cases of MDR-TB in 2006 (Forson et al., 2010).

IFN-γ an important mediator of adaptive immune response to *M. tuberculosis* infection can be unreliable as an immune correlate of protection because *M. tuberculosis* can prevent macrophages from responding adequately to IFN-γ (Ting et al., 1999). This ability of *M. tuberculosis* to limit activation of macrophages by IFN-γ suggests that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine (Flynn and Chan, 2001b). This has resulted in difficulty in the development of the right vaccine and immunotherapy for preventing and curing tuberculosis respectively. Gallegos and his colleagues in their study did infer that $CD4^+$ T cells may use IFN-γ independent mechanism in their control of *M. tuberculosis* infection in vivo (Gallegos et al., 2011). This introduces the conundrum of whether or not IFN-γ is the right marker for efficacy of BCG vaccine. It therefore questions IFN-γ's role as an important mediator of T cell response to *M. tuberculosis* infection.

Micro RNAs (miRNAs) have been documented as immune modulatory molecules that affect the development and function of both innate and adaptive immunity (Dai and Ahmed, 2011). Its role has been implicated in several diseases that include cancer and tuberculosis (Ma et al., 2011b, Dai and Ahmed, 2011). Thus knowledge of candidates that play a role in tuberculosis would be useful as drug targets, immune biomarkers and immunotherapy among others.

MiR-29a a member of the miRNA family has been documented by Steiner et al. in 2011 to affect the mouse $CD4^+$ T cell pathway that leads to the production of IFN-γ an important mediator of adaptive immune response against *M. tuberculosis* infection in both murine and human tuberculosis (Steiner et al., 2011). Thus miR-29a could be used as a potential immune biomarker for early detection of human pulmonary tuberculosis and immunotherapy to complement chemotherapy. This would act by possibly reducing the duration of treatment which is a major challenge for the treatment of the infection. MiRNA dysregulation has been associated with more than 150 human diseases (Jiang et al., 2009) and thus warrants investigation into its possible involvement in the immunopathogenesis of tuberculosis. Much work pertaining to the immunology of tuberculosis has been in animal models with data skewed towards low incidence developed countries (Caruso et al., 1999, Goren et al., 1976, Mosmann et al., 1986). Also studies in tuberculosis immunology are challenged with very small sample size coupled with much inconsistency in research findings. Although studies have been done on dsyregulation of miRNAs during acute tuberculosis, most focused on serum and sputum (Fu et al., 2011, Yi et al., 2012) whereas others considered mouse models (Ma et al., 2011b) and $CD4^-$ immune cells (Rajaram et al., 2011, Sharbati et al., 2011, Singh et al., 2013a).A current study by Kleinstueber et al. focused on T cells but in a low incidence country. Although children from high incidence country were used in the study, whole blood other than T cells was used for miRNA extraction and differential expression analysis(Kleinsteuber et al., 2013).

$CD4^+$ T cells have been documented to be central to the control of mycobacteria and subsequent infection outcome during *M. tuberculosis* infection. Cowley and Elkins reported that $CD4^+$ T cells control more than 90% of intracellular *M. tuberculosis* growth (Cowley and Elkins, 2003) thus reinforcing the crucial relevance of $CD4^+$ T cells during *M. tuberculosis* infection. $CD4^+$ T cells were currently considered in the elucidation of dysregulated

differential expression of the afore-mentioned miRNAs. This study therefore sought to define the dynamics of IFN-γ response in tuberculosis patients and their exposed but healthy household contacts. It also sought to determine miRNA dysregulation in pre-selected, immune related miRNAs and confirm the interdependency if any between miR-29a and IFN-γ.

1.2 HYPOTHESIS

It was therefore hypothesized that;

1. IFN-γ is an important mediator of $CD4^+$ T cell response to *M. tuberculosis* infection yet there exist the possibility of a T helper type 1(Th1) mediated IFN-γ independent mechanism of *M. tuberculosis* control.

2. There is alteration in differential expression of miRNAs in $CD4^+$ T cells during tuberculosis infection thus miRNAs may play an important role in the host $CD4^+$ T cell response against tuberculosis via modulation of host innate and adaptive immune responses.

1.3 GENERAL OBJECTIVES

- To elucidate the dynamics of IFN-γ production during acute pulmonary tuberculosis
- To determine the dysregulation of pre-selected, immune related candidate miRNAs differential expression in T cell response against human tuberculosis
- To confirm if miR-29a is a none redundant regulator of IFN-γ by T cell during human tuberculosis.

1.4 SPECIFIC OBJECTIVE

- To determine IFN-γ expression pattern in pulmonary tuberculosis patients before, during and after treatment.

- To identify the disparity in IFN-γ production between pulmonary tuberculosis patients and their exposed but healthy household contacts.
- To determine the influence (if any) of childhood BCG vaccination on IFN-γ response in adult tuberculosis patients.
- To determine the influence of concomitant diseases, age and sex (if any) on IFN-γ response during tuberculosis infection.
- To identify if the differential expression of any of the pre-selected immune related candidate miRNAs alter significantly during tuberculosis infection.
- To elucidate the varying expression of such miRNAs between tuberculosis patients and their exposed but healthy household contacts.
- To determine the interdependency of miR-29a and IFN-γ response using 2 TB specific antigens and 3 general antigens in tuberculosis patients and their exposed but healthy household contacts.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 OVERVIEW OF TUBERCULOSIS

Tuberculosis commonly referred to as TB is a common and deadly infectious disease in the world caused by several strains of mycobacteria mostly *M. tuberculosis* in humans (Flynn and Chan, 2001a). It is the second most deadly infectious disease next to HIV/AIDS (WHO, 2011). Tuberculosis mostly infects the lungs and is known as acute pulmonary tuberculosis. It can however spread to other parts of the body and infect other organs leading to miliary or disseminated tuberculosis. Classical symptoms of the disease include coughs, fever, night sweats and weight loss. The disturbing weight loss experience by tuberculosis patients particularly won it its name "consumption".

Tuberculosis is spread when *M. tuberculosis* is transferred via aerosols generated by an infected person by talking, sneezing or coughing. The extent of infection depends on a number of factors that include the host's immune strength, duration and frequency of exposure and the virulence of the pathogen (Cooper, 2009, Dheda et al., 2010).

There could be several outcomes to a person encountering *M. tuberculosis*. The innate immune response though not characteristic can eliminate the bacteria. The induction of acquired cell mediated immunity can in some cases revert the infection or contain it. Such individuals are considered to have Latent Tuberculosis Infection (LTBI). In this state the individual is asymptomatic and is incapable of spreading the infection. About 5% of people with LTBI would develop into tuberculosis from 1-3 years from the time of infection (Flynn and Chan, 2001a, Styblo, 1980). Others have also shown that development of active tuberculosis can be from 2-5% years following the point of infection (Dheda et al., 2010, Cooper, 2009).

Development of active TB is not associated with immunodeficiency in most cases but HIV infection, treatment with corticosteroids, ageing, alcohol or drug abuse predispose individuals to reactivation (Flynn and Chan, 2001a).

2.2 HISTORY OF TUBERCULOSIS

The earliest evidence of tuberculosis in a man and animal has been documented as far back as 8000 BC (Ayvazian, 1993). Smears from a mummified child dating back 700 BC identified TB as the cause of death (Dubos, 1982). One of the oldest legal texts in the world from the Babylonian era mentions a chronic lung disease which is likely tuberculosis (Herzog, 1998). Hippocrates (460-370 BC) introduced the term phthisis (consumption) for tuberculosis but The Hippocratic school rather thought of phthisis as a hereditary disease rather than an infectious one (Herzog, 1998).

Aristotle (384-322 BC) on the contrary held a completely different opinion. He believed that TB was contagious which was contrary to the opinion of his time that tuberculosis was hereditary (Garrison, 1913). Indian literature documents excessive fatigue, worries, hunger, pregnancy and chest wounds as the cause of tuberculosis in 1500 BC (Herzog, 1998). Thomas Willis was the first to describe miliary and chronic forms of consumption. English physician Benjamin Marten (1704-1722) was the first to speculate that consumption especially of the lungs could be caused by small living organisms that cause the symptoms once they gained entry into the body (Herzog, 1998).

Philipp Klencke demonstrated the transmitability of tuberculosis for the first time in 1843 using rabbits. He however was of the opinion that the disease was a tumour (Herzog, 1998). A year after this Jakob Henle (1809-1885) concluded that phthisis could be contagious after several experimentations and observations. He advanced 3 postulate that served as bedrock to further tuberculosis research (Herzog, 1998). They were;

1. The causative agent must be found in every case of the disease.
2. It must not occur in another disease.
3. Its application must always provoke the same disease.

It was a talented microscopist student of Henle, Robert Koch (1843-1910) who gave irrefutable proof in 1882 that a specific microbe is the fundamental cause of Tuberculosis (Sakula, 1979).

The first vaccine for tuberculosis was developed by Calmette and Guerin in 1900s who realised that *Mycobacterium bovis* (*M. bovis*) was no longer virulent and could not cause disease in laboratory animals after 231 passages in a culture medium containing glycerine and ox bile (Herzog, 1998)

2.3 DIAGNOSIS

Pulmonary tuberculosis, the most important type of tuberculosis from the public health perspective, can be diagnosed on the basis of symptoms, chest radiograph, acid fast smear by sputum microscopy, and culture or more than one of the above mentioned(Tiruviluamala and Reichman, 2002). WHO recommends sputum smear positive confirmation for tuberculosis (WHO, 2011). In practice most physicians may require *M. tuberculosis* culture from pleural or spinal fluids or sputum. There also exist Interferon gamma release assay for rapid immunodiagnosis of tuberculosis (Dheda et al., 2009).

2.4 CURRENT TREATMENT IN GHANA

Treatment of tuberculosis in Ghana follows guidelines as recommended by WHO using the directly observed treatment strategy (DOTS) for tuberculosis treatment. It has been implemented in Ghana since 1994 and led to a substantial decline in treatment failures. Before then, tuberculosis was treated without adherence to any concerted guidelines (Owusu-

Dabo et al., 2006). The current DOTS treatment regime for use in Ghana is as described in the standard treatment guidelines of the Ministry of Health, Ghana (GNDP, 2010).

According to this guideline, there are two main types of treatment:

- Standard regime
- Retreatment regime

2.4.1 Standard Regime

Six months treatment-2 months intensive phase, followed by 4 months continuation phase for both adults and children (category 1). It consists of an intensive phase of four drugs; Rifampicin (R), Isoniazid (H), Pyrazinamide (Z) and Ethanbutol (E) in a fixed dose combination taken daily for 2 months. For adults each tablet contains H75mg, R 150mg, Z 400mg and E 275mg and 2-5 tablets given a day depending on weight. For children a regime containing H 30mg, R 60mg, Z 150mg and E 100mg is recommended. For children weighing 30kg and over, give adult category 1. This is followed by a continuation phase of Isoniazid and Rifampicin for both adults (H75R150) and children (H30R60). During the continuation phase the patient must swallow all the oral drugs preferably on an empty stomach under direct observation. The patient needs to be under close supervision by a health worker or any responsible person or member of the community with support from health staff during the full duration of treatment (GNDP, 2010).

2.4.2 Retreatment Regime

This is for relapse, treatment failure, patients who default and who return after more than a month and are smear positive (and some smear negative cases who have defaulted for longer) and all other previously treated patients. Retreatment consists of initial intensive phase of 5

drugs; Rifampicin (R), Isoniazid (H), Pyrazinamide (Z) and Ethanbutol (E) daily for 3 months(doses as previously discussed) with streptomycin added for the first 2 months only.

Streptomycin should be taken as such. For adults, 500mg daily (depending on weight) and for children 15mg/kg daily for 2 months. Then follows a continuation phase with isoniazid, Rifampicin and Ethanbutol daily for a further 5 months for both adults (H75R150E275) and children (H30R60E100).

Monitoring: During the course of the treatment, all pulmonary tuberculosis patients should have repeat sputum smears examined after 2 (or 3 months if retreatment), 5 and 6(or 8) months to confirm conversion to negative smear (GNDP, 2010).

2.5 ETIOLOGY

Mycobacterium tuberculosis complex is the cause of Tuberculosis. It consists of *Mycobacterium tuberculosis* (the main cause of most human tuberculosis), *Mycobacterium africanum*, *Mycobacterium bovis* (the main cause of tuberculosis in cows), *Mycobacterium microti* and the recently discovered mycobacterium canettii (Am. Thor. Soc., 2000, Pfyffer et al., 1998).

Human tuberculosis is mostly caused by *M. tuberculosis* and *M. africanum*. In cattle *M. bovis* causes tuberculosis (Tiruviluamala and Reichman, 2002). Current tuberculosis programmes and pasteurization of milk has reduced *M. bovis* infections in humans (Am. Thor. Soc., 2000). *M. tuberculosis* is a slow growing obligatory aerobic, acid fast intracellular bacterial pathogen, which has an affinity for the lung tissue due to its richness in oxygen (Raja, 2004). It was first discovered by Koch in 1882 (Herzog, 1998). Its cell surface consists of mycolic acid and is responsible for its waxy coat appearance observed under the microscope.

2.6 EPIDEMIOLOGY

There has been a decline in absolute numbers and incidence of tuberculosis each year since 2002 (WHO, 2011). Regardless of this compelling news, there is still alarming data on the current state of the disease globally.

In 2010 WHO reported an incidence of 8.8 million out of which 13% were among people living with HIV. There are more tuberculosis coinfections in Africa alone than anywhere else in the world. Africa accounted for 82% of tuberculosis cases among people living with HIV/AIDS.

The 5 leading countries with the highest number of tuberculosis cases in the world in the order in which they appear are India, China, South Africa, Indonesia and Pakistan. Women accounted for 36% of tuberculosis incidence for 2010 (WHO, 2011).

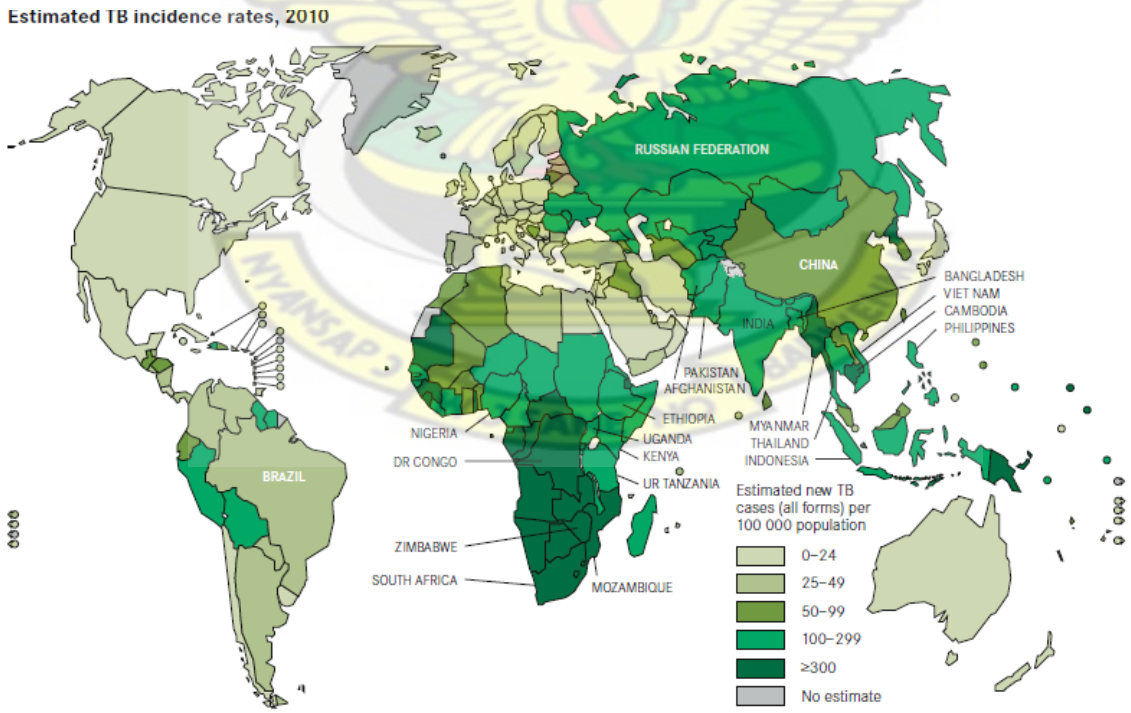


Figure 1 estimated Global tuberculosis incidence rate in 2010
source: **(WHO, 2011)**

In 2010 alone 12 million people were reported to be infected out of which 650,000 were MDR-TB. The prevalence of tuberculosis has declined steadily since 1990. An estimated 1.45 million people died from tuberculosis in 2010 out of which 24.1% of them were HIV positive. About 29.1% of the reported mortality among HIV negative tuberculosis cases in 2010 were women (WHO, 2011).This is lower than the estimated 1.7 million people who died due to tuberculosis in 2009 (WHO, 2010).

In 2009 an estimated 9.7 million children under 15 years were orphaned as a result of losing at least one of their parents to tuberculosis (www.unaids.org/en/dataanalysis/epidemiology). Ghana reported 50-99 cases of tuberculosis per 100,000 populations. This is damaging for a population of just a little over 20 million (WHO, 2011).

2.7 OVERVIEW OF IMMUNOPATHOGENESIS

The immunopathogenesis of Tuberculosis is based upon two main factors; virulence of the mycobacterium tuberculosis and the host's immunity. Though these factors are not related, yet the outcome of an infection rests on these non-related variables (Cooper, 2009).

Immunologists' interest in the immunopathogenesis of tuberculosis has been the fact that protective immune mechanisms are also responsible for pathology. This is mediated by the lymphocytes in cell- mediated immune response.

The importance of $CD4^+$ T lymphocytes were less regarded in previous studies though they were found in tuberculosis lesions. Their function was yet to be elucidated (Cooper, 2009). It was the use of mouse models that demonstrated the anti tuberculous function of T cells in aerosol challenge models (Orme and Collins, 1983) and systemic ones as well (Cooper, 2009) $CD4^+$ T Lymphocyte is the main mediator of acquired cellular response (Cooper, 2009) (Cooper, 2009). HIV patients are noted to develop a different form of the disease without granulomas. It is also not localised as seen in non-HIV tuberculosis patients (Cooper, 2009).

Experiments done using knockout mice (Orme, 1987, Mogues et al., 2001) lacking CD4 molecules also produced less granuloma if any, miliary form of the TB and reduced survival time as compared to their wild litter mates (Saunders et al., 2002).

Also disruption of the Major histocompatibility complex class II (MHC class II) genes or the beta (β) chain of the alpha/beta (α/β) T cell receptor, resulting in a deficiency of $CD4^+$ T cells renders mice more susceptible to the bacteria, even the avirulent species of the mycobacterium complex (Ladel et al., 1995). Manipulation of the immunity of experimental mice via gene knockout or use of neutralising antibodies have provided phenomenal insight that in mice, immunity correlates with a type 1 response (Mosmann et al., 1986). Type 1 response refers to the total cytokine profile released by both type 1 $CD4^+$ T cells and other cells that they interact with at the site of infection. These cells may include macrophages, dendritic cells, B cells and $CD8^+$ T cells (Cooper, 2009).

When these cells interact and give rise to a pattern of cytokine release dominated by IL-2, IL12, and IFN-γ, it is known as type 1 response (Rook G.A.W. and Hernandez-Pando R., 1996). On the other hand, if it gives rise to a pattern of cytokine release dominated by IL-4, 5, 6, 10 and 13 it is known as a type 2 responses (Salgame et al., 1991, Clerici and Shearer, 1994).

The pivot of acquired cellular immune response is the speed with which it is expressed. If for any reasons (either pathogen or host related wise) it is delayed, bacterial growth increases to a point such that expression of a potent protective response would be overwhelmed and rendered ineffective (Cooper, 2009). This aspect of tuberculosis immunopathogenesis was carefully illustrated by Rich (1944) who used the lung histopathology of tuberculosis patients from the period before manufacture of TB drugs (Rich, 1944).

2.7.1 Humans generate Th1 immunity during tuberculosis

Most humans who get tuberculosis are not immunodeficient. This observation is true because tuberculosis infected individuals are not susceptible to other infectious agents (North and Jung, 2004, Furcolow et al., 1942). There is enough evidence to suggest that humans with active tuberculosis develop *M. tuberculosis*-specific immunity. A proof of this phenomenon is given by the fact that humans with active tuberculosis generate and maintain high levels of cellular immunity against *M. tuberculosis* specific antigens (Furcolow et al., 1942, Al Zahrani et al., 2000). This type of immune response is called Delayed Type Hypersensitivity (DTH) (North and Jung, 2004). It is thought that DTH is associated with Th1 response in human tuberculosis. Evidence of Th1 mediated anti-mtb specific immunity has come from presence of $CD4^+$ and $CD8^+$ T cells in the lungs and blood of tuberculosis patient capable of responding to mtb specific antigens in vitro via replication and production of IFN-gamma and other Th1 specific cytokines (Arend et al., 2000, Ulrichs et al., 2000, Lalvani et al., 2001).

The formation of macrophage dominated granulomas at the site of infection is additional proof to the fact that individuals with active tuberculosis generate and maintain a level of immunity against mtb infection (North and Jung, 2004).

The end to the argument as to whether DTH is protective or damaging as it is responsible for pathology ended in the favour of immunity as it was discovered that the histopathological response to mtb infection in the lungs of HIV/AIDS patients were different from that of immunocompetent individuals (Chaisson et al., 1987). It is diffuse, necrotic and unable to repress mtb growth at initial site of infection in AIDS patients suffering from tuberculosis as compared to the macrophage dominated granulomas (North and Jung, 2004) observed in immunocompetent individuals with tuberculosis. Thus tuberculosis in HIV patients can involve multiple organs.

Tuberculosis in humans with excessive mutations in the genes for IFN-gamma receptor ligand-binding chain is severely systemic. The same applies for those with excessive mutations in the genes for the IL-12p40 subunit, and the IL-12 receptor 1 chain (Casanova and Abel, 2002). It goes on to show that Th1 immunity has a protective role in immunocompetent resistant individuals and potent growth restricting influence on *M. tuberculosis* and its induced pathology in immunocompetent susceptible individuals (North and Jung, 2004).

2.7.2 Route and Site of Infection

M. tuberculosis is an intracellular pathogen and thus infect macrophages and dendritic cells (Pfyffer et al., 1998). It is also an obligatory aerobe and has a preference for tissue with high oxygen supply. This explains to a larger extent its preference for lung tissues. *M. tuberculosis* enters the body via the respiratory route in nuclei of aerosols generated by an infected person. The bacteria spread from the initial point of infection in the lung to other parts of the lung or to other part for the body via the blood and/or lymphatics. Extra pulmonary/miliary tuberculosis has been reported in 15% of all tuberculosis cases (Raja, 2004).

2.7.3 Events after *M. tuberculosis* infection

Upon reaching the alveolar, *M. tuberculosis* is phagocytized by alveolar macrophages. This first step in the host-pathogen interaction determines the outcome of infection. Cell mediated immunity develops within 2 to 6 weeks of infection. There is an enormous migration of lymphocytes and macrophages to the site of infection. This leads to the formation of the caseous necrotising granuloma. Mycobacterial growth is restricted. Dead macrophages form a cheese-like caseum. *M. tuberculosis* is walled off and contained in the extra cellular caseous centre. *M. tuberculosis* may remain in the caseous centre indefinitely, be reactivated later on or may increase in number and be discharged via the airways leading to cavitations and

necrosis of the bronchi (Raja, 2004). Should all mechanisms fail, fibrosis is initiated to wall off the infection at the centre of the granuloma (Jayasankar and Ramanathan, 1999).

2.7.4 Antimycobacterial Functions

The interaction of phagocytes mostly macrophages with mycobacterium is an important determinant of infection outcome. The several forms of interaction may include binding of the bacilli to antigen presenting cells, phagolysosomal fusion among others.

2.7.5 Binding of *M. tuberculosis* to Phagocytes

Phagocytes that mediate phagocytosis of *M. tuberculosis* during tuberculosis infection include macrophages, monocytes and dendritic cells. B cells have also been documented to be involved in phagocytosis and antigen presentation (Cooper, 2009).

Binding of *M. tuberculosis* to phagocytes is mediated by cell surface receptor molecules which includes complement receptors (CR1, CR2, CR3 and CR4), Mannose receptors (Schlesinger, 1996), surfactant protein receptors, CD14 receptor (Hoheisel et al., 1995) and scavenger receptors (Gaynor et al., 1995) among others.

Mannose receptors on macrophages interact with lipoarabinomannan (LAM) (Schlesinger, 1996) to mediate its effect. Prostaglandin E2 (PGE2) and interleukin (IL)-4 are documented to up regulate CR and MR whereas interferon-gamma downregulates the receptor expression. This leads to limited ability of mtb to adhere to macrophages (Barnes et al., 1994)

2.7.6 Phagolysosome fusion

The lysosome is a complex organelle of the late endocytic pathway located in the vacuole (Bainton, 1981, De Duve and Wattiaux, 1966, Kornfeld, 1987). The lysosomal vacuole consists of potent hydrolytic enzymes capable of degrading a variety of macromolecules thus its noted antimicrobial properties (Flynn J.L. and Chan J., 2001, Flynn and Chan, 2001a). These enzymes exert their functions optimally at an acidic pH. This explains the observed

acidic pH in lysosomes (Cohn, 1963, Mellman et al., 1986, Raja, 2004). Phagosomes are produced as a result of endocytic pathway initiated by phagocytes on microbes and macromolecules (Flynn and Chan, 2001a). Phagolysosomal formation is the fusion of the phagosome and the lysosome. It is a single dynamic event which occurs with the maturation of phagosome during the process (Desjardins, 1995, Desjardins et al., 1994). Intralysosomal acidic hydrolases subject the phagocytised microbes to degradation after phagolysosomal fusion (Cohn, 1963) in a highly regulated event (Desjardins, 1995, Desjardins et al., 1994). Investigations of the interaction between phagocytes and microbes using isotopically labelled bacteria and macrophage has produced evidence that certain bacteria are degraded extensively within 2 hours after phagocytosis (Cohn, 1963).

The antimicrobial effect of the phagolysosome is accounted for by not just the hydrolytic enzymes but the direct and indirect effects of acidification as well. Nevertheless, there is very little understanding of the mechanism of phagolysosomal fusion and its effector function (Flynn and Chan, 2001a, Downey et al., 1999, Hackam, 1997, Mellman et al., 1986)

2.7.7 Macrophage initiated mechanism of Mycobacterial growth inhibition or killing

There are several mechanisms by which macrophages kill *M. tuberculosis* or inhibit its growth and spread. These mechanisms act in concert to either kill *M. tuberculosis*, inhibits its growth or/and prevent spread to other parts of the body. Lysosomal acidic hydrolases are known to be one of the most significant macrophage antimycobacterial tools employed in killing of *M. tuberculosis* upon phagolysosomal fusion (Raja, 2004).

2.7.8 Reactive Nitrogen Intermediates (RNI)

Most bacteria are killed by activated macrophages. One of the effector pathways is dependent on nitric oxide (NO) (Rook G.A.W. and Hernandez-Pando R., 1996). Thus, inhibitors of NO production has been documented to aggravate tuberculosis infection by some investigators (Chan et al., 1995, Chan et al., 1992).

There is available credible evidence that high production of NO by immunologically activated macrophages is a major antimicrobial mechanism (Chan and Flynn, 1999, Fang, 1997, MacMicking et al., 1997).

When phagocytes are activated by TNF-α and INF-γ they produce NO and related RNI via inducible nitric oxide synthase (iNOS2) (Raja, 2004) and using L-arginine as a substrate (Flynn and Chan, 2001a). The significance of these toxic nitrogen oxides in host defence against mtb in the murine system is well documented (Fang, 1997, MacMicking et al., 1997, Shiloh and Nathan, 2000). Studies have revealed that RNI play a protective role in chronic persistent infection and acute infection in mice (Flynn et al., 1998, MacMicking et al., 1997). There is compelling and accumulating evidence to support the hypothesis that RNI has an antimycobacterial role in human tuberculosis.

There have been immunohistochemical determinations of high levels of NOS2 in macrophages obtained from alveolar lavage of tuberculosis patients (Nicholson et al., 1996, Wang et al., 1998) There have also been documented increased levels of exhaled NO in tuberculosis patients (Wang et al., 1998).

2.7.9 Reactive Oxygen Intermediate (ROI)

Unlike RNI the role of ROI remains quite debatable. This controversy is justified by the fact that there is conflicting evidence over the significance of the produced ROI to host immunity against mtb infection (Flynn and Chan, 2001a). That said, there is more than a handful of work that has provided credible evidence to suggest that Reactive oxygen species (ROS) are in fact very significant to host immune defence against *M. tuberculosis*. The mycobacteriocidal effect of Hydrogen peroxide was earlier demonstrated by (Walker and Lowrie) (1981). Mice deficient in Phox have been documented to be partially challenged in their ability to control *M. tuberculosis* growth in an aerosolized infection model before the onset of specific immunity (Cooper et al., 2000), suggesting a role for ROS in the control of

M. tuberculosis early in the infectious process. Voskuil et al. also demonstrated the bactericidal effect of hydrogen peroxide on mtb in their study to better understand immune evasive mechanisms of *M. tuberculosis*. (Voskuil et al., 2011)

2.7.10 Evasion of Host immune mechanisms by *M. tuberculosis*

Mtb has inherent immune evasive mechanisms by which it is able to evade the host immune mechanism, including its ability to modulate antigen presentation. Thus, evading host immune mechanisms (Raja, 2004).

Armstrong and D'Arcy Hart hypothesised that inhibition of phagolysosomal fusion is a mechanism by which mtb evades host immune response and persist in the infected macrophages (Armstrong and Hart, 1975, Armstrong and Hart, 1971). Using electron microscopy after labelling lysosomes in a study examining the interaction of mtb with mouse macrophages. Viable bacilli were observed in only phagosomes which had not fused with lysosomes. Further evidence of phagolysosomal prevention has been documented by others in support of its use as immune evasive mechanism by *M. tuberculosis* (Russell, 1995, Deretic and Fratti, 1999). The nonfusigenic nature of mycobacterial phagosome is restricted to specific endocytic compartments (Flynn and Chan, 2001a).

Other studies have focused on mtb products that can disrupt phagolysosomal fusion. Some of the identified *M. tuberculosis* products include mycobacterial sulfatides (Goren et al., 1976), lysosomotropic polyanionic glycolipid and derivatives of multicylated trehalose 2-sulfate.

In vitro studies have demonstrated that *M. tuberculosis* generates large amounts of ammonia in culture. It is believed to be responsible for the inhibitory action of culture supernatant of virulent mycobacteria on phagolysosomal fusion (Gordon et al., 1980). This is based on the fact that ammonium chloride affects the saltory movement and raises the pH of the lysosome (Gordon et al., 1980, Hart et al., 1983). Ammonia's ability to alkalinize intracellular vacuoles cannot be attributed to its phagolysosomal fusion inhibition because other known bases

capable of raising intralysosomal pH tends to promote phagolysosomal fusion (Gordon et al., 1980). The requirement of acidification of phagosome for phagolysosomal fusion remains enervated (Mellman et al., 1986, Hackam et al., 1997, Downey et al., 1999). Thus the precise mechanism by which ammonia prevents phagolysosomal fusion remains elusive.

Yet mtb via copious production of ammonia can evade toxic environment and diminish the potency of intralysosomal acidic hydrolases by increasing the pH thereby creating sub optimal conditions (Flynn and Chan, 2001a). Attempts to better understand the relationship between phagolysosomal inhibition and intracellular survival by mtb has led to investigation of 2 mycobacterial enzyme systems related to ammonia metabolism. The mycobacterial urease, which catalyzes the conversion of urea to ammonia and carbon dioxide has been cloned, purified and characterised (Clemens and Horwitz, 1995, Reyrat et al., 1995). Creation of a mutant BCG lacking gene for urease provided vital results that showed that the mutant strain was only slightly compromised in its ability to multiply and persist in the lungs of mice (Reyrat et al., 1996). The significance of mycobacterial urease in the survival of virulent *M. tuberculosis* remains enigmatic.

Glutamine synthetase is an enzyme that has the potential to influence ammonia concentration because of its participation in nitrogen metabolism. It has been studied because of its ability to affect intracellular survival (Harth et al., 1994, Harth and Horwitz, 1999). Immunogold electron microscopy has revealed its release into phagosomes of infected monocytes and consequent abundant release in culture supernatants. Its release is associated with pathogenicity of mycobacteria under study during in vitro culture. Clemens and Horwitz (1995) documented large quantities of glutamine synthetase released by the more virulent *M. tuberculosis* and *M. bovis* as compared to lower quantities released by M. smegmatis and *M. phlei* (Clemens and Horwitz, 1995).

Lipoarabinomannan (LAM) is a glycosylphosphatidylinositol (GPI) that has the ability to modify numerous macrophage functions, including the response to IFN-gamma. LAM has the ability to inhibit antigen presentation (Ilangumaran et al., 1995). LAM and phenolic-glycolipid are known potent oxygen radical scavengers (Chan et al., 1991, Chan et al., 1989).

2.8 INNATE IMMUNITY

In the situation of no prior exposure to mtb phagocytosis and secretion of IL-12 is an important part of the innate immune response. Other components of the innate immune response includes Natural Killer (NK) Cells, Polymorphonuclear Neutrophils (PMN), macrophages (MAC), Dendritic cells (DC), natural resistance associated macrophage protein (Nramp), mast cells ,γ-δ T cells epithelial cells among others.(Raja, 2004, Palomino et al., 2007).

2.8.1 Nramp

Nramp has been documented to transport intracellular nitrite from cytosol to the phagolysosome where it is converted to NO. Defects in the production of Nramp has been noted to increase susceptibility to mycobacteria (Raja, 2004). Nramp 1 mutations have been documented to be responsible for susceptibility to mycobacteria infection among children (Newport et al., 1995).

2.8.2 Neutrophils

Although it was thought that macrophages were the only targets of mtb infection, research has documented a host of others cells which includes neutrophils (Palomino et al., 2007). Increased accumulation of neutrophil in the granuloma and increased chemotaxis has suggested a role for neutrophils (Edwards and Kirkpatrick, 1986). Characteristically, they are among the earliest cells recruited into sites where any pathogen enters into the body and/or inflammatory signals are triggered followed by NK cells, g/d cells and α-β cells. They possess well characterised microbicidal mechanisms (Urban et al., 2006). Elimination of

neutrophils before infection made mouse models more prone to infection and treatment of mice with agents that increases neutrophils led to reduction in bacillary growth rate decreases (Fulton et al., 2002).

There exist controversies surrounding the role of neutrophils. Whereas some investigators report mycobacteria killing by neutrophils (Jones et al., 1990) others could not observe this phenomenon (Denis, 1991).

There is evidence to show that granulocyte-macrophage-colony stimulating factor (GM-CSF) enhances phagocytosis of bacteria by neutrophils (Fleischmann et al., 1986). The production of defensin in human neutrophils which is lacking in macrophage mediated killing has been demonstrated in human studies (Ogata et al., 1992). In the presence of calcium, under in vivo conditions, neutrophils have been documented to kill *M. tuberculosis* (Majeed et al., 1998). It is believed that the function of neutrophils is not limited to their microbicidal ability. Therefore, these cells are thought to contribute to the control of infection through the production of chemokines (Riedel and Kaufmann, 1997), the induction of granuloma formation and the transference of their own microbicidal molecules to infected macrophages (Tan et al., 2006). Other investigators have ascribed pathological roles for neutrophils other than their protective roles (Eruslanov et al., 2005).

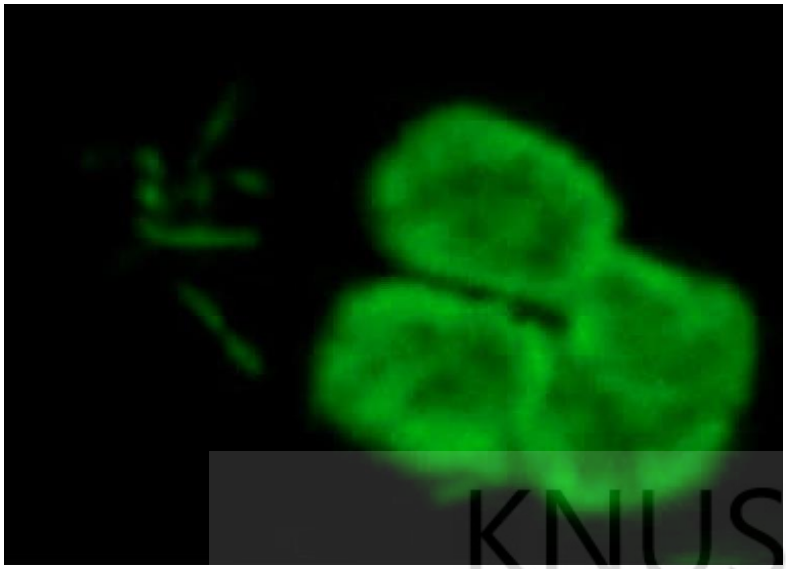


Figure 2 Neutrophils ingest *M. tuberculosis* (Palomino et al., 2007)

2.8.3 Natural Killer (NK) Cells

NK cells are very important to the development of innate immune response to mtb infection. They are among the first sells to produce INF-γ in response to *M. tuberculosis* infection. Their role is also associated with development of cytotoxicity towards targeted cells (Palomino et al., 2007). They are effector cells of the innate immunity. They can either lyse the infected cell or the pathogen itself. Work done culturing NK cells in the presence of live mtb in vitro caused expansion of the cells giving room to the fact that they may be important responders during mtb infection in vivo (Esin et al., 1996). At the onset of infection, NK cells are able to activate phagocytic cells at the site of infection and a significant reduction in NK cells is associated with Multi Drug Resistant Tuberculosis (MDR-TB). Other investigators have however shown that though NK cells are activated during the initials stages of infection, it is not essential for host resistance (Junqueira-Kipnis et al., 2003). NK cells possess the ability to regulate different aspects of the immune response to mtb infection thus its relevance to tuberculosis immunopathogenesis. They have enhanced cytotoxicity towards *M. tuberculosis* infected macrophages (Palomino et al., 2007). They have been documented by some investigators to enhance the ability of $CD8^+$ T cells to lyse *M. tuberculosis* infected

cells and produce INF-γ, thus joining the innate immune response to that of the adaptive immune response (Vankayalapati et al., 2004, Vankayalapati et al., 2002).

2.8.4 CD1d-restricted Natural killer T (NKT) cells

They are a unique set of T cells characterised by the expression of a T cell receptor that recognises the non-classical antigen-presenting molecule CD1d (Palomino et al., 2007). Once activated by alpha-galactosylceramide, the NKT cells do exert their influence in the defence against mtb infection. Upon activation, their activities include INF-γ secretion, proliferation, lytic activity and anti-mycobacterial activity. The antimicrobial molecules used is granulysin, which damages the mycobacterial surface (Palomino et al., 2007).

There exist further proof of the interaction between NKT cells and CD1d expressing cells at the disease site. This has been because CD1d can be easily detected in granulomas of tuberculosis patients (Gansert et al., 2003). However, there is proof to suggest that NKT cells play a detrimental role during the late phase of mouse experimental infection (Sugawara et al., 2002).

2.8.5 Gamma/delta (γ/δ) T-cells

They are large granular lymphocytes cells that can develop dendritic morphology in lymphoid tissues. Some γ/δ T-cells have been known to express the CD8 marker. γ/δ T-cells are non MHC restricted and they are known to function mostly as cytotoxic T cells (Raja, 2004).

There exist data to suggest that γ/δ T-cells play an important role in the host response to tuberculosis in mice and other species with humans inclusive (Izzo and North, 1992). They can be found in the blood of tuberculin positive healthy subjects. They are cytotoxic to monocytes. expressing mycobacterial antigens. They have been documented to secret granuloma forming cytokines (Munk et al., 1990). Other investigators have documented that

there exist more γ/δ T-cells in patients with protective immunity as compared to those with ineffective immunity. A study in childhood tuberculosis has revealed equal levels of T cells expressing γ/δ T-cell receptor and those lacking it, suggesting its pivotal role in early immune response against tuberculosis thus its importance in protective immunity in patients with latent tuberculosis infection (Ladel et al., 1995).

2.8.6 Macrophages (MAC)

They are by far one of the most important cells as far as mtb infection goes (Palomino et al., 2007). Alveolar macrophages are known to be the first cells to interact with mycobacterium tuberculosis. Other macrophages are later recruited from the blood stream. They are in charge of containing the infection in the host (Dannenberg, 1991).

The initial interaction of the tubercle bacilli with the macrophage takes place via cellular receptors such as receptors for Fc, complement (Schlesinger et al., 1990), mannose (Schlesinger, 1993), surfactant protein (Zimmerli et al., 1996), CD14 (Peterson et al., 1995), and CD43 (Randhawa et al., 2005). It is unclear whether the bacteria interacts with one or more receptors during in vivo infections, but in vitro experiments have shown that the nature of MAC response depends on which receptor the bacteria interacts with (Palomino et al., 2007). Their interaction with Fc receptors increases the production of reactive oxygen (ROI) intermediates and permits phagolysosomal fusion (Armstrong and Hart, 1975).

On the other hand, interaction of the bacteria with the complement receptor 3 (CR3) prevents the respiratory burst (Le Cabec et al., 2000) and blocks phagolysosomal fusion (Sturgill-Koszycki et al., 1994).

The cells, antimycobacterial molecules and cytokines act in concert to elucidate the innate immune response against mycobacterial infection. It is however noteworthy that some of the cells of the innate immune response may be involved with adaptive immune response.

Not only has complement receptors been the focus of researchers but toll-like receptors has span the agenda of investigators for some years now. TLR-2 and TLR-4 have been documented to be activated by some components of M. tuberculosis (Brightbill et al., 1999). lipoarabinomanann (LAM) has been shown to activate macrophages through TLR-2. This induces the production of IL-12 and inducible nitric oxide synthase (iNOS) (Brightbill et al., 1999).

There exists cellular cholesterol present in the macrophage cell membrane that is important for the internalisation of the bacteria (Gatfield and Pieters, 2000) regardless of the receptor with which the bacilli interacts. The cellular cholesterol functions by directly anchoring the bacterium and stabilizing its interaction with the macrophage membrane, after which the bacterium is efficiently internalised (Pieters, 2001).

M. tuberculosis generally locates itself in the mycobacterial phagosome upon entry into the macrophage (Palomino et al., 2007). The phagosome originating from the plasma membrane does express some cell surface receptors (Russell, 2007, Hasan et al., 1997). Contrary to the progression of most phagocytosis *M. tuberculosis* blocks the process (Palomino et al., 2007). Figure 2.3 shows an SEM of macrophage engulfing tubercle bacilli.

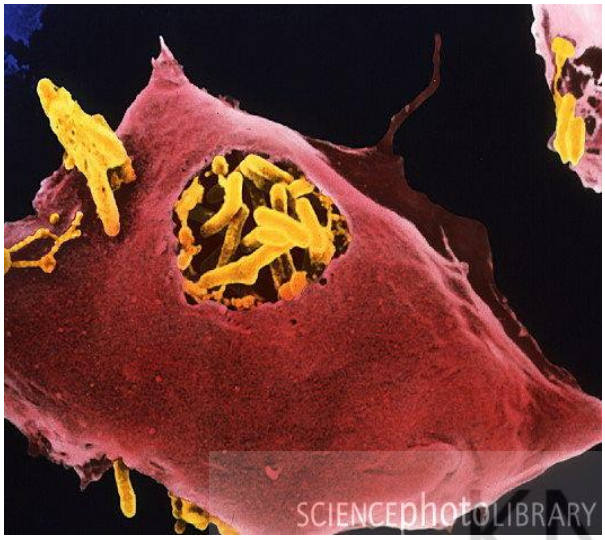


Figure 3 Coloured scanning electron micrograph of a macrophage cell engulfing a bacterium **(**http://www.sciencephoto.com**)**

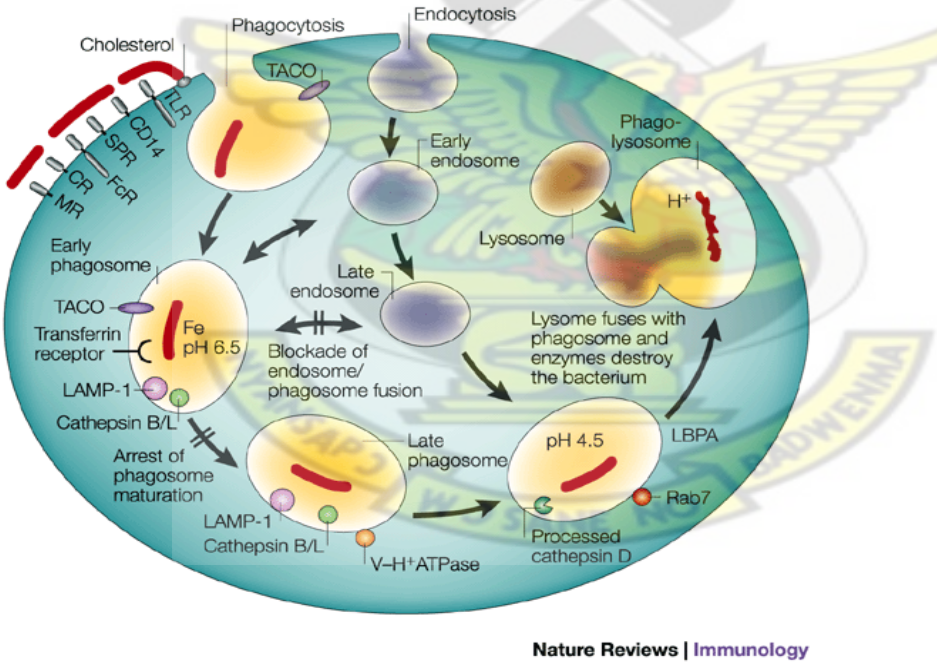


Figure 4 The intracellular lifestyle of Mycobacterium tuberculosis
(Kaufmann, 2001)

2.8.7 Dendritic Cell (DC)

Dendritic cells have been shown to be involved with the protective immunity to tuberculosis. Their high numbers in tuberculosis lesion have been documented (Sturgill-Koszycki et al., 1994, Pedroza-Gonzalez et al., 2004).

Dendritic cells are able to recognize, capture and process antigens. They then present them via Major Histocompatibility complex molecules (MHC) and CD1 (Banchereau and Steinman, 1998, Gumperz and Brenner, 2001). They bind antigens using C-type lectin receptors and Fcγ/Fcε receptors, and internalize them by endocytosis (Engering et al., 1997, Fanger et al., 1996, Jiang et al., 1995). Dendritic cells carry out endocytosis by the use of known C-type lectin receptors, such as dendritic cell-specific intercellular-adhesion-molecule-grabbing non-integrin (DC-SIGN) (Geijtenbeek et al., 2003, Tailleux et al., 2003). This molecule interacts with mannose capped-LAM, a component of the mycobacterial cell wall (Geijtenbeek et al., 2003, Figdor et al., 2002). TLR-2 and TLR-4 are known to be expressed by dendritic cells from peripheral blood and immature dendritic cells of monocyte origin (Jarrossay et al., 2001, Kadowaki et al., 2001). They have been documented to be capable of inducing a protective immune response (Palomino et al., 2007).

After phagocytizing the antigen, dendritic cells go through functional and phenotypical changes leading to their maturation. This is however accompanied by increased synthesis of MHC class I and II, expression of co-stimulatory molecules which includes CD86 and CD80 (Turley et al., 2000) and the production of IL-12 (Steinman, 2001). They efficiently migrate to the peripheral lymph nodes. (Dieu et al., 1998) has provided evidence to support in vivo transport of mtb and BCG from the lung tissue to the lymph nodes inside dendritic cells. The migration of infected dendritic cells is facilitated by chemokines via their receptors on the cell surface of dendritic cells. Chemokine receptor 7 (CCR7) has been known to make DC sensitive to chemokines (CC) CCL19 and CCL21 (Dieu et al., 1998, Gunn et al., 1998,

Kriehuber et al., 2001, Bhatt et al., 2004). DCs have been documented to facilitate protective immunity via induction of T helper 1(Th1) profile by secreting cytokines which includes IL-12, IL-18, IL-23, IFN-α and β, but not IFN-γ (Wozniak et al., 2006, Kadowaki et al., 2001, Kalinski et al., 1999, Thurnher et al., 1997).

Th1 cells have been documented to expand in response to the BCG antigens presented by the DCs in the lymphoid nodules and migrate toward infection sites, where they liberate IFN-γ, thus activating local macrophages that control bacilli replication (Humphreys et al., 2006).

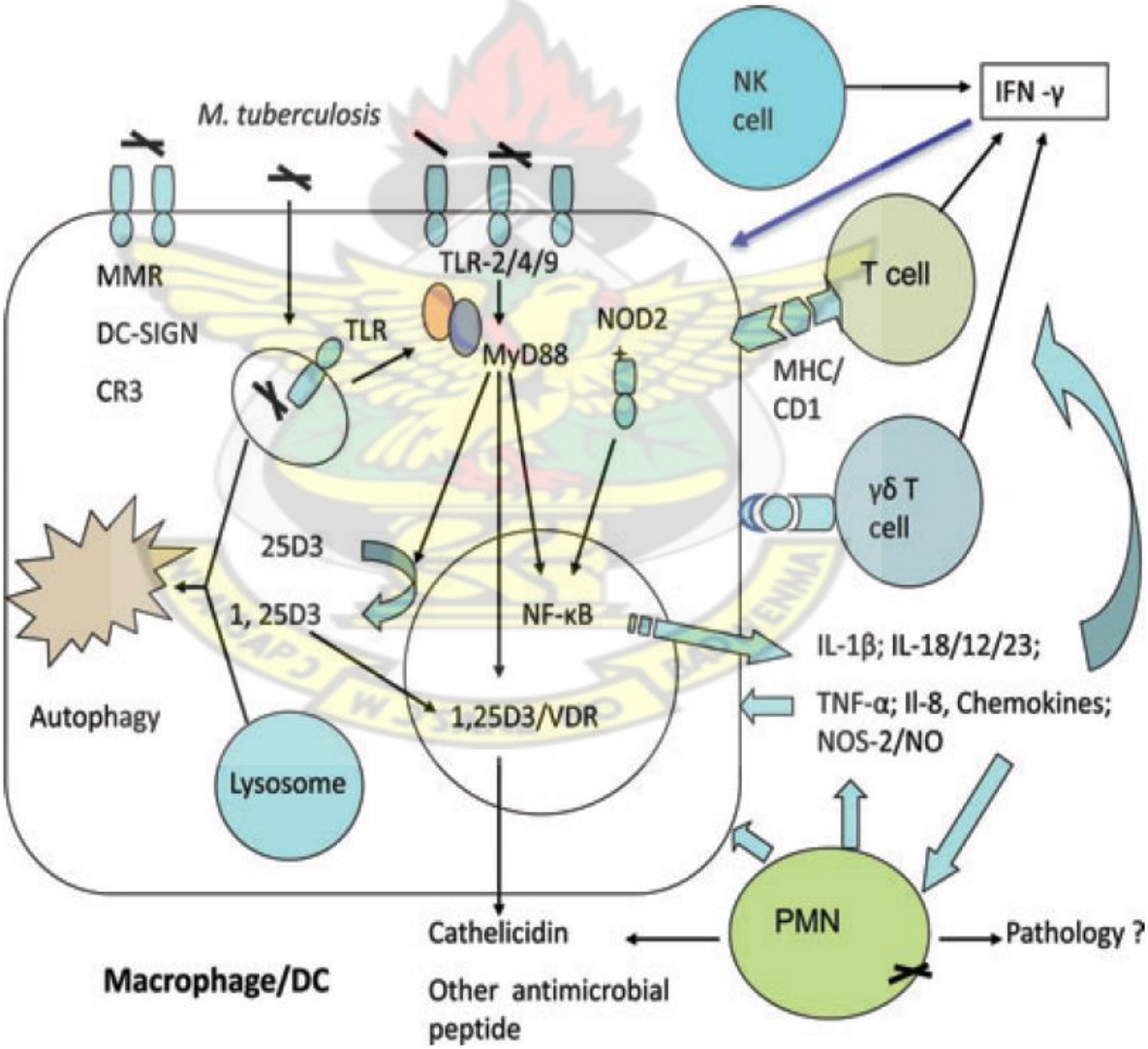


Figure 5 Innate immunity to tuberculosis infection

Innate immunity is an important first line of defence. Effector cells of the innate immune response targets direct killing of *M. tuberculosis* via recognition of non-peptide antigens whiles presenting antigens and releasing cytokines concomitantly to initiate adaptive immune response when need be. Bactericidal substances like perforin and granulysin are used to kill mtb by non-conventional subset of T cells afore mention alongside MAC, DC and PMN.

2.9 ADAPTIVE IMMUNITY

Immune response to *M. tuberculosis* infection is mainly facilitated by Cell Mediated Immune Response (CMI). Mycobacteria-infected macrophages and DC of the innate immunity present antigens to T cells that belong to adaptive immunity.

2.9.1 T cells

Studies over the past few years of anti-*M. tuberculosis* immunity in mice concluded that immunity is mediated predominantly by $CD4^+$ Th1 cells with the aid of $CD8^+$ T cells. These studies have been the subject of some reviews (Boom, 1996, Flynn and Chan, 2001a, Kaufmann, 2001). There has been documented increase in $CD4^+$ and $CD8^+$ T cells in the lung draining lymph nodes a week after exposure with virulent *M. tuberculosis* in mouse models (Feng et al., 1999)(Feng et al., 1999). Activated $CD4^+$ and $CD8^+$ T cells migrate to the site of infection and interact with APCs. The tuberculous granuloma has been documented to contain $CD4^+$ and $CD8^+$ T cells (Randhawa, 1990) that contains the infection and forestalls a potential reactivation should there be any.

2.9.1.1 $CD4^+$ T Cells

These cells are the most important in the protective immunity against tuberculosis. APCs present antigens after processing mtb in phagolysosomes via MHC class II. Murine studies using antibody depletion of $CD4^+$ T cells (Muller et al., 1987), adoptive transfer (Orme and Collins, 1984) or the use of gene knockout mice (Caruso et al., 1999) have demonstrated that $CD4^+$ T cell is prerequisite for controlling mtb infection. In humans, the immunopathogenesis

of HIV infection has provided evidence that loss of $CD4^+$ T cells increases susceptibility to mtb infection (Selwyn et al., 1989). $CD4^+$ Tcells depletion caused rapid re-activation of infection in chronic persistent mtb infection in murine models (Scanga et al., 2000). The primary effector function of $CD4^+$ T cells is the production of cytokines especially IFN-γ, sufficient to activate macrophages. In a study, levels of IFN-γ were severely diminished very early in infection when MHC class II or $CD4^-$ T cells deficient mice were used (Caruso et al., 1999). Apoptosis or lysis of infected cells by $CD4^+$ T cells have been hypothesized to play a role in controlling mtb infection (Oddo et al., 1998).

Apart from the conventional role of $CD4^+$ T cells, other functions have been ascribed these subsets of T cells. They have been documented to help in the development of $CD8^+$ T cell mediated immune response (Scanga et al., 2000, Serbina et al., 2001).

2.9.1.2 $CD8^+$ T Cells

The role of $CD8^+$ T cells in the control of infection is well established (Palomino et al., 2007). Mice unable to develop molecules such as CD8α, transporter associated with antigen Processing (TAP), and perforin are more susceptible to mtb infection that those able to produce these molecules (Flynn et al., 1992, Behar et al., 1999). These cells use cytokine production and bacterial lysis as mechanism of bacterial control (Palomino et al., 2007). $CD8^+$ T cells are capable of producing IFN-γ and IL-4 and hence may play a likely role in the balance between the Th1 and Th2 cells in the site of infection in the lungs of patients infected with pulmonary tuberculosis. $CD8^+$ T cells are able to secrete IFN-γ via activation of T-cell receptor or by interaction with infected dendritic cells (Serbina and Flynn, 1999). Though not much is understood as to how mycobacterial antigens get access to MHC class I molecules, yet macrophages can present mycobacterial antigens to $CD8^+$ T cells 12 hours post infection (Raja, 2004).

$CD8^+$ T cells are documented to be effective in lysing of infected cells thus reducing the intracellular bacilli population (Stenger et al., 1997). The mechanism by which $CD8^+$ T cells are able to reduce mycobacterial burden appears to be associated with granular exocytosis involving perforin and granzymes. Nevertheless, it is granulysin (found in $CD8^+$ T granules) which kills the mycobacterium (Stenger et al., 1998).

2.10 CYTOKINES

Cytokines do regulate all the cells of the immune system. *M. tuberculosis* is intracellular bacilli that induce cell mediated immune response. This is primarily facilitated by the secretion of cytokines. The inflammatory response to *M. tuberculosis* is critical to containing the infection but is also responsible for chronic infection and observed pathology during tuberculosis (Flynn and Chan, 2001a).

2.10.1 Interleukin 12 (IL-12)

Interleukin 12 (IL-12) is a very important and crucial in controlling *M. tuberculosis* infection. Interleukin 12 (IL-12) is secreted following phagocytosis of *M. tuberculosis* by MAC and DC (Ladel et al., 1997, Henderson et al., 1997), this polarises CMI to *M. tuberculosis* infection towards Th1 response with the production of IFN-γ.

Decreased bacterial numbers and increased mean survival time was observed in *M. tuberculosis* infected BALB/c mice when they were given IL-12 early though the mouse eventually succumbed to the infection (Chan et al., 1995, Flynn et al., 1995b). Concrete proof of the role of IL-12 in protection against tuberculosis was given in a study using IL-12p40–gene deficient mice. The mice were susceptible to infection and had a greatly increased bacterial load and decreased mean survival time compared to control mice (Cooper et al., 1997). Humans with mutations in IL-12p40 or the IL-12 receptor genes present with reduced but not necessarily absent IFN-γ production from T cells. They are more susceptible to disseminated BCG and *M. avium* infections (Flynn and Chan, 2001a). A study particularly

proved the role of IL-12 in the immune response against tuberculosis. In the study, it was observed that administration of IL-12 DNA reduced mycobacterial load in mice with a chronic *M. tuberculosis* infection (Lowrie et al., 1999).

2.10.2 Interferon gamma (IFN-γ)

IFN-γ is a key cytokine in controlling *M. tuberculosis* infection. The cytokine is produced by $CD4^+$ and $CD8^+$ T cells (Lyadova et al., 1998, Lalvani et al., 1998, Orme et al., 1992, Orme et al., 1993, Serbina and Flynn, 1999, Barnes et al., 1993) as well as NK cells. Others have also reported IL-12 dependent IFN-γ production by alveolar MACs infected with mycobacteria (Wang et al., 1999, Fenton et al., 1997).

IFN-γ knockout (GKO) mice have been known to be most susceptible to virulent *M. tuberculosis* (Cooper et al., 1993, Flynn et al., 1993). Bacilli grew basically unchecked in the organs of GKO mice, although granulomas formed they quickly became necrotic. Macrophage activation was noted to be defective in these mice and NOS2 expression was low among them (Flynn et al., 1993, Dalton et al., 1993). Persons defective in the gene for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections, including *M. tuberculosis* infections (Ottenhoff et al., 1998). Although IFN-γ production alone is not enough to control mtb infection. Nevertheless it is required for protective immune response to *M. tuberculosis* (Flynn and Chan, 2001a).

Some studies have suggested that IFN-γ levels are depressed in patients with active tuberculosis (Lin et al., 1996, Zhang et al., 1995). (Hirsch et al., 1999) also did provide compelling results to show that *M. tuberculosis* induced IFN-γ production by PBMCs from tuberculosis patients were decreased compared with findings in healthy tuberculin reactors. IFN-γ can be unreliable as an immune correlate of protection because *M. tuberculosis* can prevent macrophages from responding adequately to IFN-γ as demonstrated in a study (Ting et al., 1999). This ability of *M. tuberculosis* to limit activation of macrophages by IFN-γ

suggests that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine (Flynn and Chan, 2001a).

2.10.3 Tumour Necrosis Factor α (TNF- α)

The duality of TNF- α in both tuberculosis protective immune response and pathology has been the subject and has a long standing history in tuberculosis research. *M. tuberculosis* induces the production of TNF- α by dendritic cells, T cells and Macrophages (Ladel et al., 1997, Henderson et al., 1997, Serbina and Flynn, 1999, Barnes et al., 1993). TNF-α is required for the control of *M. tuberculosis* infection via granuloma formation (Raja, 2004, Flynn and Chan, 2001a).

In studies using mouse models, mice lacking TNF-α or the 55kDa TNF receptor rapidly succumbed to *M. tuberculosis* infection with higher mycobacterial burden compared to the control mice (Flynn et al., 1995a, Bean et al., 1999). There also exist possible role in mediating macrophage activation during *M. tuberculosis* infection (Flynn and Chan, 2001a).

TNF-α has been reported by some investigators to synergise with IFN-γ to induce NOS2 expression. Thus activating macrophage bactericidal mechanism leading to macrophage dependent killing of *M. tuberculosis*.

There exists substantial data to validate the importance of this cytokine in granuloma formation in mtb infection and most likely other mycobacterial diseases (Flynn et al., 1995a, Bean et al., 1999, Ehlers et al., 1999, Kindler et al., 1989, Garcia et al., 1997, Raja, 2004, Flynn and Chan, 2001a). NOS2 expressions in granulomas of TNF receptor lacking mice when infected with *M. tuberculosis* was delayed by Flynn and his colleagues in their investigation (Flynn et al., 1995a). Other investigators however did not see similar delays when they did a similar study with TNF-α deficient mice instead (Bean et al., 1999). Granulomatous response is lacking following mtb infection in murine models in the absence of 55-kDa TNF receptor and TNF-α. In the few disorganised granulomas formed, there were

observed impairment in lymphocyte and macrophage co-localisation as well as fewer activated macrophages (Flynn and Chan, 2001a, Flynn et al., 1995a, Bean et al., 1999) . TNF-α affects cell migration to granulomas at the site of infection. It also affects localisation within tissues during mtb infection.

TNF-α induces the expression of chemokines and chemokine receptors in addition to adhesion molecules. This leads to the formation of granulomas within the infected tissues. The mechanism by which TNF-α exerts this influence is yet to be elucidated by researchers (Flynn and Chan, 2001a).

TNF-α is often referred to as the inducer of host-mediated pathology often related to the lungs (Rook et al., 1987, Rook, 1990, Moreira et al., 1997). (Flynn and Chan, 2001b) suggested from a study using mice that, it is the absence of a well organised granuloma leading to diffused infection through the body that accounts for 100% mortality in mice lacking TNF-α or its receptor and not necessarily the increase in bacterial load. Thus TNF-α contributes substantially to modulating inflammatory response and pathology during *M. tuberculosis* infection.

2.10.4 Interleukin 10 (IL-10)

Evidence from human tuberculosis studies have shown that IL-10 is elevated in the lungs (Almeida et al., 2009) and serum (Verbon et al., 1999) of active pulmonary tuberculosis (PTB) patients. Neutralisation of endogenous IL-10 in studies using peripheral blood mononuclear cells (PBMCs) from pulmonary tuberculosis patients have revealed and increase in IFN-γ and T cell proliferation (Zhang et al., 1994).

IL-10 has been shown to be produced by both myeloid and lymphoid cells. Toll like Receptor ligation due to massive influx of pathogen products induces the production of IL-10 (Kaiser et al., 2009, Redford et al., 2011).

IL-10 has also been known to be produced by innate cells like DCs via none TLR related stimuli like C-type lectin receptors (Rogers et al., 2005). Other immune cells capable of producing IL-10 include neutrophils, $CD4^+$ T cells, $CD8^+$ T cells, B cells, mast cells and eosinophils. It is important to note that the pathways differ in these cells (Redford et al., 2011).

Following phagocytosis of *M. tuberculosis* by macrophages, IL-10 production is a consequence of natural antimicrobial response from the host. Pathogen induced immune evasive mechanism can also induce MAC to produce IL-10. IL-10 has been proposed to block phagosome maturation (O'Leary et al., 2010) and IFN-γ induced activation of MAC to initiate mycobacterial killing (Moore et al., 2001) leading to the survival of *M. tuberculosis* in host

In addition to this it has been shown to down regulate major histocompatibility complex molecules during phagocytosis to block antigen presentation (Moore et al., 2001) leading to disease progression. A review by (Redford et al., 2011) concluded that IL-10 was functioning to limit the immune response to mtb and may contribute to tuberculosis pathogenesis. Other cytokines do play important roles in the development of this disease and has been the subject of some studies. This includes Interluekin-6(VanHeyningen et al., 1997), Interluekin-4 (Lin et al., 1996) and transforming growth factor-β (TGF-β) (Hirsch et al., 1997, Toossi et al., 1995, Dahl et al., 1996).

2.11 MICRO RNAS (MIRNAS)

MiRNAs are newly discovered, small non-coding ribonucleic acids that have been documented to regulate host genome expression post transcriptionally. They have been discovered to regulate various biological processes which include immune cell lineage commitment, differentiation, maturation and maintenance of immune function. In line with this, dysregulation of miRNA expression patterns have been associated with several diseases including autoimmune diseases (Dai and Ahmed, 2011).

The discovery of miRNAs changed our understanding about immune regulation and its relevance to diseases.

It was not until 1993 that the first miRNA (lin-4) was identified by Lee and his team (Lee et al., 1993).

It took years after the first discovery to prompt investigations and discovery into the diverse and abundant nature of miRNAs in different species (Lau et al., 2001, Bartel, 2004, Lagos-Quintana et al., 2001, Lee and Ambros, 2001). This was precipitated by the identification of the 2nd miRNA (let-7) (Reinhart et al., 2000).

The development of cutting edge bioassays has made it possible for further development into the discovery, biogenesis and function of miRNA. It was the recognition of aberrant expression and or function of miRNAs in a broad range of human diseases that gave miRNA research widespread attention (Zhang and Farwell, 2008, Zhang et al., 2007, Krutzfeldt and Stoffel, 2006, Eacker et al., 2009, Pauley et al., 2009).

2.11.1 Biogenesis

Most mammalian RNA genes have been identified in the intron region of either protein or non-protein coding transcripts. It is only a small number that has been identified in exon regions of non-coding RNAs (Rodriguez et al., 2004, Kim et al., 2009, Kim and Nam, 2006).

It is therefore noteworthy that some miRNA genes can either be intronic or exonic miRNAs (Rodriguez et al., 2004, Kim et al., 2009).

The last decade has seen an escapade of investigations into miRNA biogenesis pathway and regulation receiving extensive review in some recent publications (Bartel, 2004, Kim et al., 2009, Carthew and Sontheimer, 2009, Winter et al., 2009, Chan and Slack, 2007, Kim, 2005). During the process of biogenesis as shown in figure 2.4 miRNA genes are transcribed by RNA polymerase II into primary miRNA transcripts (pri-miRNA). Most of these transcripts are thousands of nucleotides long, containing various hairpin structures (Kim et al., 2009, Kim, 2005, Lee et al., 2004).

During the canonical miRNA biogenesis pathway, the pri-miRNAs are cleaved in the nucleus by a microprocessor complex. The microprocessor complex is composed of the nuclear RNase III enzyme, Drosha and the double-stranded-RNA-binding protein, DiGeorge syndrome critical region protein 8 (DGCR8). cleaving of the pri-miRNAs by the microprocessor complex forms the precursor miRNA (pre-miRNAs) (Gregory et al., 2004, Lee et al., 2003).

There exists an alternative miRNA biogenesis pathway known as non-canonical miRNA biogenesis pathway (Dai and Ahmed, 2011). In this pathway a different precursor, mirtrons are rather produced irrespective of microprocessor processing (Okamura et al., 2007, Berezikov et al., 2007, Ruby et al., 2007). Branched pre-mirtrons go through lariat-mediated debranching to generate pre-miRNAs after splicing of mirtrons by spliceosome (Chan and Slack, 2007, Ruby et al., 2007, Okamura et al., 2007).

Pre-miRNAs are exported to the cytoplasm from the nucleus by the nucleocytoplasmic shuttle protein Exportin-5.They are then processed by the RNAIII enzyme dicer to yield a 22 nucleotide long miRNA duplex which is imperfectly matched (miRNA/miRNA*). They are then loaded into the Argonaute (Ago) protein to generate RNA-induced silencing complex

(RISC). The guided strand of the miRNA/miRNA* duplex remains in the RISC as a mature miRNA. The complementary strand is however degraded (Kim et al., 2009, Carthew and Sontheimer, 2009). The mature miRNA loaded unto RISC interacts with the 3' UTR of its target messenger RNA (mRNA) to regulate gene expression. The seed region of the miRNA is about 2-8 nucleotides long. It is important for recognition of target (Dai and Ahmed, 2011). The mechanism of miRNA mediated gene regulation (translation repression or miRNA cleavage and degradation) is determined by the degree of complementation between the miRNA seed region and the target mRNA 3' UTR (Bartel, 2004, Carthew and Sontheimer, 2009). MiRNA-regulated genes are mainly subjected to translational inhibition as revealed by High-throughput proteomic analysis (Baek et al., 2008).

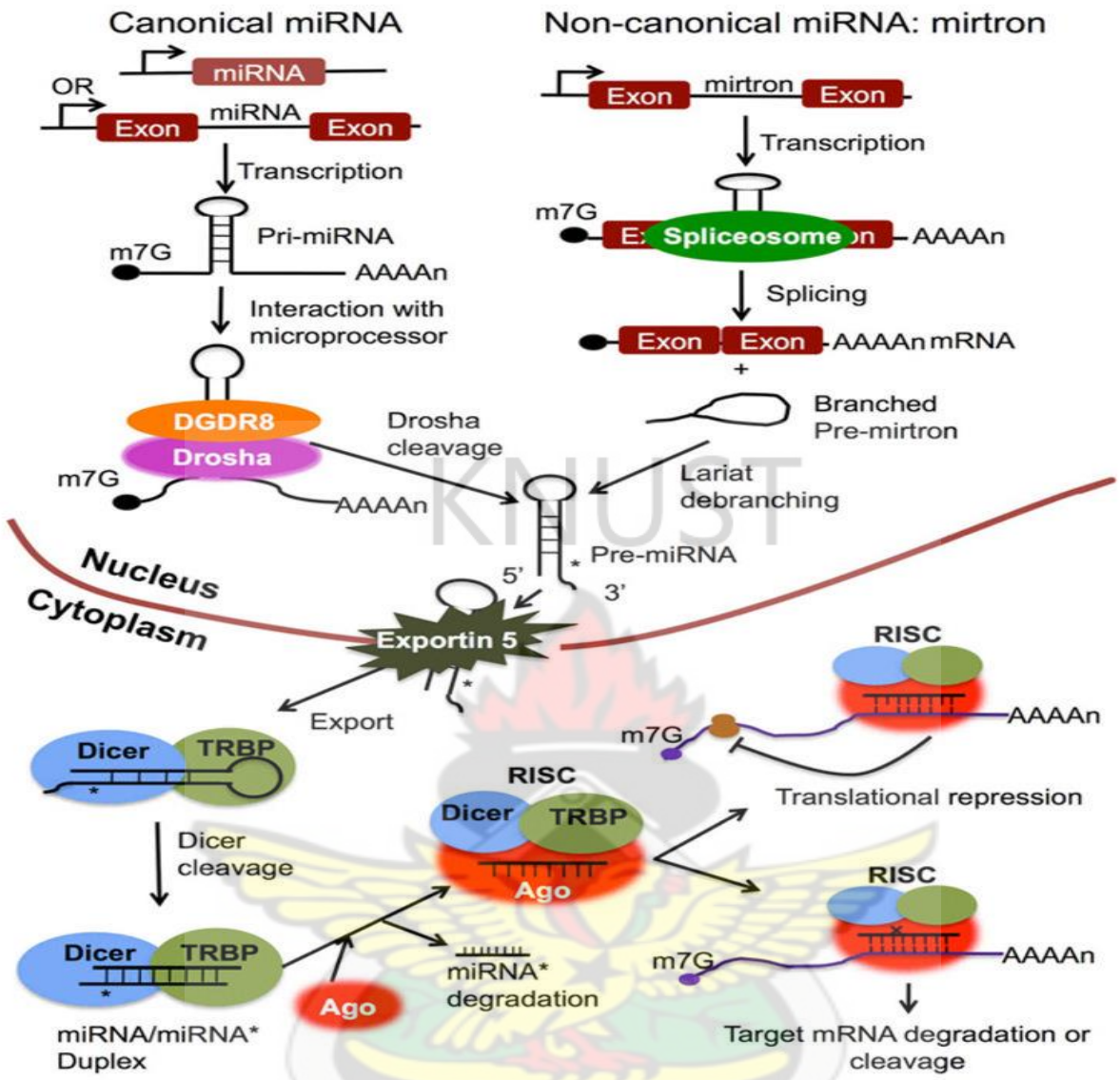


Figure 6 miRNA biogenesis and action in animal cells
(Dai and Ahmed, 2011)

2.11.2 Role of miRNAs in immune system development and function

There has been an unprecedented rise in evidence to support the claim that miRNAs are important for the developed and function of both innate and adaptive sectors of the immune system (Taganov et al., 2007, Xiao and Rajewsky, 2009)

2.11.3 MiRNAs in the regulation of innate immunity

Recent studies have brought to light that, miRNAs in addition to regulating the development of the innate immune cells also fine tunes the innate immune response sometimes via

negative feedback regulation of toll-like receptor signalling (Taganov et al., 2007, Gantier et al., 2007).

miR-223 was highly induced by the transcription factor CCAAT enhancer binding protein α which resulted in enhanced granulocyte differentiation (Fazi et al., 2005, Pulikkan et al., 2010). On the other hand another study with miR-223 knockout mice revealed that miR-223 acted as a negative regulator of granulocyte differentiation by targeting a different transcription factor, myocyte-specific enhancer factor 2C (Johnnidis et al., 2008).

Monocytopoiesis has been documented to be controlled by a circuitry loop which consists of miR-17-5p, miR20a, miR-106a, acute myeloid leukaemia-1(AML-1) and macrophage colony-stimulating factor receptor (M-CSFR) (Fontana et al., 2007). During monocytopoiesis, the expression of miR-17-5p, miR20a, and miR-106a was reduced. On the other hand, their target gene, AML-1, was upregulated to promote the expression of M-CSFR, which is crucial in differentiation and maturation of monocytes and macrophages.

MiR-424 was shown to also enhance monocyte differentiation (Rosa et al., 2007). Other miRNAs involved with toll-like signalling regulation of the innate immune system includes miR-146a (Taganov et al., 2006), miR-155 (O'Connell et al., 2007), miR-21 (Dai and Ahmed, 2011), miR-147(Liu et al., 2009) and miR-9 (Bazzoni et al., 2009).

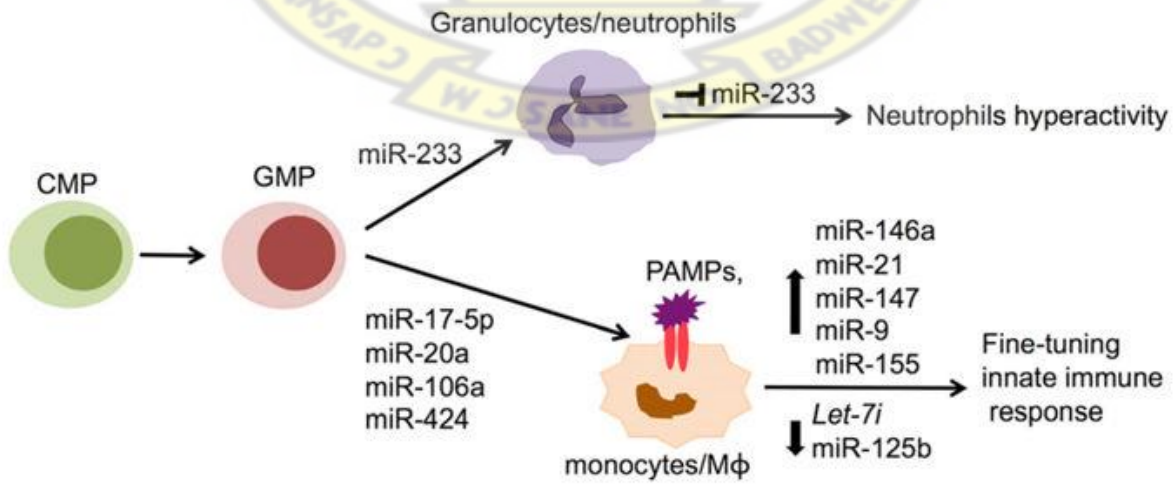


Figure 7 miRNAs in the regulation of innate immunity (Dai and Ahmed, 2011)

2.11.4 MiRNAs in the regulation of adaptive immunity

There have been documented roles of miRNA in the regulation of lymphocyte development and function. MiR-155 is upregulated in T and B lymphocytes upon activation. It is required for lymphocyte homeostasis and normal immune function (Rodriguez et al., 2007, Thai et al., 2007, Vigorito et al., 2007, Dorsett et al., 2008). MiR-155 knockout mice have defective T and B immunity, including abnormal Th1/Th2 differentiation ratio with increased Th2 polarisation and cytokine production, a reduced number of Treg cell, decreased germinal centre response and low numbers of IgG class-switched plasma cells and memory cells (Rodriguez et al., 2007, Thai et al., 2007, Vigorito et al., 2007, Calame, 2007, Kohlhaas et al., 2009).

MiR-181a also plays an important role in the regulation of both T and B cell development (Dai and Ahmed, 2011). Expression of miR181a in hematopoietic progenitor cells in vitro resulted in an increase in B-lineage cells (Chen et al., 2004). This miRNA was found to target negative regulators of TCR signalling and therefore plays an important role in T cell maturation by regulating T cell receptor signalling strength and then tuning T cell sensitivity to positive and negative selection. Inhibiting of miR-181a expression in immature T cells impaired both positive and negative selection during T cell development (Li et al., 2007).

MiR-150 has been documented to be selectively expressed in mature resting T and B cells but not in their progenitors (Monticelli et al., 2005, Xiao et al., 2007). Over expression of miR-150 prematurely resulted in blockage of B cell development (Xiao et al., 2007, Zhou et al., 2007).

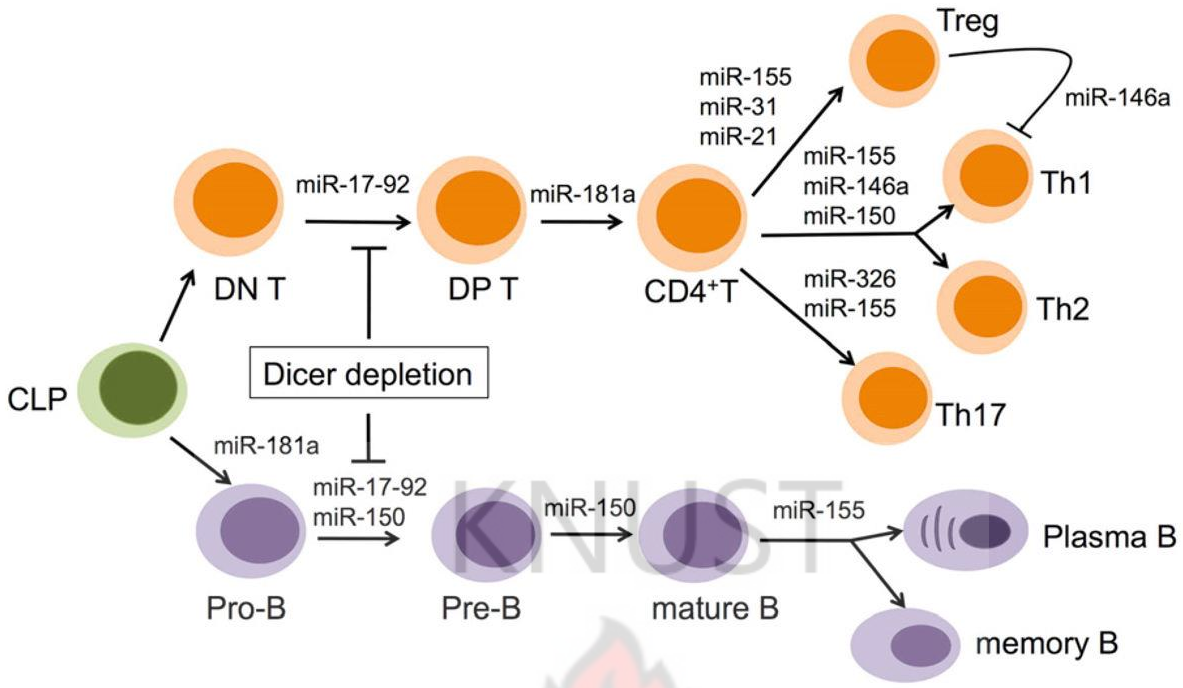


Figure 8 miRNAs in the regulation of adaptive immunity

2.11.5 MiRNAs and bacterial infection

With the background knowledge that miRNAs regulate the expression of a number of genes, the relationship between dysregulation of miRNAs and infectious diseases became the subject of extensive investigation for a number of such diseases (Singh et al., 2013a). Early studies focused mostly on the role of miRNAs in parasitic and viral infections (Ding and Voinnet, 2007, Cullen, 2011, Hakimi and Cannella, 2011). Recently the significance of miRNAs in host bacterial interaction has been demonstrated (Eulalio et al., 2012). It was this leap that generated rise in interest to dissect the relationship between deadly bacterial diseases like tuberculosis and miRNAs. MiR-393 was the first documented miRNA involved in bacterial infection. It was reported to contribute to resistance against extracellular bacteria Pseudomonas syringae in Arabidopsis by repressing auxin signalling (Navarro et al., 2006). *H. pylori* infections have also been documented to alter the expression of miRNAs(De Flora and Bonanni, 2011). Salmonella has also been reported to significantly induce miR-155, miR-146a and miR-21(Schulte et al., 2011). Expression of miR-155, miR132 and miR-146

was induced by lipopolysaccharide from salmonella and *Escherichia coli* (Tili et al., 2007). Significant changes in the miRNA profile of bone marrow derived macrophages have been reported to be induced by *Listeria monocytogenes*. These miRNAs included miR-155, miR-146a, miR-125a-3p/5p and miR-149 (Schnitger et al., 2011).

2.11.6 MiRNAs and Mycobacterial infection

MiRNAs have been discovered in plasma, body fluids and serum in stable forms protected from endogenous Rnase activity. This brings to light their promising potential for use as disease biomarkers and targets for therapeutics (Jackson, 2009). The first clarification concerning the involvement of miRNAs in the pathogenesis of pulmonary tuberculosis was reported in 2011 by Fu and his group in which they investigated the relationship between circulating serum miRNAs and active pulmonary tuberculosis using micro-array based expression profiling method. In this study, 92 miRNAs were significantly detected out of which 59 were down regulated and 33 were upregulated in serum from tuberculosis patients as compared to serum from controls. Upon RT-PCR confirmation of microarray results, miR-29a and miR-93 was found in significantly higher amount in serum and sputum from active pulmonary tuberculosis patients as compared to controls (Fu et al., 2011). Another study identified overexpressed miR-147 and under suppressed miR-19b-2 in sputum of tuberculosis patients as compared to healthy controls (Yi et al., 2012). Sharbati et al. demonstrated in 2011 after infecting macrophages with *M. avium* that several miRNAs were differentially regulated. Many of these differentially regulated miRNAs decreased in expression. Some of which includes miR-20a, miR-191, miR-378 and miR-185. On the other hand other miRNAs were up regulated upon manipulation of this immune cell. These included miR-146a/b, miR-29a and Let-7a. Caspases 3 and 7 were identified as targets for Let-7e and miR-29a after integrated analysis of miRNA and mRNA expression as well as target prediction and reporter assays. This study demonstrated that the inhibition of apoptosis after mycobacterial infection

is controlled by miRNAs (Sharbati et al., 2011). The instrumental role of miR-29 was also demonstrated in murine T cells after infection with Listeria Monocytogenes or Mycobacterium bovis bacillus Calmette-Guerin (BCG). Contrary to the afore mentioned up regulation of miR-29, Ma and co-workers observed down regulation of miR-29 expression in IFN-γ-producing natural killer cells,$CD8^+$ T cells and $CD4^+$ T cells. Contrary to earlier studies they showed that miR-29 directly targets IFN-γ mRNAs and suppresses their production (Ma et al., 2011b). Recently, alteration in differential expression of miRNAs was reported in infected human macrophages in response to high virulent *M. tuberculosis* as compared to less virulent strain, *M smegmatis* (Rajaram et al., 2011). Finally, Kleinsteuber et al. shows for the first time decreased expression of miR-21, miR-26a, miR-29a, and miR-142-3p in $CD4^+$ T Cells and Peripheral Blood from Tuberculosis Patients(Kleinsteuber et al., 2013).

Although the pleiotropic nature and heterogeneous diversity of miRNAs and their function in different cells makes it difficult to elucidate the exact mechanism of their involvement in host pathogen interaction, it has been established empirically that several miRNAs are in no doubt involved in bacterial infections especially mycobacterial infections.

CHAPTER THREE

3.0 METHODOLOGY

3.1 STUDY POPULATION

A total of 32 clinically confirmed tuberculosis patients yet to start chemotherapy and 56 of their exposed but healthy household contacts were consecutively recruited from 3 periurban hospitals (Komfo Anokye Teaching Hospital (KATH), Kumasi South Hospital (KSH) and Kwame Nkrumah University of Science and Technology (KNUST) Hospital). These hospitals are situated in Kumasi, the 2nd largest city in Ghana, West Africa with a population of over 2 million. KATH is the second largest teaching hospital in Ghana. It has a chest clinic that attends to persons living with tuberculosis and has a 24-bed capacity ward that admits patients with severe cases of tuberculosis and for whom medical admission may be necessary at the onset of the disease. It sees as many as approximately 548 new tuberculosis cases per year (2008/2009). Kumasi South Hospital and KNUST Hospital attends to about 60 tuberculosis patients each year. Severe cases from these hospitals are referred to KATH.

3.2 STUDY AREA

Kumasi, otherwise known as the Garden City is the 2nd largest city in Ghana which is on latitude N 06°41.37' and longitude W 001°36.65'. It is located in the southern central part of Ghana in the Ashanti Region. It is about 480km north of the equator and 160km north of the Gulf of Guinea. There are close to 2 million people living in Kumasi.

3.3 STUDY DESIGN

We conducted a nested case control study in prospectively newly recruited cases in three periurban hospitals previously described (see 3.1) between February 2011 and November 2012. There were 3 clearly defined time points where blood samples were taken from patients and controls. Time points 1, 2 and 3 were before antituberculous chemotherapy, a month into antituberculous chemotherapy and 6 months from start of chemotherapy (after treatment) respectively.

A case for this study was defined as a clinically confirmed tuberculosis patient yet to start antituberculous chemotherapy and a control for this study otherwise referred to as latently *M. tuberculosis* infected (LTBI) is a household contact of the tuberculosis patient who lives under the same roof and or spends at least 12 hours with the tuberculosis patient with a positive IFN-γ response to purified protein derivative (PPD) antigen (as described under 3.9.3).

3.4 DIAGNOSIS AT THE HOSPITAL

All individuals who were clinically suspected to have tuberculosis and had been referred to hospital for treatment, underwent *M. tuberculosis* sputum smear test, and for sputum negative cases; laboratory confirmation by *M. tuberculosis* sputum culture. *M. tuberculosis* sputum smear test was routinely performed and was applied independently of their decision to participate in the study and to donate additional samples for research.

3.5 INCLUSION CRITERIA

- Clinically diagnosed and confirmed tuberculosis patients who are yet to start chemotherapy.
- Household contacts exposed to *M. tuberculosis* who spend at least 12 hours with the tuberculosis patient or and live under the same roof with tuberculosis patient.

3.6 EXCLUSION CRITERIA

- Suspected tuberculosis but not clinically confirmed patients.
- Clinically confirmed tuberculosis patients who have already started chemotherapy.
- Persons who do not spend at least 12 hours with tuberculosis patient

3.7 FIELD WORK

Patients reporting to the hospital for the first time upon suspicion of tuberculosis (mostly with at least two weeks of persistent cough) were given a spot test. For smear positive cases, it was confirmed by use of early morning sputum. For smear negative cases, sputum culture was

used for confirmation. Clinical diagnosed and confirmed tuberculosis patients were consecutively enrolled on the study after the study was carefully explained to them and their informed consent sought via thumbprint or and signature. Their blood samples and demographics were taken and they were followed up home to take samples from eligible contacts. Blood was transported by the field team to the laboratory to commence laboratory assays immediately. The laboratory assays were in 6 phases. In phase 1, the peripheral blood mononuclear cells (PBMCs) were isolated from whole blood from tuberculosis patients and controls. Total cell count of PBMCs was then performed. In phase 2, a specified number of PBMCs were restimulated using 6 different antigens for 5 days. In phase 3, the culture supernatant from stimulated cells was used to quantify the amount of IFN-γ produced by T cells. In phase 4, PBMCs from phase 1 were used to enrich $CD4^+$ T cells after which their purity was determined by fluorescent activated cell sorting (FACS) analysis. MiRNAs were extracted from the isolated $CD4^+$ T cells in the 5^{th} phase. Finally, miRNAs' differential expressions were quantified using a singleplex and multiplex uantitative real time-polymerase chain reaction (qRT-PCR) system in the 6^{th} phase. This was repeated for all study participants for the second time point (a month into treatment) and third time point (6 months from start of treatment). For subsequent time points, patients were followed up to their homes where samples were taken.

3.8 SAMPLE COLLECTION

For all study participants (both cases and controls), 30mls peripheral blood were placed into heparinized monovettes (refer to figure 48 in appendix) for immunologic and molecular analyses prior to chemotherapeutic treatment (time point 1), after a month of chemotherapy (time point 2) and after treatment (time point 3).

3.9 LABORATORY ASSAY

3.9.1 PBMC isolation

PBMCs were isolated from whole blood of patients and their contacts to be used for in vitro antigen restimulation.

3.9.1.1 Preparing samples

Monovettes were mildly shaken after which blood was pipetted into a 50ml Falcon tubes. It was then diluted with 1× Dulbecco's phosphate buffered saline (1× DPBS) (GIBCO, USA) at room temperature to the 50ml mark as shown in figure 9.

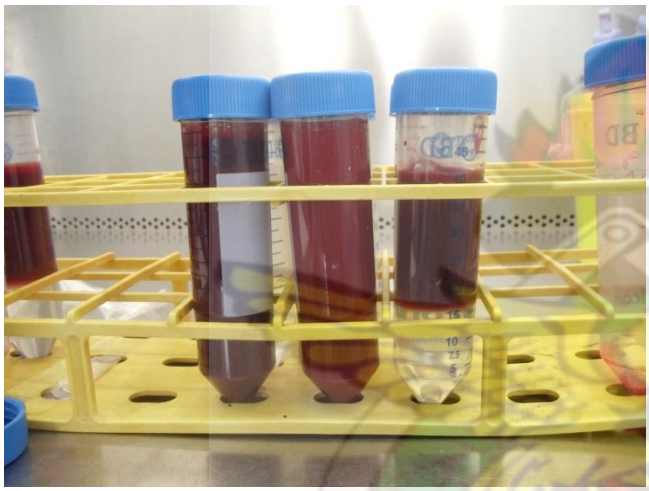


Figure 9 The first 2 tubes on the left contains blood diluted with PBS and the right tube contains biocoll layered with blood.

3.9.1.2 Blood Layering

A 50ml Falcon tube containing 15mls of Biocoll separating Solution (BIOCHROM AG, Germany) was carefully layered with 25mls of blood. This was repeated for each patient sample as each sample after dilution has a total volume of 50mls.

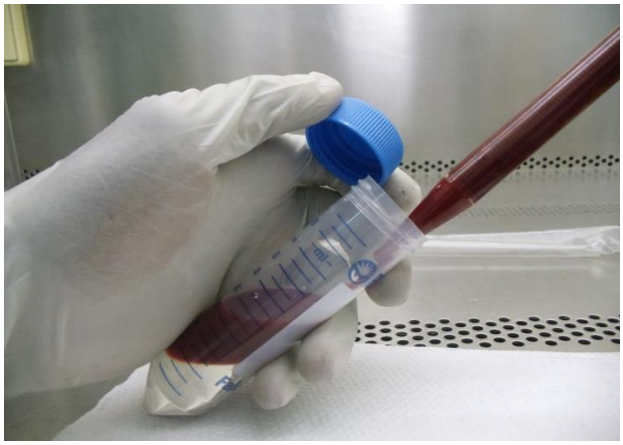


Figure 10 Layering biocoll with blood.

3.9.1.3 PBMC separation

The biocoll layered with blood (2 for each sample) was then centrifuged at 1600rpm for 30 minutes without breaks at room temperature. There were 4 layers formed. The bottom layer contained the pelleted red blood cells and the next on top was the biocoll. After the biocoll was the buffy coat which contained the PBMCs and after the buffy coat was the top layer which was the plasma. The buffy coat was carefully removed from the middle layer with a Pasteur's pipette into a fresh 50mls Falcon tube.

3.9.1.4 1st washing

The isolated PBMC was washed by diluting via a factor of 1:3 dilutions using 1×DPBS (GIBCO, USA) kept on ice. It was then centrifuged at 1200rpm for 20minutes at 4°C.

3.9.1.5 2nd washing

Supernatants were poured off the pelleted cells. The pelleted cells from one tube were re-suspended in 1×DPBS (GIBCO, USA) kept on ice and transferred into the next tube. The contents of the 2nd tube were then resuspended using the 1000µl pipette to ensure cells were adequately mixed in the PBS. The volume was topped to the 10ml mark and centrifuged at 1200rpm for 10 minutes at 4°C. The supernatant was discarded. The cell pellets were resuspended in 1ml 1×DPBS (GIBCO, USA) and kept on ice for counting.

Figure 11 cells pelleted at the base of the falcon tube after 2nd washing.

3.9.2 Cell Counting

Cells were counted to enable the determination of precise quantity to be used for each assay per the protocol's recommendation. Cells were counted using the Neubauer hemacytometer. A well on a 96 well plate was filled with 195µl of trypan blue (GIBCO, USA). After, 5µl of cell suspension was added (dilution factor=40) and resuspended gently using the 5µl pipette. Then, 10µl of the trypan blue and cell suspension was pipetted unto the Neubauer hemacytometer for counting via the Zeiss Axiovert 25 inverted microscope (Carl Zeiss, Germany).

The formula used for computing the total cell count is as follows

Total number of cells= #cells counted × 10^4 × dilution factor × volume of cell suspension

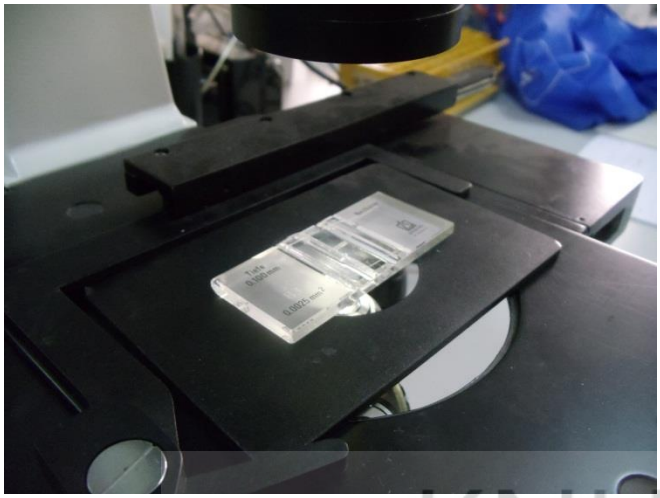


Figure 12 Neubauer hemacytometer loaded with samples for counting on the Zeiss Axiovert 25 inverted microscope

3.9.3 *In vitro* stimulation of PBMC with *M. tuberculosis* specific antigens

In vitro restimulation was used to induce PBMCs to produce INF-γ with the use of tuberculosis specific antigens and controls. In all, 6 antigens were used for the stimulation of PBMCs. Out of these, 4 were positive controls and the remaining 2 were tuberculosis specific antigens. The 2 tuberculosis specific antigens were Purified Protein Derivative tub. (PPDtub) and Early Signs of Antigenic Target 6 KDa (ESAT-6) (Paediatric Infectious Diseases Group, Department of General Paediatrics, Neonatology, and Paediatric Cardiology, Düsseldorf, Germany). The positive controls were Phorbol 12-myrstate 13-acetate (PMA) used together with Ionomycin (Io), Phytohaemagglutinin (PHA) and Staphylococcal Enterotoxin B (SEB) (Paediatric Infectious Diseases Group, Department of General Paediatrics, Neonatology, and Paediatric Cardiology, Düsseldorf, Germany)

For each sample 6 wells were used on a 96 well plate. Each well contained 2×10^5 cells

An aliquot of the PBMC cell suspension equivalent to 1.4×10^6 was pipetted into a 15ml tube containing 1.4mls of X-VIVO media. Each of the 6 labeled wells was filled with 200µl of X-VIVO cell suspension. The first well served as a negative control. The 2nd and 3rd wells were infected with PPDtub and ESAT6 respectively. The 4th well was infected with Io and PMA

whereas the 5th and 6th wells were infected with PHA and SEB respectively. The table below gives the wells, antigens, dilutions and volumes used

Table 1 ANTIGENS AND THEIR VOLUMES USED PER WELL

WELL	ANTIGEN	FINAL CONCENTRATION/WELL
1	UNSTIMULATED	N/A
2	PPDtub	5 µg/ml
3	ESAT-6	5 µg/ml
4	PMA/Io	5 µg/ml each
5	PHA	5 µg/ml
6	SEB	5 µg/ml

The plates containing the cells stimulated with various antigens were then placed in the incubator at 37°C with 5% CO_2 concentration for 5 days.

3.9.6 Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA assay was used to determine antigen specific INF-γ concentration in culture supernatants. The INF-γ concentration in culture supernatants were quantified using the ELISA for Human INF-γ kit (MABTECH, Sweden) with minor modifications to the manufacturer's recommended protocol. Serial dilution of human recombinant INF-γ standard was started from 500ng/ml instead of 1000ng/ml. This was done to eliminate the parabolic nature of standard curve observed. Culture supernatants from wells with tuberculosis specific antigens were diluted in 1:5 ratio whereas culture supernatants from wells with positive controls were diluted in 1:100 ratio. This was done to maintain the INF-γ concentration within detectable range.

3.9.6.1 Antibody Coating

Monoclonal antibody 1-D1K (mAb 1-D1K) coating antibody was diluted to 2µg/ml in 1×PBS using a dilution factor of 1:500. A high protein binding ELISA plate was coated with the antibody by adding 100µl per well. The plate was incubated overnight at 4°C.

3.9.6.2 Blocking of unspecific binding sites

The coating solution was discarded on the second day after which it was washed twice using 1×PBS with 200µl per well. The plate was dried on paper towel by blotting. The unspecific binding sites were then blocked by adding 200µl per well of incubation buffer (1×PBS, 1% Bovine Serum Albumin (BSA), 0.05% Tween 20). It was then incubated at room temperature for an hour.

3.9.6.3 Incubation of Culture supernatant

The plate was washed 5 times with washing buffer (1×PBS, 0.05% Tween 20) using 200µl per well. For the tuberculosis specific antigens a total of 50µl culture supernatant (diluted 1:10 with incubation buffer) was used and for the positive controls a total of 50µl culture supernatants (diluted 1:100 with incubation buffer) was used. The samples were all ran in triplicates. The standard used was diluted 1:1000 in incubation buffer. The starting concentration was 2ng/ml running in duplicates. It was serially diluted by filling 100µl incubation buffer in subsequent wells and by transferring 100µl from well to well. The last 2 wells were used as blanks by adding 100µl of incubation buffer only. It was then incubated at room temperature for 2 hours.

3.9.6.4 Detection

The supernatant was discarded and the plate was washed 5 times using washing buffer by adding 200µl per well. The stock solution of Biotinylated monoclonal antibody 7-B6-1 was

diluted in incubation buffer (1:1000). 100 µl of which was added to each well and incubated at room temperature for an hour.

It was washed 5 times using the washing buffer by adding 200µl per well. Streptavidin-Horse Radish Peroxidase (diluted with incubation buffer at 1:250) was added 100µl per well and incubated for an hour.

The contents were discarded after the hour and washed 5 times with washing buffer by adding 200µl per well. The plate was dried by blotting on paper towel and placed on a white paper background. 100µl per well of substrate solution (for a plate: 12mls of substrate buffer (0.1M phosphate buffer, pH 5.5: 13.8g $NaH_2PO_4 \times H_2O$ in 1L Aqua dist.), 200µl TetraMethylBenzidin (TMB) in Dimethyl sulfoxide (DMSO), 1.2µl H_2O_2) was added. The colour changed from colourless to blue for positive wells after it was allowed to develop for 5minutes.The reaction was stopped by adding 25 µl 2M H_2SO_4 per well. All positive wells changed from blue to yellow

3.9.6.5 Elisa Reading

The Optical density (OD) was measured at 450nm and 570nm using the SUNRISE TECAN ELISA machine (Austria). The actual concentration was computed from the ODS using the graphpad Prism version 5.0.

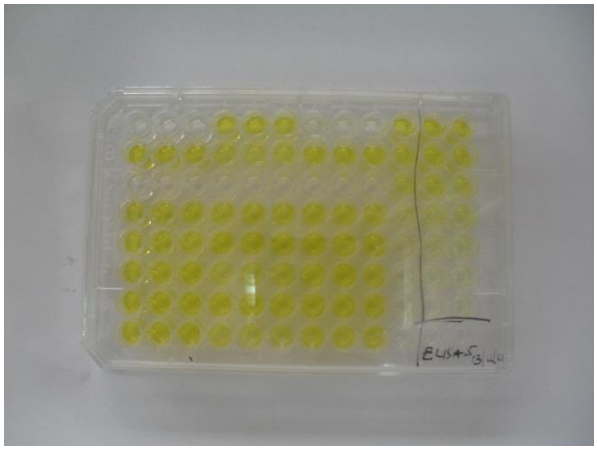


Figure 13 Colour of positive wells changes from blue to yellow upon addition of sulphuric acid just before reading with SUNRISE ELISA machine

3.9.7 MiRNA Isolation

MiRNAs were isolated from the pelleted $CD4^+$ T cells from the $CD4^+$ T cell enrichment assay using the Mirvana miRNA isolation kit (life Technologies, USA).

Cell pellets were palm thawed and 500 µl of Lysis/Binding solution was added to each sample. The resulting solution was thoroughly mixed by vortexing. Then, 50 µl of miRNA homogenate additive was added after which the contents of the eppendorf tubes containing the samples were briefly vortexed and incubated on ice for 10 minutes. After that, 550 µl of Acid Phenol Chloroform was added to each sample and vortexed for 60 seconds. The resulting solution was centrifuged at 12,000 rpm for 5 minutes. Twox200 µl of the aqueous layer was harvested into a second eppindorf tube. Exactly 500 µl of 100% ethanol was added to the harvested layer for each sample and thoroughly mixed with the pipette. A cartridge was placed into the first collection tube after which 700 µl of ethanol suspension was pipetted unto it. It was centrifuged for 60 seconds at 10,000 rpm. The flow through was discarded and the step was repeated using the remaining ethanol suspension. Then, 700 µl of wash solution one was pipetted unto the cartridge after which it was centrifuged for 60 seconds at 10,000 rpm as before. The flow through was discarded and this step was repeated twice using 500 µl wash solution 2/3. The empty tube and cartridge was centrifuged for 90 seconds at 10,000

rpm. The cartridge was placed in the second collection tube after which 100 µl of Ampuwa water of 95°C was incubated for 2 minutes at room temperature. The contents were centrifuged at 12,000 rpm for 60 seconds. At this time the filter cartridge was rather discarded and the ampuwa water containing the miRNAs were transferred into cryotubes and stored in the -80 freezer

3.9.8 RNA Quantification

Extracted RNA was quantified for single assay and plate array for miRNA screening. The isolated RNA sample was palm thawed. Then, 1 µl of the RNA extract of interest was pipetted unto the nanodrop and measured using the RNA measurement option selected on the programme. For each sample measured 500ng was needed for reverse transcription.

3.9.9 TaqMan Reverse Transcription for Plate assay

Taqman Plate array requires cDNA to perform. It is therefore needful to convert the miRNA to cDNA using this assay. The equivalent volume corresponding to 500ng was computed and pipetted from the RNA extract from the sample of interest. Mastermix of the reaction were added in each tube for each sample according to the scheme below. For each sample two tubes were prepared one for primer pool A and the other for primer pool B (Applied Biosystems, Germany). There were 32 targets of interests out of which 3 were housekeeping genes the remainder being miRNAs.

Table 2 SCHEME OF MASTERMIX FOR PRIMER POOL A

Component	**Volume used**
RNase-free H_2O	8.6-xµl
10x RT Buffer	1.5 µl
$MgCl_2$ (25mM)	1.8 µl
100mM dNTPs	0.4 µl
RNase Inhibitor	0.2 µl

Multiscribe RT	1.5 µl
MegaPlex Primer-Pool A	1.0 µl
miRNA	x µl
TOTAL	**15 µl**

Table 3 SCHEME OF MASTERMIX FOR PRIMER POOL B

Component	**Volume used**
RNase-free H_2O	8.6-xµl
10x RT Buffer	1.5 µl
$MgCl_2$ (25mM)	1.8 µl
100mM dNTPs	0.4 µl
RNase Inhibitor	0.2 µl
Multiscribe RT	1.5 µl
MegaPlex Primer-Pool B	1.0 µl
miRNA	x µl
TOTAL	**15 µl**

The two tubes for each sample, one containing primer Pool A and the other primer Pool B are placed in the thermal cycler. The reverse transcription is started using the programme below:

Table 4 PCR PROGRAMME

Time	**Temperature**
30mins	16°C
30mins	42°C
5mins	85°C
Infinity	4°C

The tubes are kept in the refrigerator for the RT-PCR later on.

3.9.10 TaqMan Reverse Transcription for Single assay

RNAs from samples of interest were thawed on ice after which dilutions were prepared to obtain a final concentration of 2ng/ µl.

The mastermix per sample were prepared on ice as follows:

Table 5 SCHEME OF MASTERMIX FOR MIR-29A

Components	**Volume used**
RNase free water	4.16 µl
100mM dNTPs	0.15 µl
10x RT Buffer	1.5 µl
RNase Inhibitor	0.19 µl
Reverse Transcriptase	1 µl
Primer (miR29a)	3 µl

Table 6 SCHEME OF MASTERMIX FOR RNU48

Components	**Volume used**
RNase free water	4.16 µl
100mM dNTPs	0.15 µl
10x RT Buffer	1.5 µl
RNase Inhibitor	0.19 µl
Reverse Transcriptase	1 µl
Primer (RNU48)	3 µl

For each sample one mastermix was prepared for the target of interest and the other for the corresponding housekeeping gene. Then, 10 µl of the mastermix for the target was pipetted into a tube of an 8 strip tube followed by 10 µl of mastermix for the House keeping gene in the next tube for the same sample. Again, 5 µl (prepared dilutions of final concentration 2ng/ µl) of RNA was added to the master mix for target and an equal volume was added to the mastermix for housekeeping gene for the same sample. It was gently mixed by pipetting up and down. The tubes were sealed and transferred to the thermal cycler using the programme earlier on stated for taqman reverse transcription with plate assay (see section 3.9.9).

3.9.11 qRT-PCR for Plate Array

QRT-PCR for Plate Array was used to quantify the differential expression of miRNA targets to determine which of the selected targets were involved in the immune modulation of T cell response to *M. tuberculosis* infection. Real Time PCR was done on the products of reverse

transcription using the TaqMan Universal PCR Mastermis, No AmpErase UNG (Applied Biosystems, Germany) following manufacturer's recommended protocol.

For each sample 2 different master mixes were prepared. One for cDNA (product of reverse transcription) containing primer pool A and the other for cDNA containing primer pool B. It was prepared using the scheme below. The mastermix which contained all reagents for PCR with the exception of cDNA was prepared 10% higher than the number of samples being tested for. So, 5ng of cDNA was placed in each well of the optical 96 well plate used for the RT-PCR.

Table 7 SCHEME FOR RT-PCR MASTERMIX

SAMPLE=	1X(µl)	A(95X)	B(10X)	H_2O(3.5X)
Rnase free water	9.7	921.5	97	40
Taqman 2x MM	10	950	100	35
cDNA(5ng/well)	0.15	14.3	1.5	
TOTAL	**19.85**	**1885.8**	**198.5**	**75**

The pre coated optical 96 well PCR plate was filled with 20 µl/well of the master mix prepared using the scheme shown below:

Table 8 SCHEME FOR PIPPETING UNTO AN OPTICAL 96 WELL PLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	A	A	A	A	A	A	A	A	A	A
B	A	A	A	A	A	A	A	A	A	A	A	A
C	A	A	A	A	A	A	A	A	A	A	A	A
D	A	A	A	A	A	A	A	A	A	A	A	A
E	A	A	A	A	A	A	A	A	A	A	A	A
F	A	A	A	A	A	A	A	A	A	A	A	A
G	A	A	A	A	A	A	A	A	A	A	A	A
H	B	B	B	B	B	B	B	B	B	H_2O	H_2O	H_2O

The optical plate was sealed and centrifuged for 5 minutes at 1200 rpm at 4°C after which it was placed in an Applied Biosystem's 7500 fast RT-PCR system. PCR was started using the programme listed below.

Table 9 PLATE ARRAY PCR PROGRAMME

Time	Temperature	
10mins	95°C	40 cycles
15 secs	95°C	
60secs	60°C	

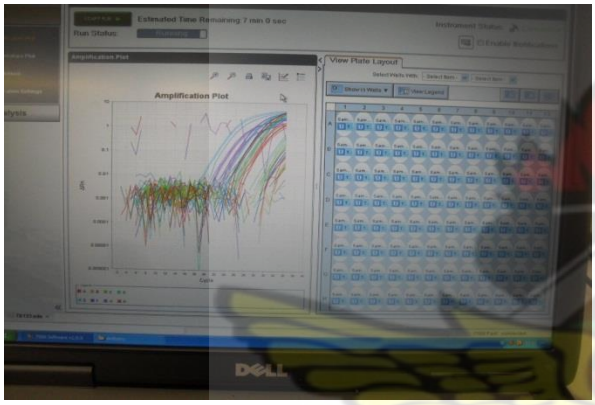


Figure 14 monitor displaying Amplification plot from a ran sample using the 7500 software

Expression of target miRNAs relative to housekeeping gene RNU48 was computed from mean Ct (cycle threshold) values.

3.9.12 qRT-PCR for Single Assay

QRT-PCR for Single Assay was used to determine the differential expression of miR-29a using cDNA earlier prepared. cDNA from single assay reverse transcription for both sample and housekeeping gene of interest were diluted in a 1:5 ratio with RNase free water.

Master Mix for the single assay was prepared for 48 samples using the scheme below. The mastermix which contained all reagents for PCR with the exception of cDNA was prepared 10% higher than the number of samples being tested for to compensate for loss due to pipetting errors

Table 10 SCHEME FOR PREPARING MASTERMIX

SAMPLE	miR-29a		RNU48	
	x1(µl)	**x60(µl)**	**x1(µl)**	**x60(µl)**
Taqman MM	10	600	10	600
Rnase free H_2O	7	420	7	420
Taqman assay	1	60	1	60
TOTAL	**18**	**1080**	**18**	**1080**

For each sample 18 µl from target mastermix was pipetted in a well for miR-29a and the same was repeated for housekeeping gene. This was repeated for all samples being used. Diluted cDNA was mixed thoroughly by vortexing briefly and 2µl was added to the master mix in wells for target and housekeeping gene per each sample. Plate was sealed using an optical adhesive film and centrifuged at 1200 for 5 minutes at 4°C Plate was placed in RT-PCR system (same as used for plate array) and run was started using the same programme as plate array (section 3.9.11).

3.10 STATISTICAL ANALYSIS

Analysis of data from miRNA expression was performed using Microsoft excel. All miRNA targets were ran in triplicates, thus mean Ct values were computed and used. Data were normalised by finding the difference in Ct values between the target miRNA of interest and their house keeping gene for each patient (RNU48 was used as housekeeping gene). The change in Ct values between target and housekeeping gene (ΔCt) was used in a formula where 2 was raised to the power of ΔCt to obtain the fold change of the gene expression with respect to RNU48 (housekeeping gene)($2^{\Delta Ct}$). The resultant values were used in statistical analysis performed below.

Data were analysed using Graph Pad Prism version 5.0 (Graph Pad software San Diego California, USA). Confidence interval was set at 95%. In all statistical tests a *p value* (two

tailed) of <0.05 was considered statistically significant. The D'Agostino & Pearson omnibus normality test was used to test for normality of all groups. Also all graphs shown in this thesis were drawn using this software. Comparisons of two groups were performed using the Mann-Whitney U test. Comparisons of three or more groups were performed using the Kruskal Wallis test. Test for correlation between miR-29a expression and IFN-γ expression was performed using the spearman correlation test.

3.11 DATA HANDLING

All data and information obtained from patients have been anonymised and cannot be linked to the patient in anyway. Names or any identifier have not been used in my thesis, any publication or reports from this study. The principal investigator and co-investigators have access to the data. All samples collected in this study have been given code numbers. For processing of these samples or analysis of any results obtained from these samples, only these code numbers were used. Likewise, all data and information obtained from patients will not appear anywhere with names on it and cannot be linked to the patient in anyway.

3.12 ETHICAL APPROVAL

Ethical approval for this study was obtained from the Committee on Human Research, Publication and Ethics (CHRPE) at the School of Medical Sciences (SMS) at the Kwame Nkrumah University of Science and technology (KNUST) in Kumasi, Ghana. A copy of the approval letter can be found in Appendix O. Additionally all patients gave their informed consent given by signature or thumbprint as and when necessary.

CHAPTER FOUR

4.0 RESULTS

Out of the 32 tuberculosis patients recruited for the study, 24(75%) were males. All cases were new cases diagnosed with pulmonary tuberculosis with a mean age of 36.3. All patients were on the same treatment regime per DOTS protocol. Concomitant diseases included HIV, hernia, dysmenorrhea and hypertension. The details of basic demographics of the tuberculosis patients and LTBIs are summarised in Table 11 below.

Table 11 DEMOGRAPHIC CHARACTERISTICS OF TUBERCULOSIS PATIENTS AND LTBIS

Demographic Characteristics	**Frequencies**
Active Pulmonary TB Group	
Number of Participants	32
Age(years, average; SD)	36.3± 10.58
Sex(male: female)	24:8
BCG vaccination	
Yes	14/32
No	18/32
Therapy	
HRZE	32/32
Classification	
New Case	32/32
Diagnosis	
Pulmonary TB	32/32
Concomitant Disease	
HIV	1/32
Hernia	1/32
Dysmenorrhea	1/32
Hypertension	2/32
None	27/32
LTBIs	
Number of Participants	19
Sex(male: female)	5:14
Age(years, average; range)	35.70 ± 13.76

4.1 DYNAMICS OF INTERFERON-GAMMA RESPONSE

4.1.1 Interferon-gamma response over treatment period

PPD induced IFN-γ response did increase slightly after a month of chemotherapy but remained steady for the remainder of treatment. Comparison of PPD induced IFN-γ response

for all time points (before antituberculous chemotherapy, a month into chemotherapy and 6 months into chemotherapy) did not show significant changes (p= 0.8922) as shown in Figure 15. SEB induced IFN-γ response increased after a month of chemotherapy but declined back to baseline level after chemotherapy (6 months from start of chemotherapy). Comparison of SEB induced IFN-γ response for all time points (before antituberculous chemotherapy, a month into chemotherapy and 6 months into chemotherapy) did not show significant changes (p= 0.8922) as shown in Figure 16.

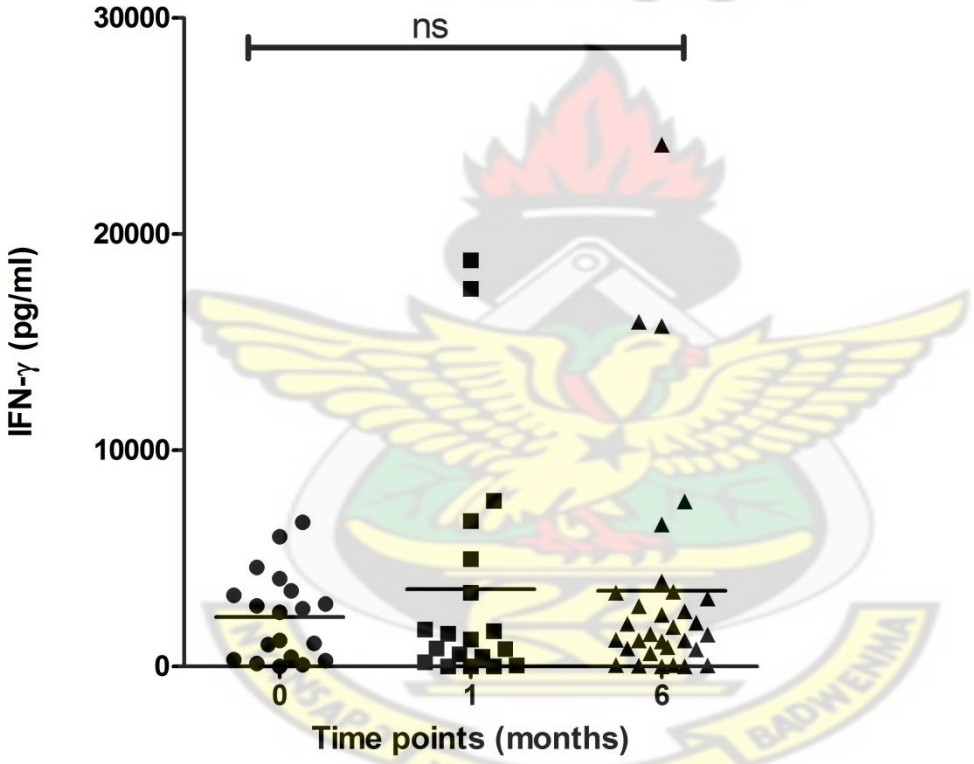


Figure 15 shows PPD induced IFN-γ response of Tuberculosis patients at 3 time points (Time points 0(n=19), 1 (n=19), and 6 (n=31)).

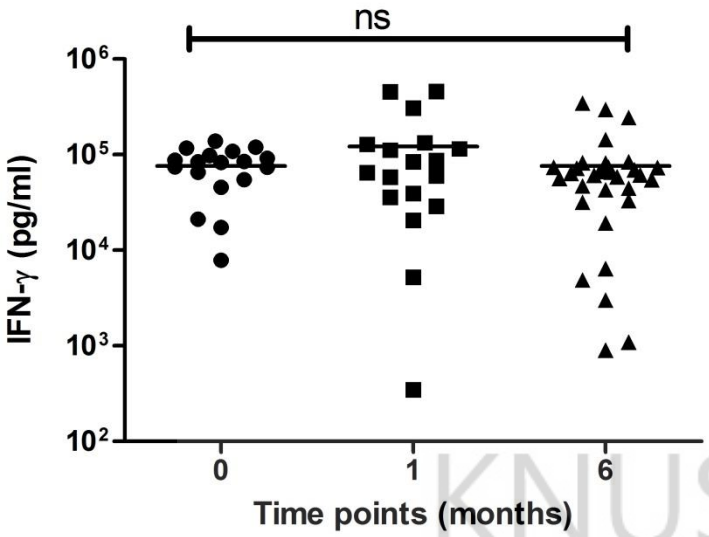


Figure 16 shows SEB induced IFN-γ response of TB patients at 3 time points (time point 1(n=18), 2 (n=18), 3 (n=31))

4.1.2 Interferon-gamma response between patients and their contacts

Comparison of PPD induced IFN-γ response between Tuberculosis patients and Latent Tuberculosis Infected household contacts of the patients did show significantly higher response in latently infected individuals than patients (p= 0.0183) as shown in figure 17. This pattern was similar with SEB induced IFN-γ response (p= 0.0008) as shown in figure 18

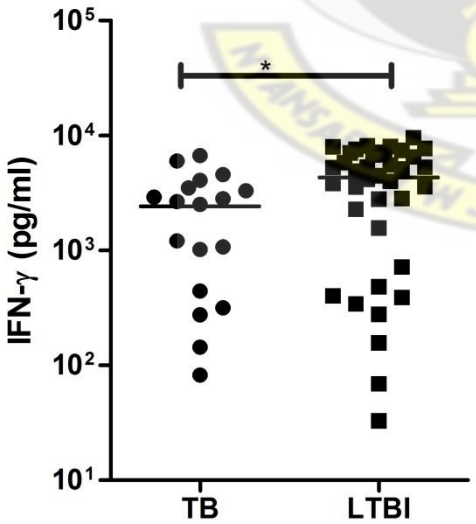


Figure 17 shows PPD induced IFN-γ response between Tuberculosis (TB) patients (n= 18) and Latent Tuberculosis Infected contacts (LTBIs) (n= 38)

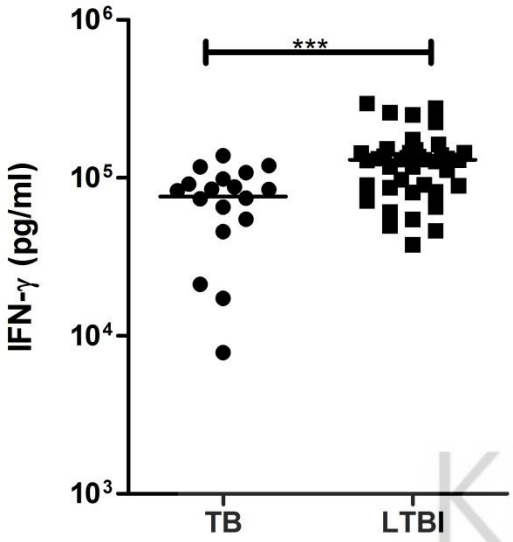


Figure 18 shows SEB induced IFN-γ response between Tuberculosis (TB) patients (n= 18) and Latent Tuberculosis Infected contacts (LTBIs) (n= 40)

4.1.3 Interferon-gamma response between vaccinated and non-vaccinated patients

Although PPD induced IFN-γ response was slightly higher in prior BCG vaccinated patients than none vaccinated ones it was not significant (p= 0.1314) as shown in figure 19. A similar pattern was observed for SEB induced IFN-γ response for the earlier stated groups of patients (p= 0.7333) as shown in figure 20.

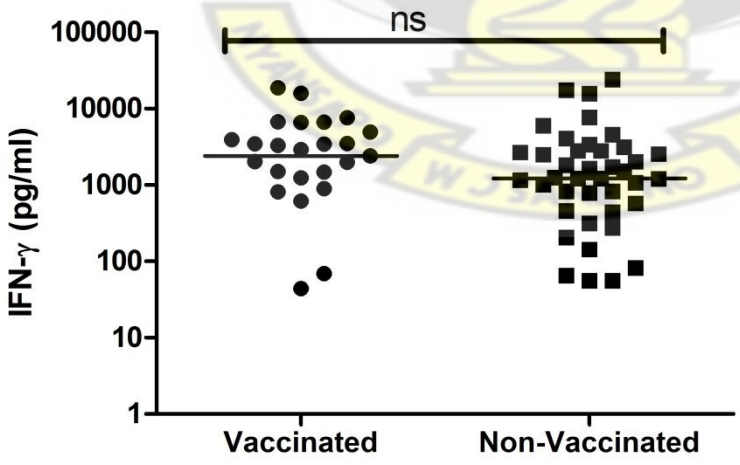


Figure 19 shows PPD induced IFN-γ response between BCG vaccinated Tuberculosis patients (n= 27) and non-vaccinated Tuberculosis patients (n= 43)

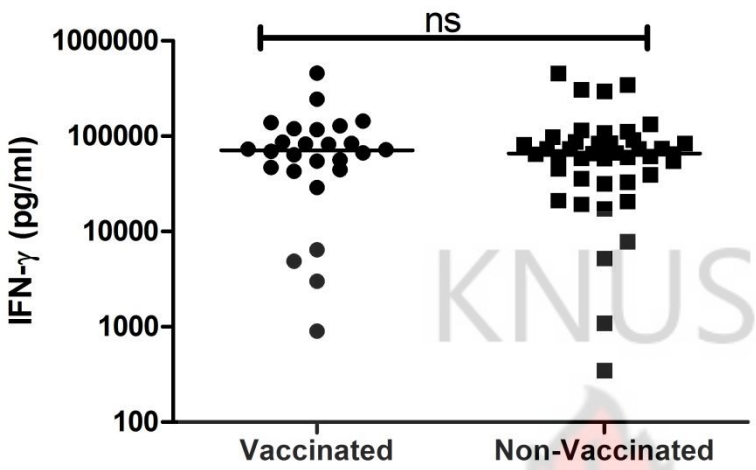


Figure 20 shows SEB induced IFN-γ response between BCG vaccinated Tuberculosis patients (n= 26) and non-vaccinated Tuberculosis patients (n= 42)

4.1.4 Influence of age, sex and concomitant diseases on interferon-gamma response among patients.

Ageing of the patients showed the tendency to repress PPD IFN-γ response but this negative correlation was not significant for responses before antituberculous chemotherapy (SR= -0.07143; p= 0.7782), a month into chemotherapy (SR= -0.3690; p= 0.1596) and 6 months from start of chemotherapy (SR= -0.04420; p= 0.8199). This observation was similar for PPD IFN-γ response for the first (SR= -0.1387; p= 0.5830) and second time points (SR= -0.05694; p= 0.8224) except for the third time point (SR= 0.1727; p= 0.3527) where the observed tendency deviated, yet these observations at all the time points were statistically insignificant (see figure 21).

There was a slightly higher median IFN-γ response in males than females for PPD (p= 0.1175) and SEB (p= 0.1215) induced responses but they were not statistically significant (see figure 22).

Tuberculosis patients without any known concomitant disease did show slightly higher PPD induced IFN-γ response than Tuberculosis patients with known concomitant disease. This difference was however not significant (p= 0.3397). There was observed similar trend for SEB induced IFN-γ response (p= 0.2863) (see figure 23)

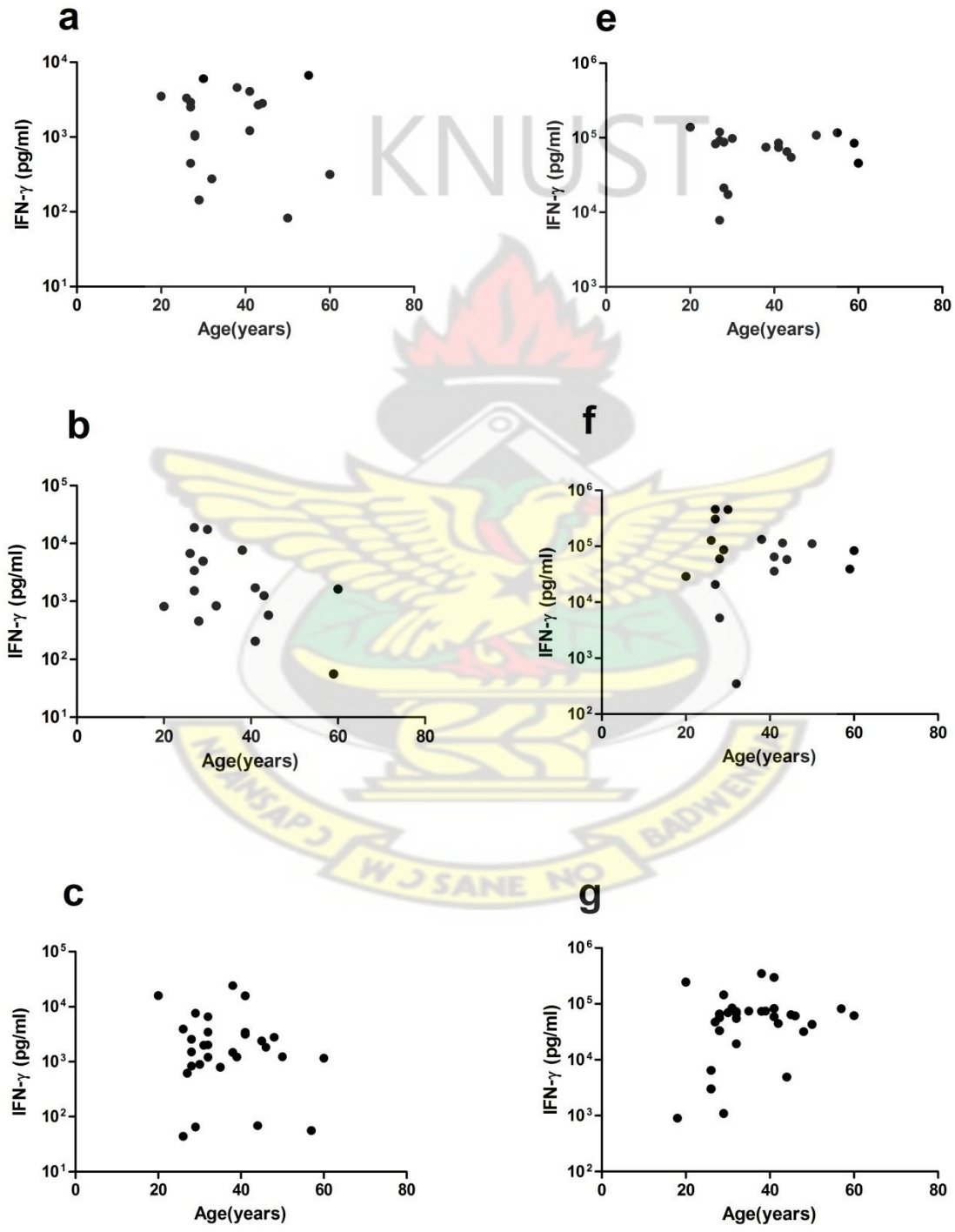


Figure 21 Correlations between age of patients and PPD induced IFN-γ responses are shown on the left for time point 0 (a) (n=18), time point 1(b) (n=16) and time point 6(c) (n=29). On the right of the graph are Correlations between age of patients and SEB induced IFN-γ responses for time point 0 (e) (n=18), time point 1(f) (n=18) and time point 6(g) (n=31)

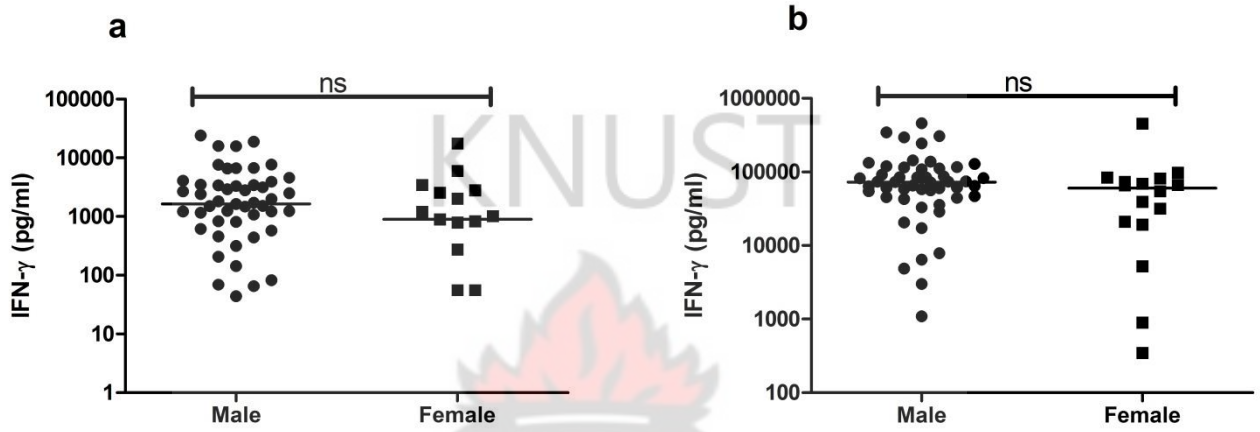


Figure 22 (a) shows PPD induced IFN-γ responses between male (n=51) and female (n=17) patients (b) shows SEB induced IFN-γ responses between male (n=50) and female (n=16) patients

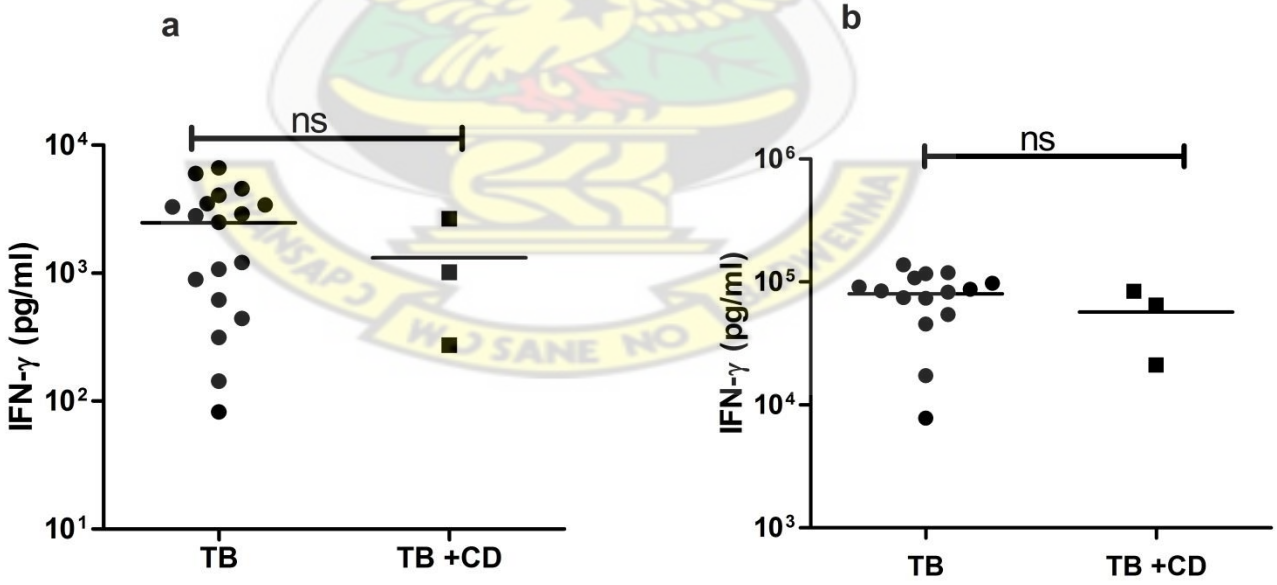


Figure 23 (a) shows PPD induced IFN-γ responses between Tuberculosis patients (TB) (n=18) and Tuberculosis patients with concomitant disease (TB+ CD) (n=3) (b) shows SEB induced IFN-γ responses between Tuberculosis patients (TB) (n=18) and Tuberculosis patients with concomitant disease (TB+ CD) (n=3)

4.2 CANDIDATE MIRNA SCREENING

29 preselected immune related miRNAs were screened in Tuberculosis patients and LTBIs. Out of these miRNAs, 9 were not detectable in more than 50% of the samples using the taqman assay thus could not be included in the analysis. Out of the 20 detectable miRNAs 7 miRNAs' differential expression were altered significantly during antituberculous chemotherapy. A summary of the miRNAs and successful candidates are shown in figure 24. The five patients consecutively used throughout in the analysis were used because of available data for all three time points. A to E refers to the same set of patients as used throughout results.

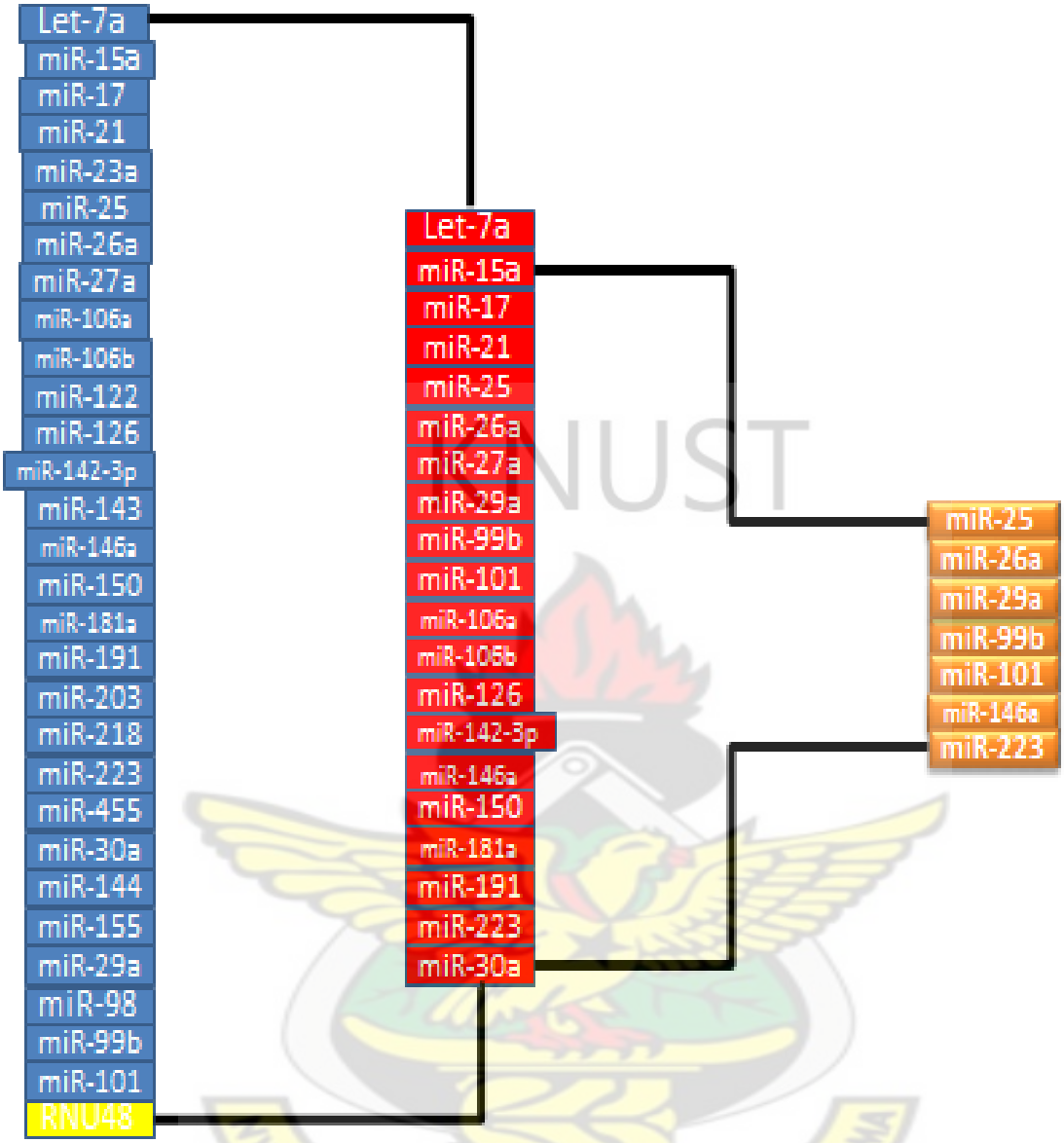


Figure 24 shows preselected candidate miRNAs and successful candidates. Blue tabs on the left shows all 29 candidates, yellow tab shows the endogenous housekeeping gene used, middle red tabs shows detectable miRNAs and orange right tabs show significantly altered candidates.

4.2.1 miR-25

MiR-25 differential expression increased steadily a month into chemotherapy through to the end of treatment (as shown in figure 25). Although it increased significantly (p= 0.0270) throughout treatment there were no clearly observed trends for individual responses from patients (see figure 26).

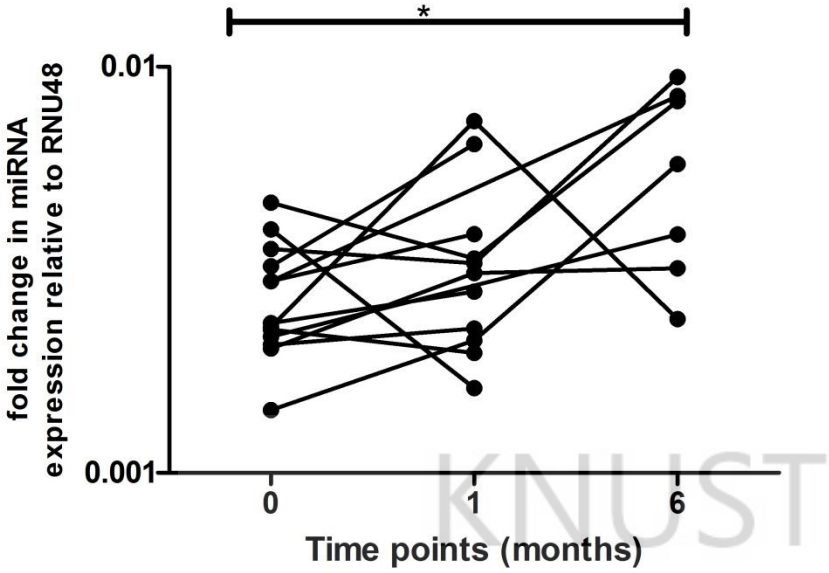


Figure 25 Shows differential expression of miR-25 during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).

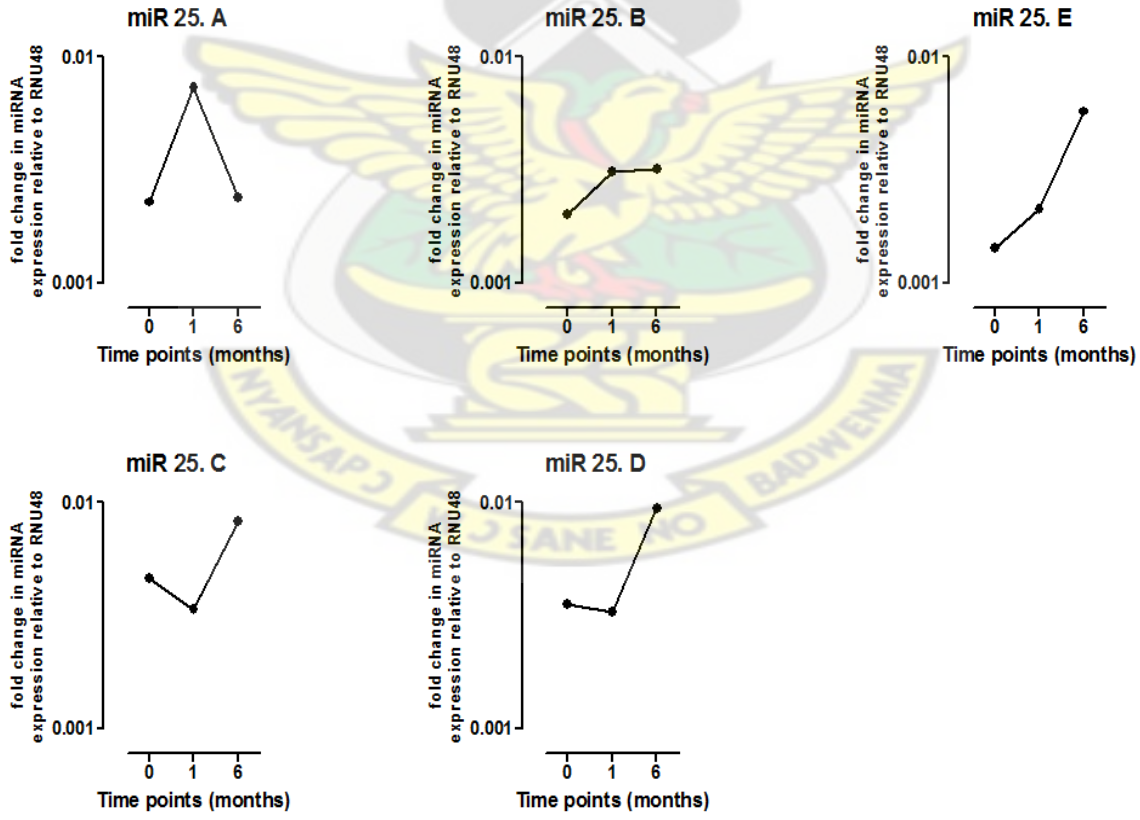


Figure 26 shows differential expression pattern of miR-25 for 5 different patients during antituberculous chemotherapy indicated by letters A-E.

4.2.2 miR-26a

MiR-26a differential expression increased steadily a month into chemotherapy through to the end of treatment (as shown in figure 27). Although it increased significantly (p= 0.0248) throughout treatment there were no clearly observed trends for individual responses from patients (see figure 28).

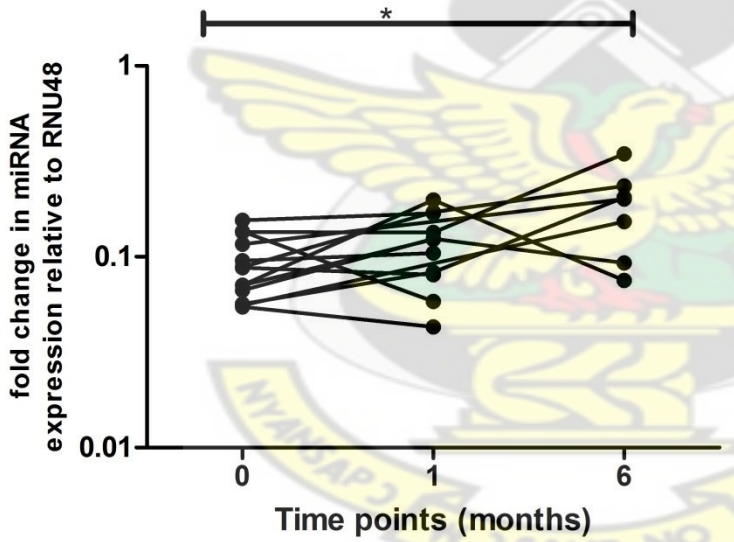


Figure 27 Shows differential expression of miR-26a during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).

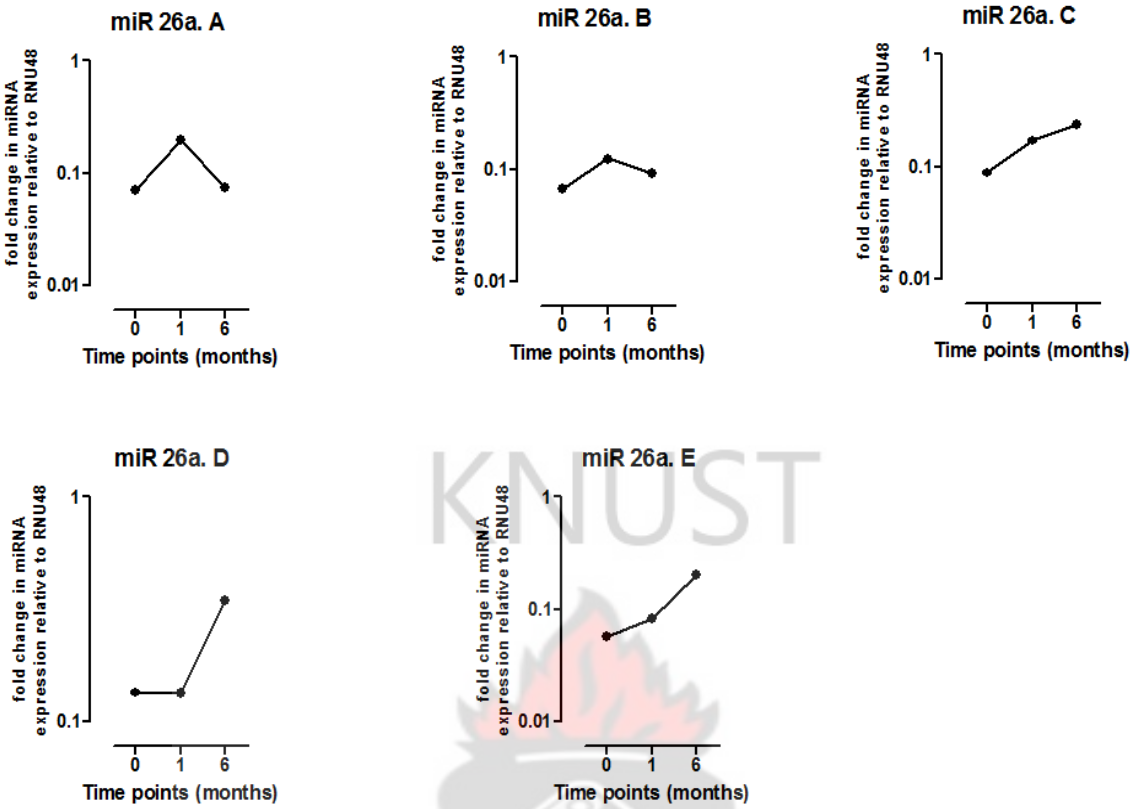


Figure 28 shows differential expression pattern of miR-26a for 5 different patients during antituberculous chemotherapy indicated by letters A-E.

4.2.3 miR-29a

MiR-29a differential expression increased steadily a month into chemotherapy but decreased then after at end of treatment (as shown in figure 29). Although its differential expression was significantly altered (p= 0.0205) throughout treatment there were no clearly observed trends for individual responses from patients (see figure 30).

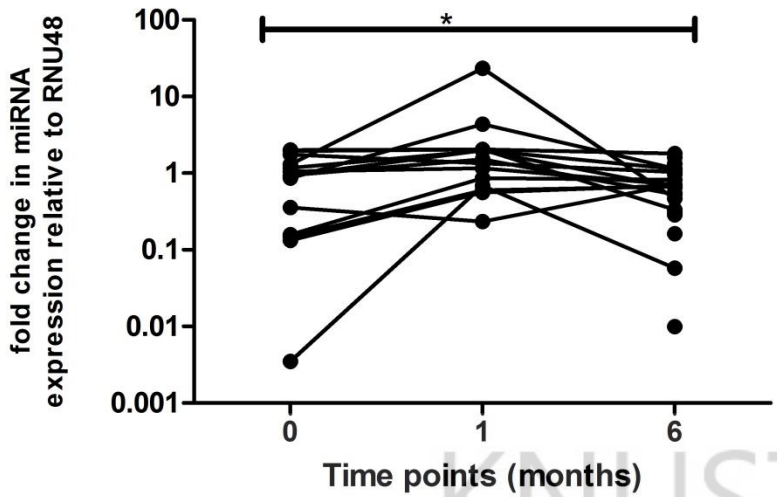


Figure 29 Shows differential expression of miR-29a during antituberculous chemotherapy. Time point 0 (n=14), time point 1 (n=13) and time point 6 (n=25).

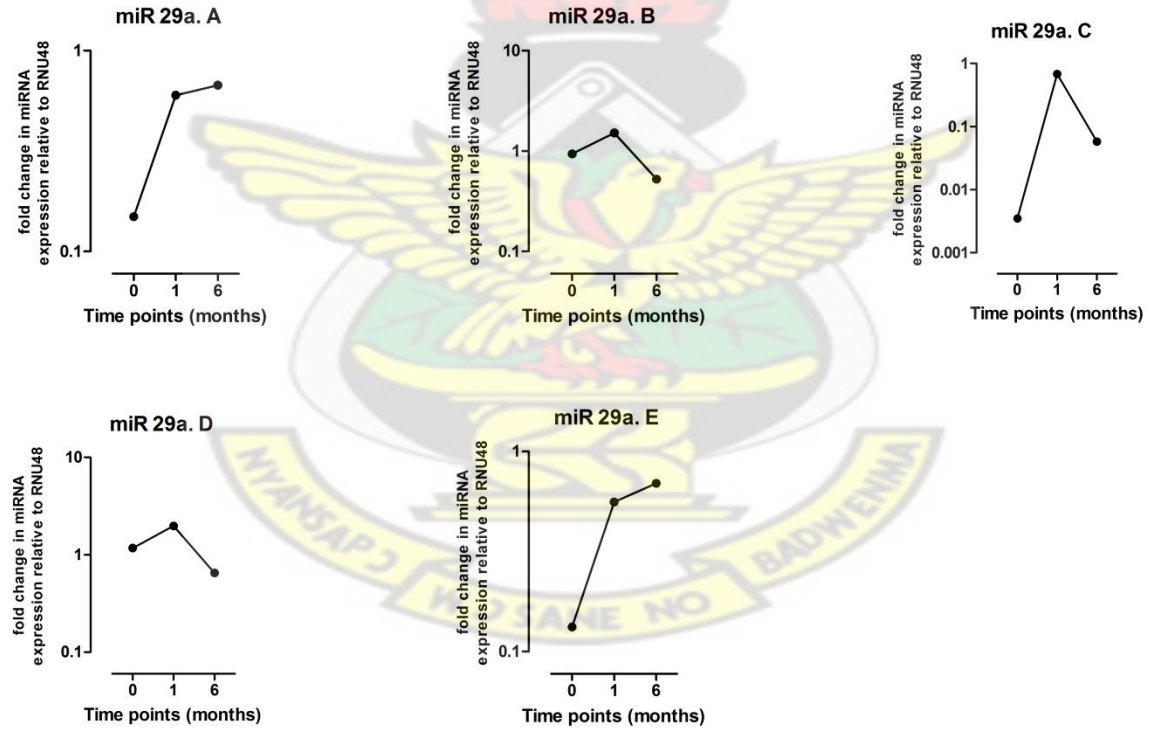


Figure 30 shows differential expression pattern of miR-29a for 5 different patients during antituberculous chemotherapy indicated by letters A-E.

4.2.4 miR-99b

MiR-99b differential expression increased steadily a month into chemotherapy through to the end of treatment (as shown in figure 31). Although it increased significantly (p= 0.0027)

throughout treatment there were no clearly observed trends for individual responses from patients (see figure 32).

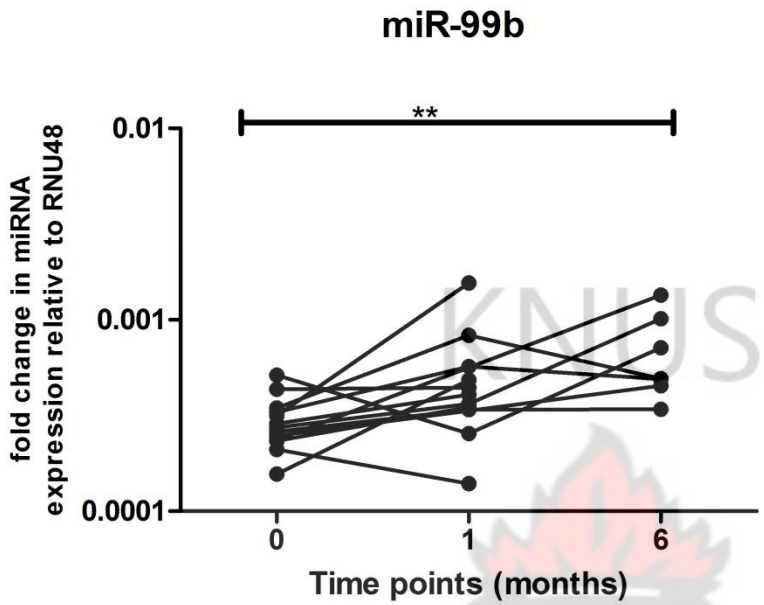


Figure 31 Shows differential expression of miR-99b during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).

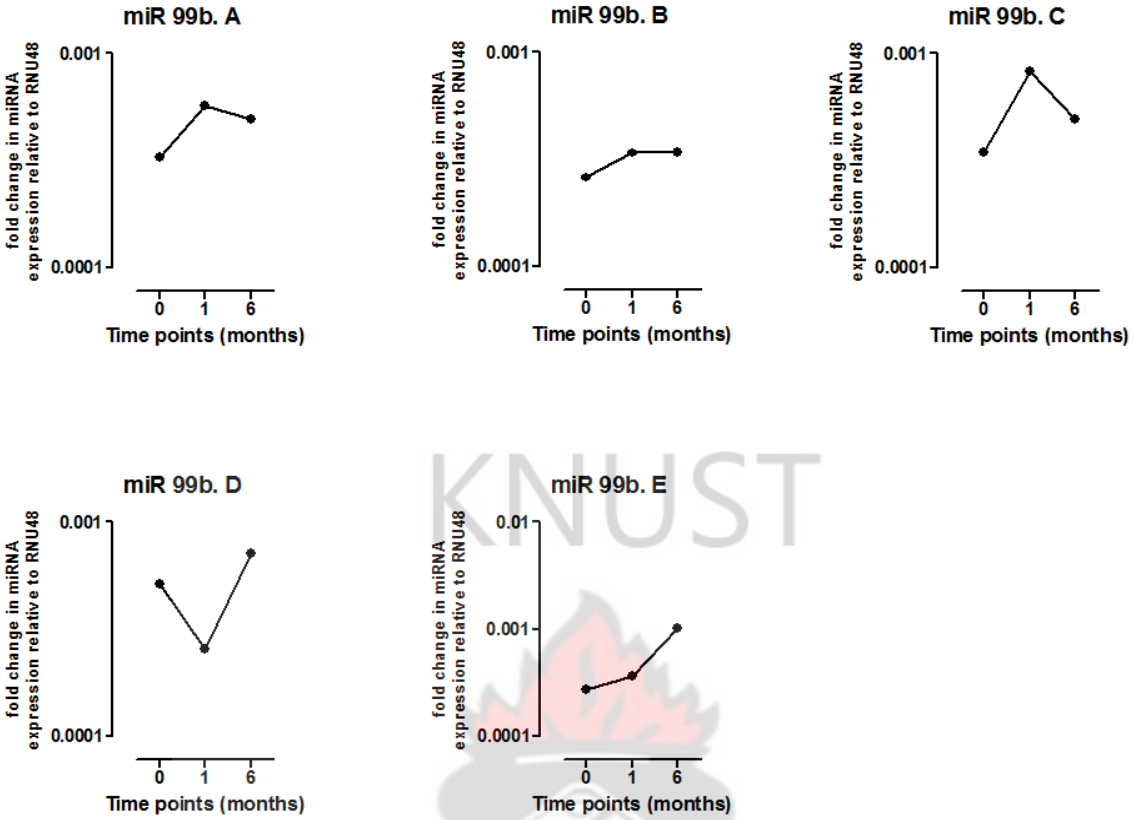


Figure 32 shows differential expression pattern of miR-99b for 5 different patients during antituberculous chemotherapy indicated by letters A-E.

4.2.5 miR-101

MiR-101 differential expression increased steadily a month into chemotherapy through to the end of treatment (as shown in figure 33). Although it increased significantly (p= 0.0227) throughout treatment there were no clearly observed trends for individual responses from patients (see figure 34).

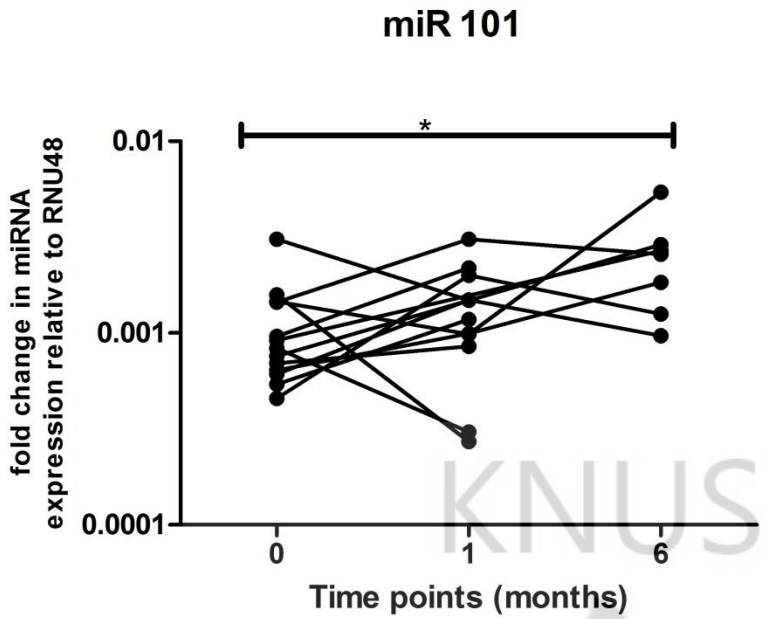


Figure 33 Shows differential expression of miR-101 during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).

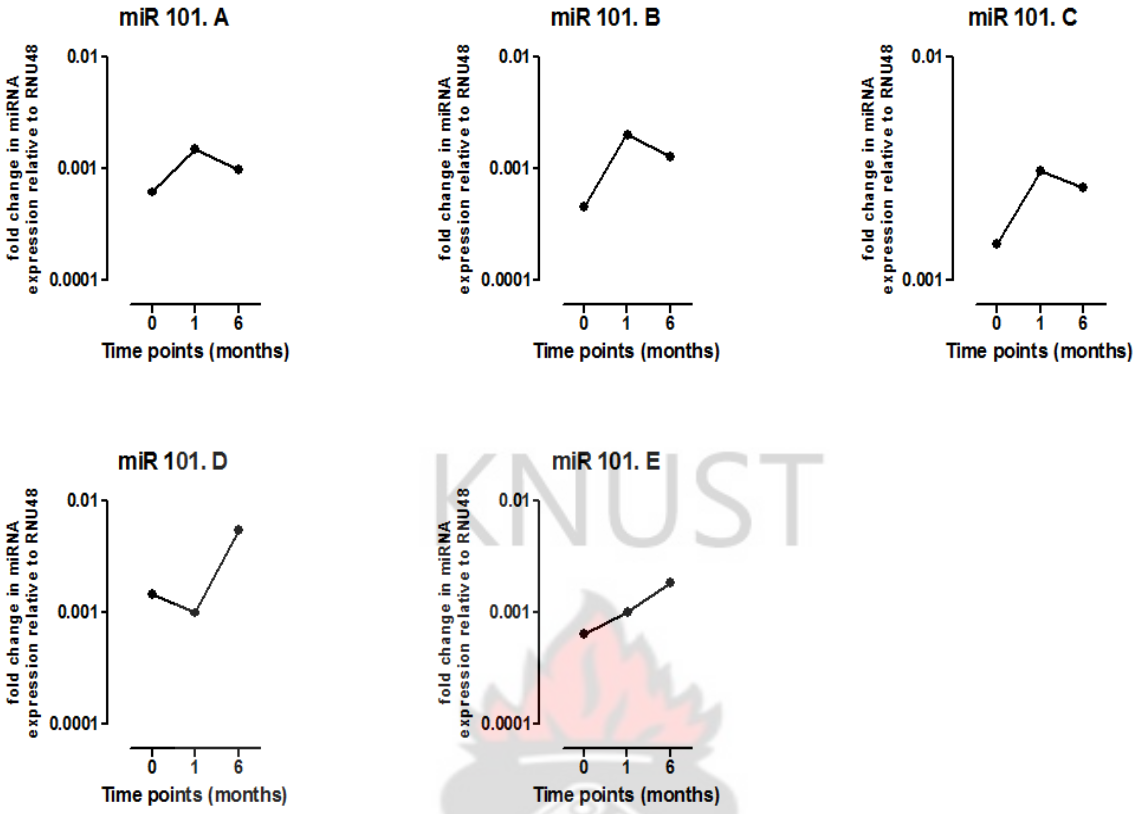


Figure 34 shows differential expression pattern of miR-101 for 5 different patients during antituberculous chemotherapy indicated by letters A-E.

4.2.6 miR-146a

MiR-146a differential expression increased steadily a month into chemotherapy through to the end of treatment (as shown in figure 35). Although it increased significantly (p= 0.0375) throughout treatment there were no clearly observed trends for individual responses from patients (see figure 36).

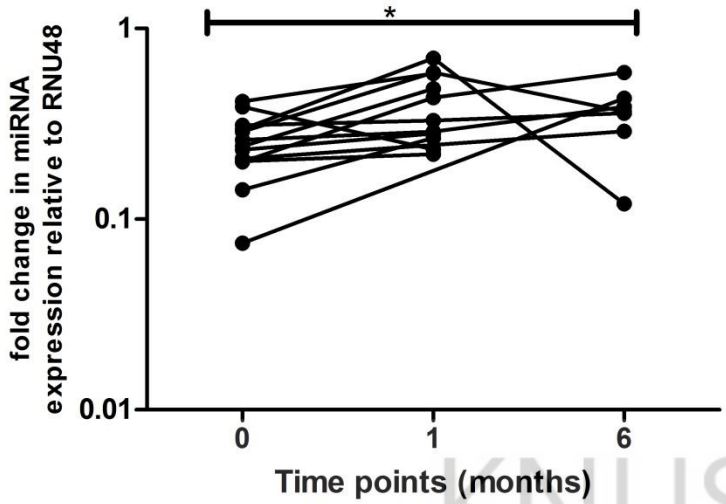


Figure 35 Shows differential expression of miR-146a during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).

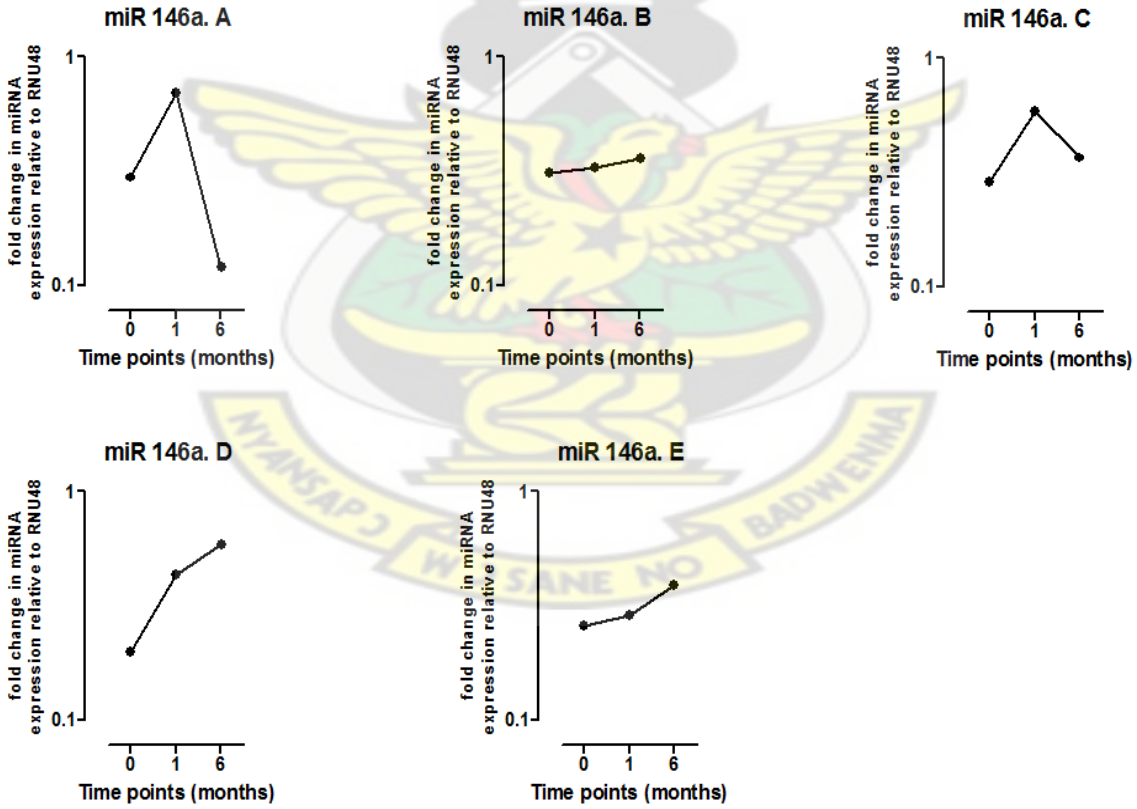


Figure 36 shows differential expression pattern of miR-146a for 5 different patients during antituberculous chemotherapy indicated by letters A-E.

4.2.7 miR-223

MiR-223 differential expression increased steadily a month into chemotherapy but decreased then after at end of treatment (as shown in figure 37). Although its differential expression was significantly altered (p= 0.0434) throughout treatment there were no clearly observed trends for individual responses from patients (see figure 38).

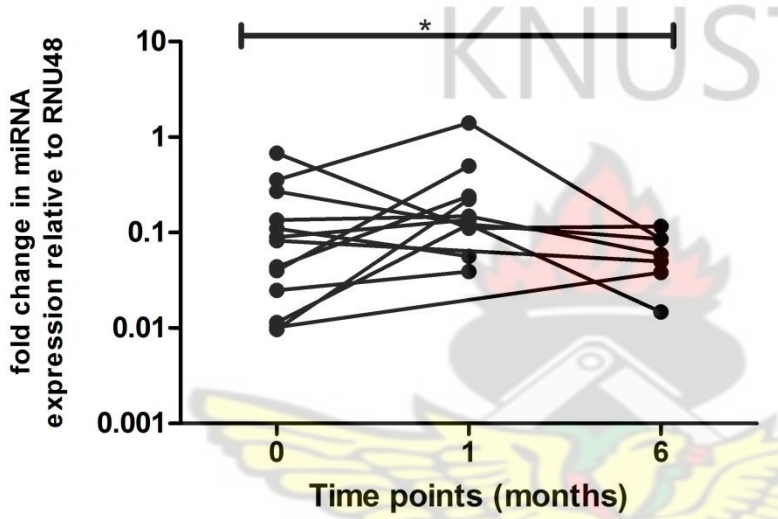


Figure 37 Shows differential expression of miR-223 during antituberculous chemotherapy. Time point 0 (n=11), time point 1 (n=9) and time point 6 (n=7).

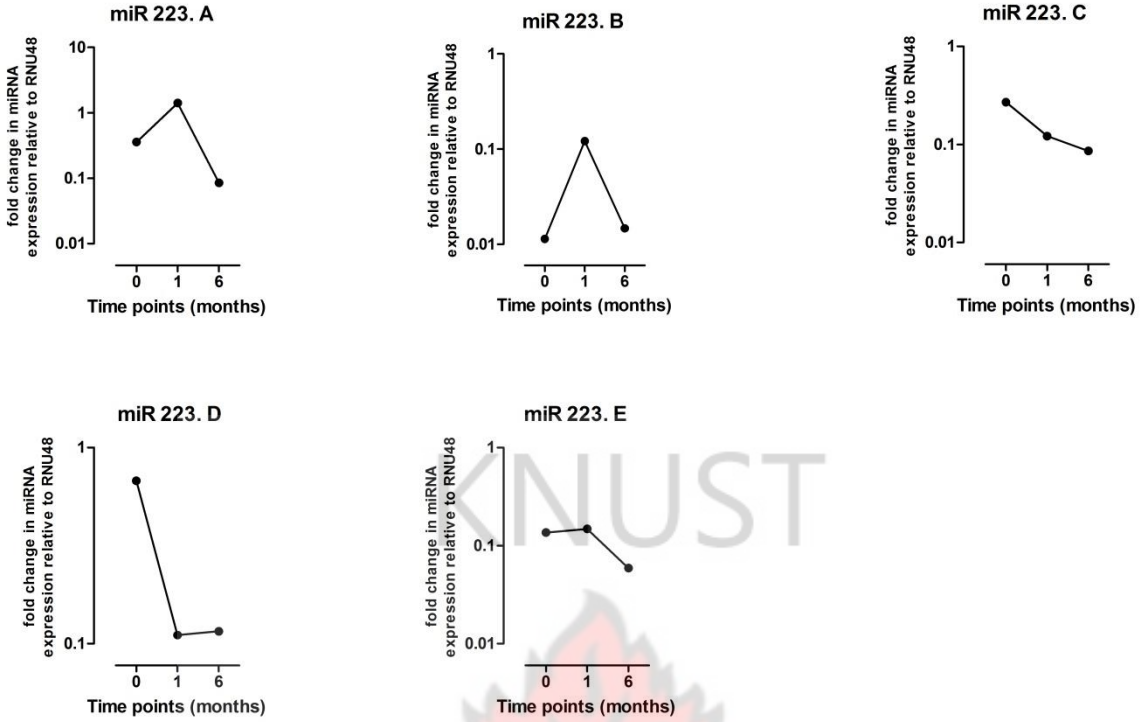


Figure 38 shows differential expression pattern of miR-146a for 5 different patients during antituberculous chemotherapy indicated by letters A-E.

4.2.8 MiRNA expression between patients and contacts

The differential expression of the successful preselected candidate miRNAs of Tuberculosis patients were compared to the LTBIs. The same was done for the other miRNAs that were not significantly altered during antituberculous chemotherapy. There were no statistically significant difference in median differential expression between tuberculosis patients and LTBIs for miR-25 (p=0.2928), miR-26a (p=0.4173), miR-29a (p=0.1435), miR-99b (p=0.3806), miR101 (p=0.3848), miR-146a (p=0.0501) and miR-223(p=0.9538) as shown in figure 39. A summary of comparison between Tuberculosis patients and LTBIs for miRNAs whose differential expression were not altered during anti-tuberculous chemotherapy are shown in table below.

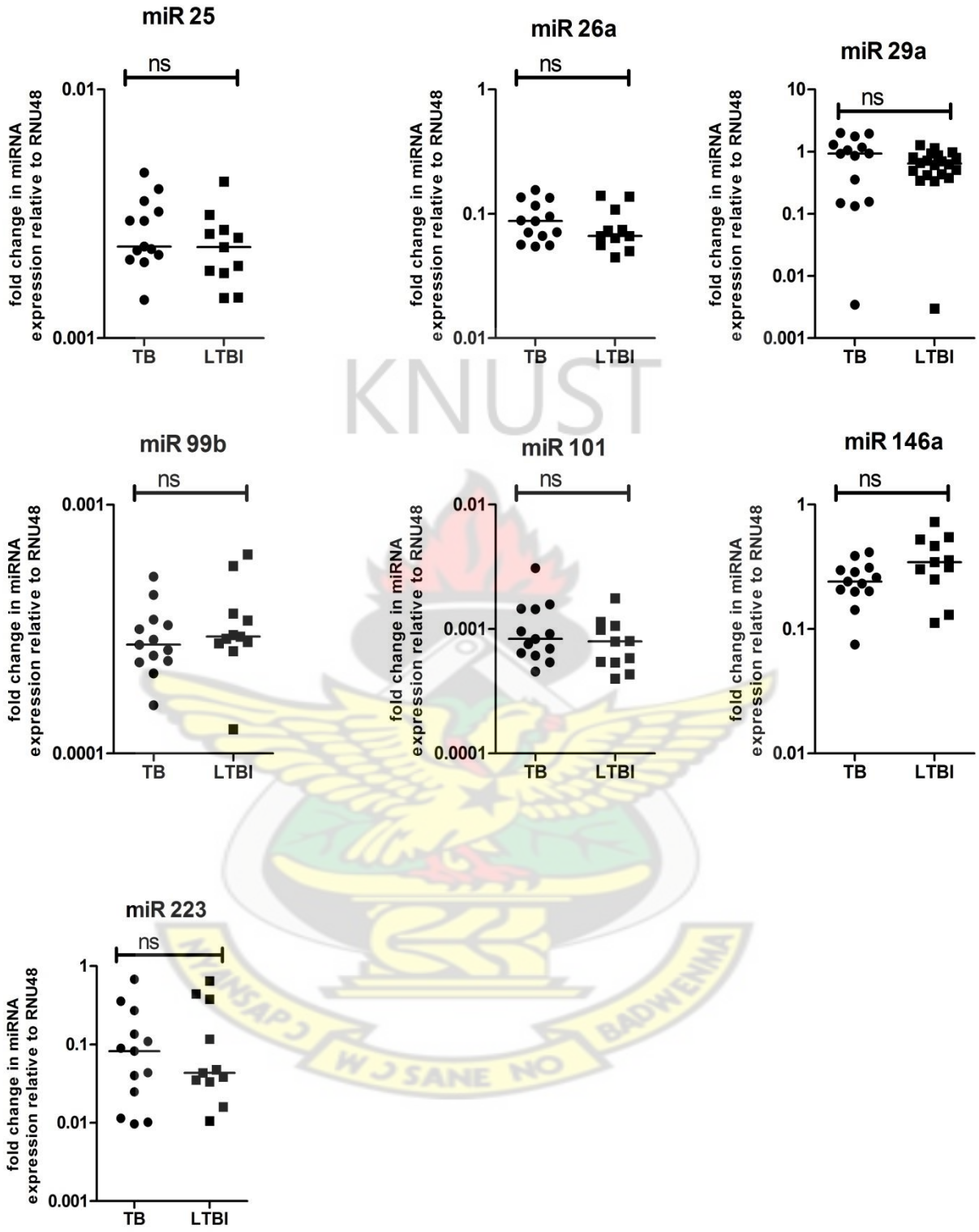


Figure 39 shows relative expressions of preselected candidate miRNAs (that were significantly altered during treatment) of patients (n=13) and LTBIs (n=11).

Table 12 MEDIAN DIFFERENTIAL EXPRESSION OF NONE ALTERED MIRNAs IN LTBI AND TB PATIENTS

Candidate miRNAs	**fold change in miRNA expression relative to RNU48 Median(range)**		**p-value**
	TB	**LTBIs**	
Let-7a	0.0008775 (6.311e-005- 0.004218)	0.0009288 (4.061e-005- 0.001974)	0.9476
miR-15a	0.0002083 (2.005e-005- 0.0009020)	0.0002793 (2.941e-005- 0.0006098)	0.7169
miR-17	0.1085 (0.06768- 0.2400)	0.08091 (0.04818- 0.2200)	0.0822
miR-21	0.07012 (0.02871- 0.1731)	0.06092 (0.04000- 0.1562)	0.7721
miR-27a	0.001321 (0.0004864- 0.002118)	0.001027 (0.0003841- 0.002097)	0.6181
miR-106a	0.1086 (0.06728- 0.2722)	0.08889 (0.05238- 0.2167)	0.0822
miR-106b	0.003541 (0.002065- 0.007799)	0.002482 (0.001800- 0.01350)	0.5239
miR-126	0.0005366 (8.087e-005- 0.007025)	0.0003803 (0.0001393- 0.003303)	0.4869
miR-142-3p	0.8902 (0.3214- 2.136)	0.6637 (0.2822- 1.843)	0.2970
miR-150	3.018 (1.781- 8.686)	3.175 (1.666- 8.654)	0.0501
miR-181a	0.001408 (0.0004035- 0.002520)	0.001079 (0.0006507- 0.002483)	0.9814
miR-191	0.06307 (0.03186- 0.1513)	0.06104 (0.03123 -0.1385)	0.6021
miR-30*	0.007281 (0.003700- 0.01271)	0.005842 (0.003215- 0.01555)	0.9545

4.3 MODULATION OF INTERFERON GAMMA RESPONSE BY MIR-29A

To determine if miR-29a has any influence on IFN-γ response miR=29a differential expression was correlated with IFN-γ response for 5 different antigens. 2 Tuberculosis specific antigens namely PPD and ESAT-6 as well as 3 different general antigens namely PMA with Ionomycin, PHA and SEB. This was performed for both LTBIs and TB patients. Although there was an observed seemingly positive correlation it was not positive for Tuberculosis patients for IFN-γ responses induced by PPD (SR= 0.2506; p= 0.0968), ESAT-6 (SR= 0.1225; p= 0.4228), PMA/Io (SR= 0.2129; p= 0.2342), PHA (SR= 0.1338; p= 0.3983) and SEB (SR= 0.2672; p= 0.0760) as shown in figure 40. A similar trend was observed for LTBIs with IFN-γ responses for PPD (SR= 0.3437; p= 0.1626), PMA/Io (SR= 0.2990; p= 0.2437), PHA (SR= 0.3643; p= 0.1372) and SEB (SR= 0.1001; p= 0.6927) except ESAT-6 (SR= 0.6593; p= 0.0142) which showed statistically significant positive correlation (see figure 41).

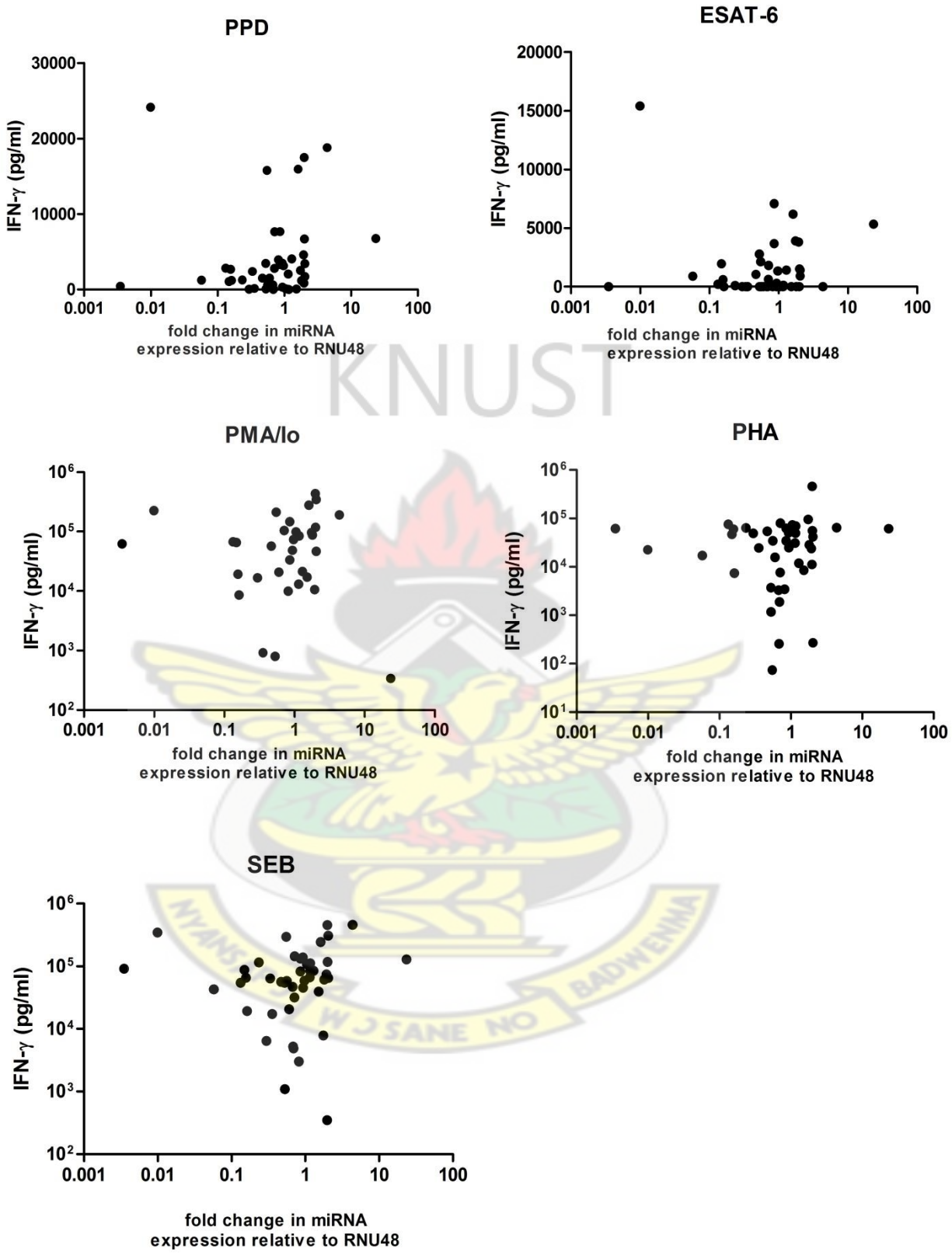


Figure 40 shows correlation between miR-29a and IFN-γ responses induced by 5 different antigens for Tuberculosis patients (n=45).

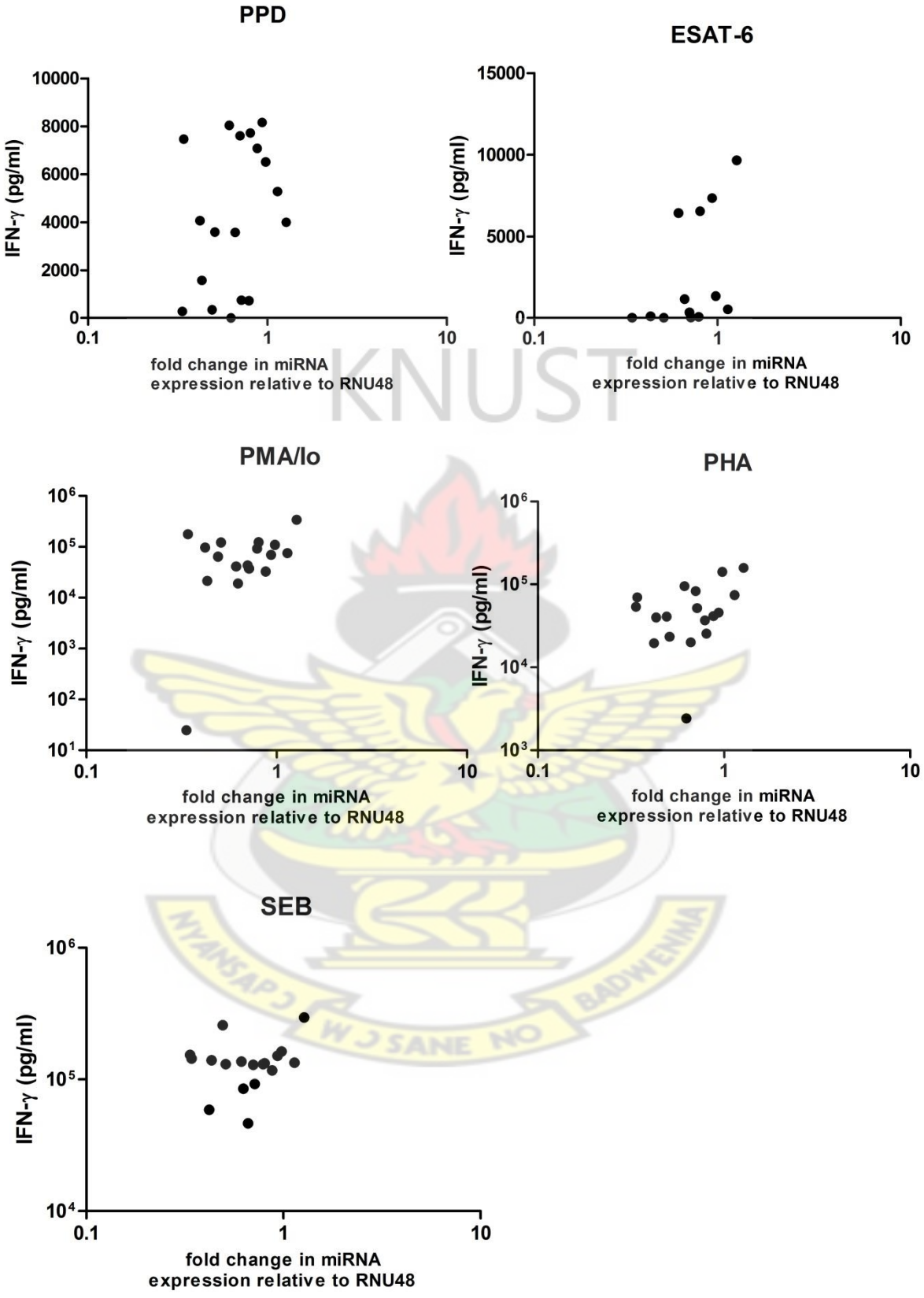


Figure 41 shows correlation between miR-29a and IFN-γ responses induced by 5 different antigens for LTBIs (n=18).

CHAPTER FIVE

5.0 DISCUSSION

Findings from this study show clearly that PPD and SEB induced IFN-γ responses in tuberculosis patients during antituberculous chemotherapy were not significantly altered but there were significantly higher IFN-γ responses in LTBIs than Tuberculosis patients for PPD and SEB. IFN-γ responses were not influenced by prior BCG vaccination, age, sex of patients and concomitant diseases. Out of the 29 preselected immune related candidate miRNAs screened, 7 of them were significantly altered during antituberculous chemotherapy. The differential expressions of the candidate miRNAs however were not significantly different between Tuberculosis patients and LTBIs. Lastly there were no correlation between miR-29a and IFN-γ responses for PPD, ESAT-6, PMA/Io, PHA and SEB restimulation of PBMCs from Tuberculosis patients. This trend was similar for LTBIs except for ESAT-6 induced IFN-γ response which was significantly correlated with miR-29a positively.

5.1 INTERFERON-GAMMA RESPONSE OVER TREATMENT PERIOD

Control of infection in both mice and humans critically relies on *M. tuberculosis* specific $CD4^+$ Th1 cell responses, which includes the production of IFN-γ as shown by (Wolf et al., 2008) and (Cooper, 2009). There was an increase in IFN-γ responses a month into antituberculous chemotherapy which decreased by end of treatment although the changes were not significant. Chemotherapy kills bacteria. This results in more bacteria for antigen processing and presentation by macrophages and the other antigen presenting cells. The likely sequence of events would be increased $CD4^+$ T cell activation and an increase in IFN-γ production observed a month into chemotherapy. Immune response and chemotherapy may act concomitantly to reduce bacterial load which may account for the decreased inflammation typified by the slight reduction in IFN-γ response between a month into chemotherapy and end of therapy. It is tempting to speculate that there is the possibility of a Th1 ($CD4^+$ T cell) dependent, IFN-γ independent mechanism of control of *M. tuberculosis* complex during

Tuberculosis which could account for the none significant increase in *M. tuberculosis* observed during treatment. This is reinforced by Cowley Elkins' work using mouse model. They demonstrated that $CD4^+$ T cells control > 90% of intracellular *M. tuberculosis* growth in vitro in the complete absence of IFN-γ. They showed that *M. tuberculosis* control was conferred through $CD4^+$ T cell rather than IFN-γ pathway of the afore-mentioned cells (Cowley and Elkins, 2003). Results shown are consistent with a recent study by Kleinsteuber et al. in humans when they demonstrated a none significant IFN-γ responses during antituberculous chemotherapy in adult and children tuberculosis patients (Kleinsteuber et al., 2013). Findings from this study, however do not agree with Jo et al.'s study findings. They showed that most patients with active pulmonary tuberculosis have repressed IFN-γ secretion by PBMCs in response to tuberculosis specific antigen stimulation which improves significantly after successful antituberculous therapy. In this study, they proposed an "anti-inflammatory cytokine mediated reduced antigen presentation leading to IFN-γ repression" as a possible explanation for the initial repression of IFN-γ observed, which increases significantly after chemotherapy (Jo et al., 2003).

5.2 INTERFERON-GAMMA RESPONSE BETWEEN PATIENTS AND THEIR CONTACTS

The pattern of response observed between patients and their exposed but healthy household contacts (LTBIs) do support the theory that IFN-γ is important for control of *M. tuberculosis* infection and may determine the outcome of infection (Wolf et al., 2008, Raja, 2004). This is supported by the fact that household contacts of the patients who were exposed to *M. tuberculosis* infection did produce significantly higher IFN-γ about twice as compared to the median IFN-γ production of the patients. There were no significantly different responses observed when IFN-γ produced by tuberculosis patients after treatment with LTBIs was compared (data not shown). The results shown are consistent with an earlier study using experimental model that suggested that IFN-γ is critical for activation of macrophages which

in turn is required for *M. tuberculosis* killing (Roche et al., 1995). It is also in agreement with Jouanguy et al.'s work from 1999 which showed extreme susceptibility of people unable to produce IFN-γ to mycobacteria infection (Jouanguy et al., 1999). It is also consistent with findings of studies showing susceptibility of mice with disruptions in IFN-γ or p40 gene to *M. tuberculosis* infection (Flynn et al., 1993, Cooper et al., 2002). However, the findings of our studies are not consistent with that of (Bennekov et al., 2006) who suggested that IFN-γ levels during *M. tuberculosis* infection may simply be a measure of inflammatory status rather than a protective immune response. Cowley et al. (2003) challenged the view that IFN-γ secreted by $CD4^+$ T cells is a marker for protective immunity (Cowley and Elkins, 2003). A recent study by Kleinsteuber et al. in humans demonstrates no significant difference in IFN-γ responses between patients and LTBIs (Kleinsteuber et al., 2013). These findings also do not agree with the results from this present study. Contrary to the opinion by Yu et al. that IFN-γ is not useful to distinguish latent from active disease (Yu et al., 2012),It is shown that exposed but healthy household contacts of the tuberculosis patients do produce significantly higher IFN-γ when their PBMCs are stimulated with tuberculosis specific antigen PPD as compared to the tuberculosis patients. IFN-γ response to PPD antigen among exposed but healthy house hold contacts do suggest that some exposed people in areas of endemicity develop an immune response without any clinical disease. Zhang and Hirsch in separate studies also did provide compelling results to show that PPD induced IFN-γ production by PBMCs from tuberculosis patients were decreased compared with findings in healthy tuberculin reactors (Hirsch et al., 1999, Zhang et al., 1995). These studies are consistent with our results and support the findings of our study. It is demonstrated in this study that despite the current evidence supporting a Th1 mediated $CD4^+$T cell dependent, IFN-γ independent mechanism of *M. tuberculosis* control, IFN-γ secretion is repressed in acute pulmonary

Tuberculosis patients. Thus, reinforcing the importance of IFN-γ in control of human *M. tuberculosis* infection.

5.3 INTERFERON-GAMMA RESPONSE BETWEEN VACCINATED AND NON-VACCINATED PATIENTS.

Protective immunity by BCG is documented to be dependent upon the induction of $CD4^+$ T cells that produce IFN-γ which subsequently induce bactericiding via macrophage activation (Hanekom, 2005, Hussey et al., 2007). In the case of Tuberculosis, (Abebe, 2012) documents that IFN-γ produced by $CD4^+$ T cells have been used as a yardstick against which BCG or other Tuberculosis vaccine induced immunity to Tuberculosis is assessed. It was shown in this present study that BCG vaccination does not confer better IFN-γ response among acute Pulmonary Tuberculosis patients compared with none vaccinated acute Pulmonary Tuberculosis patients. This could be explained by the fact that prior BCG vaccination during childhood in a Tuberculosis endemic area loses its efficacy in adults.

Though the general school of thought is that BCG induced immunity is lost in adults, some studies do report a lack of correlation between BCG induced immune protection and IFN-γ secreted by $CD4^+$ T cells (Connor et al., 2010, Hussey et al., 2007, McShane, 2011). This throws more light on the theory that challenges the use of IFN-γ response as an indicative of protection and supports an IFN-γ independent T cell control of *M. tuberculosis* infection. Cowley and Elkins (2003) examined the mechanism of BCG-induced immune protection in IFN-γ knock-out mice. They concluded from their empirical data that, $CD4^+$ T cells control more than 90% of intracellular *M. tuberculosis* growth in vitro in the complete absence of IFN-γ. More so, the BCG-induced $CD4^+$ T cell control of *M. tuberculosis* growth was lost upon depletion of the cells afore mentioned (Cowley and Elkins, 2003). Hoft et al. in their study used several assays to evaluate the immune responses against BCG in vaccinated humans. They found that mycobacterial growth inhibition did not correlate with IFN-γ

responses (Hoft et al., 2002). This was also in accordance with Mittrucker et al.'s findings in 2007 that provided strong evidence that IFN-γ does not correlate directly with BCG-induced protection (Mittrucker et al., 2007).

5.4 INFLUENCE OF AGE, SEX AND CONCOMITANT DISEASES ON INTERFERON-GAMMA RESPONSE AMONG PATIENTS.

The pattern of IFN-γ response, when PBMCs from patients were stimulated in vitro with PPD was not influenced by the age of the patients and sex before chemotherapy (time point 0) and a month into chemotherapy (time point 1). This clearly shows that the pattern of IFN-γ production in response to Tuberculosis specific antigens for the patients earlier discussed were not affected or biased by the age of the patients.

The extent of IFN-γ production depends on how much activated T cells are available. This in principle can be influenced by either host or the bacillus. The stepwise commitment from hematopoietic stem cells in the bone marrow to T lymphocyte-restricted progenitors in the thymus represents an important homeostatic process that maintains and replenishes T cell population. Age induced repression of blood cell haematopoiesis due to the shrinking of the thymus can affect the available T cell present. The consequential result may be reduced IFN-γ production due to limited availability of T cells. Our data of T cell population among the PBMCs isolated (data not shown) rules out the likelihood of this phenomenon affecting IFN-γ production thus evidenced by the insignificant association between age and IFN-γ production.

IFN-γ production from Isolated PBMCs of tuberculosis patients did not show statistically significant difference between males and females upon restimulation of PBMCs with PPD and SEB patients. Silva et al in their study also showed significantly higher IFN-γ response in treated tuberculosis patients than active Tuberculosis patients (Silva et al., 2012). This lays more emphasis on the ability of antituberculous chemotherapy to restore clinically relevant

levels of IFN-γ response other than the influence of sex. It appears that susceptibility to tuberculosis and response to *M. tuberculosis* infection may have nothing to do with the sex of the patient other than immune manipulation by the tuberculosis bacillus.

Immune response to *M. tuberculosis* infection is predominantly Th1 response. The induction of Th2 immune response not only represses the Th1 response but nullifies the overall effect of Th1 mediated immune response. Concomitant diseases during tuberculosis may influence immune responses to *M. tuberculosis* infection. Findings from this study show that tuberculosis patients with concomitant infection did not have a significant variation in IFN-γ response as compared with tuberculosis patients without concomitant diseases. Although such scientific speculation is yet to receive attention from investigators, enrolment of tuberculosis patients with concomitant diseases as part of our study population could have modulated Th1/Th2 paradigm shift. Our analysis however shows no such influence. This observation could be explained by the enhanced persistent increase in antigen presentation as a result of antituberculous chemotherapic mycobactericiding effect. This may influence T cell polarisation during T cell activation and differentiation predominantly towards a Th1 response thus limiting the possible influence of other concomitant diseases. This may have to be confirmed with a higher sample size.

5.5 CANDIDATE MIRNA SCREENING

Out of the panel of 29 preselected immune related candidates, 7 of them were altered during antituberculous chemotherapy although they were not significantly differentially expressed when compared to LTBIs (refer to figure 24 and 39). Emerging evidence suggests that miRNAs play a very important role in host-pathogen interactions. Although miRNAs had been implicated in the pathogenesis of several diseases including cancer, not much attention had been given to miRNAs' involvement in bacterial infection until recently (Eulalio et al., 2012). Implication of miRNAs in the response of mammalian cells to bacterial infection was

inferred from studies involving TLRs. In this study miR-146a among other miRNAs were documented to be induced after stimulation of TLR4 (Taganov et al., 2006). The modulation of these miRNAs could be due to mycobacteria immune modulatory mechanisms thus influencing the outcome of Tuberculosis infection.

MiR-25 differential expression increased significantly after treatment. This could be explained by the fact that alteration of miR-25 was influenced by *M. tuberculosis* thus bactericiding effect of antituberculous chemotherapy during treatment significantly increased the differential expression of this miRNA. The non-significantly observed differential expression between patients and control suggests that although miR-25 may influence disease outcome it may not be the only determinant of disease susceptibility in immunocompetent individuals. A recent study using similar candidates including miR-25 did not report it as one of the significantly altered candidates (Kleinsteuber et al., 2013). Increased plasma levels of miR-25 have been reported in a pancreatic cancer patient (Ren et al., 2012). MiR-25 has also been implicated in Type 1 diabetes (Nielsen et al., 2012) and breast cancer (Jonsdottir et al., 2012). The implication of miR-25 in tuberculosis is reported for the first time in this study.

The differential expression pattern of miR-26a was similar to that of miR-25. It increased significantly after anti-tuberculous chemotherapy. This places emphasis on the possible pathogen induction of miRNA modulation in host immune cells. There were no differences in expression between patients and LTBIs. This could be explained by heterogeneity of host susceptible factors not limited to this miRNA. Kleinsteuber however reports contrary results in their study where they show high levels of miR-26a in LTBIs than TB patients. Contrary to this finding is that shown by a study in China (Qi et al., 2012) which demonstrates higher differential expression of miR-26a in Tuberculosis patients than healthy tuberculin reactors. MiR-26a has also been implicated in cancer (Zhang et al., 2013, Zhang et al., 2011, Schetter et al., 2010).

MiR-29a increased a month into chemotherapy but its differential expression decreased thereafter. The decrease was not below the median differential expression before anti-tuberculous chemotherapy. The median expression during treatment differed significantly for all time points examined. A study finding consistent with this study reports miR-29a to be induced upon mycobacteria infection in humans (Sharbati et al., 2011). Fu et al. reports upregulated levels of miR-29a in serum and sputum of tuberculosis patients and proposes miR-29a as a potential biomarker for tuberculosis (Fu et al., 2011). Further, it is tempting to speculate that miR-29a repression and uncontrolled up regulation may be induced by *M. tuberculosis* infection leading to immune evasion thus persistence of *M. tuberculosis* in infected cells. In this study miR-29a was slightly higher in tuberculosis patients than LTBIs although this was not significant. Another study reports higher differential expression in LTBIs than tuberculosis patients (Kleinsteuber et al., 2013). This could be explained by difference in ethnic background and decreased start levels of miR-29a in the low incidence country where study was conducted. In addition to the above mentioned, the later study was done in a low incidence country with a lower baseline levels of miR-29a than reported for this study. Ma et al. on the other hand shows repression in miR-29a expression upon infection of mice T cells with *M. bovis* (Ma et al., 2011a). These results are consistent with the findings of this study.

The increase in differential expression of miR-99b was highly significant after anti-tuberculous chemotherapy. This unfolds a novel immune evasive mechanism by *M. tuberculosis* by modulating miR-99b. It has been reported by Singh et al. that up regulation of miR-99b in infected dendritic cells represses pro inflammatory cytokine production thus leading to persistence of *M. tuberculosis* in infected APCs (Singh et al., 2013b). Although the findings of this study proposes potential immune evasion via increase in expression of miR-99b in infected dendritic cells, findings from this study infers a potential immune evasive

mechanism in $CD4^+$ T cells via miR-99 repression. This is important as the same miRNA may play different roles in different immune cells. This could be possible especially due to their pleiotropic nature. Although miR-99b was screened in a recent study, no significant difference of differential expression between patients and exposed but healthy controls were reported (Kleinsteuber et al., 2013). Their findings are consistent with that from this study as we report no significant variation in differential expression of miR-99b between patients and LTBIs (refer to figure 39). MiR-99b has also been implicated in breast cancer (Turcatel et al., 2012).

Differential expression of miR-101 was repressed before treatment but increased significantly after treatment. This places emphasis on miR-101 as one of the afore mentioned miRNAs in $CD4^+$ T cells used by *M. tuberculosis* to modulate host immune response during *M. tuberculosis* infection. miR-101 was reported to be upregulated in sera of Tuberculosis patients as compared to healthy controls (Fu et al., 2011). Differential expression of miR-101 was slightly higher in Tuberculosis patients than in LTBIs although the difference was not statistically significant; the pattern is similar to that reported by Fu et al. in their study. MiR-101 has also been reported to be involved in the pathogenesis of human breast cancer (Wang et al., 2012).

Differential expression of miR-146a increased significantly by the end of antituberculous chemotherapy. Although the differential expression of the above mentioned miRNA was lower in Tuberculosis patients than LTBI. This was however not statistically significant. It appears that *M. tuberculosis* may induce the down regulation of miR-146a as part of a complex immune modulatory system to persist in host. This could explain the significant increase in differential expression after mycobactericiding effect of anti-tuberculous chemotherapy. Contrary to this finding, another study reports high differential expression of miR-146a in Tuberculosis patients. They postulated that it was induced by *M. avium* infection

(Sharbati et al., 2011). This difference in expression pattern may be due to a number of reasons including host genetics and ethnic background, mycobacterium species causing infection and source of miRNA investigated among others. MiR-146a has been implicated in other bacterial infections including Helicobacter pylori infection (Xiao et al., 2009, Liu et al., 2010), Salmonella enterica infection (Schulte et al., 2011, Sharbati et al., 2011) and Listeria monocytogenes infection (Schnitger et al., 2011). It has also been implicated in gastric cancer (Kogo et al., 2011) and autoimmunity (Chan et al., 2013, Boldin et al., 2011).

It is reported for the first time in Ghana in this study that miR-223 is implicated in M. tuberculosis infection. Induction of *M. tuberculosis* infection appears to up regulate differential expression of miR-223 in Tuberculosis patients than LTBIs although the difference reported is not significant. Tuberculosis patients after a month of anti-tuberculous chemotherapy show a steady increase in differential expression of miR-223. However, this dropped from a month into chemotherapy till end of treatment, suggesting a possible repressive effect of antituberculous chemotherapy and subsequent reduced bacteraemia on miR-223. In Kleinsteuber et al.'s study they examined miR-223 among other candidates but reported no significant difference between tuberculosis patients and controls although it was highly expressed in LTBIs than tuberculosis patients (Kleinsteuber et al., 2013). These findings are contrary to that from the present study and may be explained by population where samples were pooled from. Whereas our study was conducted in a Tuberculosis endemic region, Kleinsteuber et al's work was done in a low incidence region. Other than that environmental, genetic and ethnic differences could have greatly influenced observed differences. A recent study shows that miR-223 is associated with *M. tuberculosis* infection. In the study miR-223 was upregulated in blood and lung parenchyma of tuberculosis patients and during murine tuberculosis. Deletion of miR-223 rendered tuberculosis-resistant mice highly susceptible to acute lung infection. They identified leukocyte chemotaxis via direct

regulation of CXCL2, CCL3, and IL-6 in myeloid cells as the mechanism by which it exerts its biological function during tuberculosis (Dorhoi et al., 2013). The median differential expression of miR-223 was higher in tuberculosis patients than LTBIs although not statistically significant in this study. In addition to this, median differential expression of miR-223 was higher after anti-tuberculous chemotherapy than before. These are all consistent with Dorhoi et al.'s study findings. Although Dorhoi et al.'s study focuses on myeloid cells and its regulation by miR-223 this study reports a possible regulation via antimycobacterial $CD4^+$ T cell responses. It is tempting therefore to speculate that repression of miR-223 differential expression may be a mechanism by which *M. tuberculosis* evade $CD4^+$ T cell responses thus up regulation of this miRNA may be involved in appropriate host mycobactericidal $CD4^+$ T cell responses to *M. tuberculosis* infection.

A careful look at individual patients reveals varied differential expression patterns for significantly altered candidate miRNAs. This may infer that it might not be possible to consider such candidates as markers for disease prognosis. It also shows that for immunocompetent individuals, changes in miRNA differential expression during the same antituberculous chemotherapy may differ. Possible explanations to these observations still remain the subject of on-going investigations.

In an attempt to characterise the effect of miR-29a differential expression, this current study sought to elucidate the association between differential expression of miR-29a and an empirically documented target, IFN-γ. IFN-γ production by PBMCs was induced using two TB specific antigens PPD and ESAT-6 in addition to three general antigens PMA/Io, PHA and SEB. The differential expression of the said miRNA was correlated with the IFN-γ response. Although not statistically significant, a tendency towards positive correlation was reported. The findings shown in this work is consistent with Kleinsteuber et al.'s study where they showed a likely positive but insignificant correlation between miR-29a and IFN-γ

(Kleinsteuber et al., 2013). This intensifies the discussion on miR-29a being a none redundant repressor of IFN-γ because these results findings are contrary to the pioneering work by (Ma et al., 2011b) and (Steiner et al., 2011). The current findings rather show a tendency to up regulate IFN-γ response during tuberculosis and other infections rather than repress it. This may be due to the difference in regulation of IFN-γ by miR-29a in animal models and humans. A similar trend was observed for LTBIs in this current study (Figure 41). This particularly reinforces the debate against negative regulation of IFN-γ by miR-29a.Thus one may speculate several inferences from data shown.

Firstly, miR-29a may not be a none redundant repressor of IFN-γ as suggested by empirical data from this study. Secondly miR-29a may not be the only miRNA regulating IFN-γ response in T cell thus masking the effect by miR-29a. Lastly, miR-29a may be using other mechanisms other than IFN-γ to modulate $CD4^+$ T cell response to *M. tuberculosis* infection.

5.6 LIMITATIONS OF THE STUDY

This work however had some limitations notwithstanding. An independent confirmation via culture was not carried out before recruitment. This was not possible because patients had to start treatment immediately and it took time to get results from bacterial culture. Clinicians at the hospitals however concluded their diagnosis based on laboratory test (microscopy, culture or and PCR), clinical symptoms and radiographic results. Also there were defaulters for 2^{nd} and 3^{rd} time points. Prominent among reasons given included poor health, recovery and lack of interest. This did not affect our results as we used the appropriate statistical test to adjust for missing time point data. There could have been more samples included in this study. This was due to high cost of experiment and limited funds. It goes without saying that the sample size used was higher than most studies involving miRNAs.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

IFN-γ mediated Th1 response is critical for control of *M. tuberculosis* infection. However there exists the possibility of a Th1 mediated ($CD4^+$ T cell dependent) IFN-γ independent mechanism of *M. tuberculosis* control. IFN-γ repression may be a likely immune evasive mechanism employed by *M. tuberculosis* to overwhelm the host immunity. Gender and age of the patients, concomitant diseases as well as prior BCG vaccination did not influence IFN-γ expression in tuberculosis patients. The current study is considered as an initial step to characterize the role of microRNAs in tuberculosis. Consistent pattern of significantly altered differential expression of miR-25, miR-26a, miR-29a, miR-99b, miR-101, miR-146a and miR-223 suggests a role of these candidates in $CD4^+$ T cell immunity during tuberculosis disease and recovery. These candidates may represent future candidates for immunotherapies and vaccines against *M. tuberculosis* infections. Although miR-29a may be involved with $CD4^+$ T cell response to *M. tuberculosis* infection there is no evidence to support interdependency of IFN-γ production in tuberculosis patients and LTBIs.

6.2 RECOMMENDATIONS

Further work needs to be carried out to investigate the effect of IFN-γ receptor anergy on the ability of tuberculosis patients to respond to tuberculosis specific antigens in vitro. The likely counteractive role of anti-inflammatory cytokines like IL-10 among others on the capacity of IFN-γ to suppress mtb infection in acute pulmonary tuberculosis patients undergoing chemotherapy should be examined to give a good immunological profile of the patients. Such a study should compare multiple Th1 and Th2 in a cohort of acute tuberculosis patients before and during anti-tuberculous treatment. Further studies will have to be performed to elucidate the processes targeted by candidate miRNAs and to reveal if miRNAs are possible targets for disease intervention strategies and new targets for vaccine developments in

tuberculosis. Lastly on-going work would look at other possible targets for successfull candidates with significantly altered differential expression and varying expression in $CD4^+$ T cell sub population.

6.3 NOVELTY AND LESSONS LEARNT

It has been shown for the first time to the best of my knowledge in this study that the 7 afore mentioned miRNA candidates are significantly altered during acute pulmonary tuberculosis and may be implicated in the immunopathogenesis of tuberculosis. Whereas there has been a growing opinion vouching for IFN-γ as an empirically determined candidate for miR-29a, data from this study does not support this. In agreement with another concomitant study (previously described in the discussion), a strong case is being put forward against the none redundant modulation of IFN-γ by miR-29a. Although, others have done studies on miRNAs in tuberculosis, no one has carefully used qRT-PCR system to examine the dysregulation of the candidate miRNAs in a cohort study. Studies have been limited to a case control study using serum or sputum as opposed to my work with $CD4^+$ T cells, an important immune cell that is central to the outcome of infection. Also presented in this study is a merging view of the pivotal role of IFN-γ that merges the extreme opinions about the role of this cytokine during active tuberculosis. Whereas empirical data that shows no significant changes in this cytokine during anti-tuberculous chemotherapy is presented, yet I show a significant difference in IFN-γ expression in patients as compared to LTBIs. This presents an immunological elucidation of a somewhat enigmatic immunopathogenesis of this disease. It is being postulated empirically that, not only does IFN-γ play central role in the outcome of infection from latency to reactivation but present a possible IFN-γ independent, yet $CD4^+$ T cell dependent pathway which are not mutually exclusive to clearing infection by host. This seemingly contrasting data, although revealing, provides the basis for a paradigm shift in our understanding of host immune response to *M. tuberculosis* infection.

The investigator has learnt the dynamics underlying cell mediated immune response to intracellular bacteria like *M. tuberculosis* and its modulation using immunological tools and technology available to us in molecular immunology. Some of these included flow cytometry, ELISA, Imag system, singleplex and multiplex RT-PCR systems just to mention a few. In addition to this, I have also learnt transferable skills in research that includes study design, project management, good laboratory practice, biostatistics, scientific writing and publication among others.

6.4 CONTRIBUTION TO KNOWLEDGE

Whereas it is accepted that immune modulatory molecules (be it of host or pathogen origin) may be employed by pathogen in host pathogen interaction, in an immune evasive mechanism to oust host's defence and pathogen containment, empirical evidence has been provided to suggest in this study that not only are miRNAs significantly altered during disease state but may be employed by *M. tuberculosis* in this case to evade or and repress host immunity as part of a myriad of mechanisms employed to overwhelm host immunity.

Although it still remains uncertain which miRNA dysregulation or combination of altered miRNAs may be responsible (if so) for the significantly repressed IFN-γ responses for tuberculosis patients, it has been empirically shown that, miR-29a does not regulate IFN-γ production in the study subjects used in this study.

Lastly, a strong basis for understanding contrasting opinions about the candid role of IFN-γ during acute pulmonary tuberculosis has been provided.

These information provided and proven in this thesis will significantly add to the existing understanding of the immunopathogenesis of tuberculosis.

REFERENCES

Abebe, F. 2012. Is interferon-gamma the right marker for bacille Calmette-Guerin-induced immune protection? The missing link in our understanding of tuberculosis immunology. *Clin Exp Immunol,* 169**,** 213-9.

Al Zahrani, K., Al Jahdali, H. & Menzies, D. 2000. Does size matter? Utility of size of tuberculin reactions for the diagnosis of mycobacterial disease. *Am J Respir Crit Care Med,* 162**,** 1419-22.

Almeida, A. S., Lago, P. M., Boechat, N., Huard, R. C., Lazzarini, L. C., Santos, A. R., Nociari, M., Zhu, H., Perez-Sweeney, B. M., Bang, H., Ni, Q., Huang, J., Gibson, A. L., Flores, V. C., Pecanha, L. R., Kritski, A. L., Lapa E Silva, J. R. & Ho, J. L. 2009. Tuberculosis is associated with a down-modulatory lung immune response that impairs Th1-type immunity. *J Immunol,* 183**,** 718-31.

Am. Thor. Soc. 2000. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999. *Am J Respir Crit Care Med,* 161**,** 1376-95.

Arend, S. M., Andersen, P., Van Meijgaarden, K. E., Skjot, R. L., Subronto, Y. W., Van Dissel, J. T. & Ottenhoff, T. H. 2000. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. *J Infect Dis,* 181**,** 1850-4.

Armstrong, J. A. & Hart, P. D. 1971. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *J Exp Med,* 134**,** 713-40.

Armstrong, J. A. & Hart, P. D. 1975. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *J Exp Med,* 142**,** 1-16.

Ayvazian, F. L. 1993. *History of tuberculosis, In L. B. Reichman & E. S. Hershfield (Eds.),* New York, Marcel Dekker.

Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P. & Bartel, D. P. 2008. The impact of microRNAs on protein output. *Nature,* 455**,** 64-71.

Bainton, D. F. 1981. The discovery of lysosomes. *J Cell Biol,* 91**,** 66s-76s.

Banchereau, J. & Steinman, R. M. 1998. Dendritic cells and the control of immunity. *Nature,* 392**,** 245-52.

Barnes, P. F., Abrams, J. S., Lu, S., Sieling, P. A., Rea, T. H. & Modlin, R. L. 1993. Patterns of cytokine production by mycobacterium-reactive human T-cell clones. *Infect Immun,* 61**,** 197-203.

Barnes, P. F., Modlin, R. L., Bloom, B. R. & Ellner, J. J. 1994. *Tuberculosis: Pathogenesis, protection and control,* Washington DC, ASM Press

Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell,* 116**,** 281-97.

Bazzoni, F., Rossato, M., Fabbri, M., Gaudiosi, D., Mirolo, M., Mori, L., Tamassia, N., Mantovani, A., Cassatella, M. A. & Locati, M. 2009. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A,* 106**,** 5282-7.

Bean, A. G., Roach, D. R., Briscoe, H., France, M. P., Korner, H., Sedgwick, J. D. & Britton, W. J. 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *The Journal of Immunology*, 162**,** 3504-3511.

Behar, S. M., Dascher, C. C., Grusby, M. J., Wang, C. R. & Brenner, M. B. 1999. Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. *J Exp Med,* 189**,** 1973-80.

Bennekov, T., Dietrich, J., Rosenkrands, I., Stryhn, A., Doherty, T. M. & Andersen, P. 2006. Alteration of epitope recognition pattern in Ag85B and ESAT-6 has a profound influence on vaccine-induced protection against Mycobacterium tuberculosis. *Eur J Immunol,* 36**,** 3346-55.

Berezikov, E., Chung, W. J., Willis, J., Cuppen, E. & Lai, E. C. 2007. Mammalian mirtron genes. *Mol Cell,* 28**,** 328-36.

Bhatt, K., Hickman, S. P. & Salgame, P. 2004. Cutting edge: a new approach to modeling early lung immunity in murine tuberculosis. *J Immunol,* 172**,** 2748-51.

Boldin, M. P., Taganov, K. D., Rao, D. S., Yang, L., Zhao, J. L., Kalwani, M., Garcia-Flores, Y., Luong, M., Devrekanli, A., Xu, J., Sun, G., Tay, J., Linsley, P. S. & Baltimore, D. 2011. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med,* 208**,** 1189-201.

Boom, W. H. 1996. The role of T-cell subsets in Mycobacterium tuberculosis infection. *Infect Agents Dis,* 5**,** 73-81.

Boussiotis, V. A., Tsai, E. Y., Yunis, E. J., Thim, S., Delgado, J. C., Dascher, C. C., Berezovskaya, A., Rousset, D., Reynes, J. M. & Goldfeld, A. E. 2000. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *J Clin Invest,* 105**,** 1317-25.

Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J. & Modlin, R. L. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science,* 285**,** 732-6.

Calame, K. 2007. MicroRNA-155 function in B Cells. *Immunity,* 27**,** 825-7.

Carthew, R. W. & Sontheimer, E. J. 2009. Origins and Mechanisms of miRNAs and siRNAs. *Cell,* 136**,** 642-55.

Caruso, A. M., Serbina, N., Klein, E., Triebold, K., Bloom, B. R. & Flynn, J. L. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol,* 162**,** 5407-16.

Casanova, J. L. & Abel, L. 2002. Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol,* 20**,** 581-620.

Chaisson, R. E., Schecter, G. F., Theuer, C. P., Rutherford, G. W., Echenberg, D. F. & Hopewell, P. C. 1987. Tuberculosis in patients with the acquired immunodeficiency syndrome. Clinical features, response to therapy, and survival. *Am Rev Respir Dis,* 136**,** 570-4.

Chan, E. K., Ceribelli, A. & Satoh, M. 2013. MicroRNA-146a in autoimmunity and innate immune responses. *Ann Rheum Dis,* 72 Suppl 2**,** ii90-5.

Chan, J., Fan, X. D., Hunter, S. W., Brennan, P. J. & Bloom, B. R. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. *Infect Immun,* 59**,** 1755-61.

Chan, J. & Flynn, J. L. 1999. *Nitric oxide in Mycobacterium tuberculosis infection,* New York, Plenum.

Chan, J., Fujiwara, T., Brennan, P., Mcneil, M., Turco, S. J., Sibille, J. C., Snapper, M., Aisen, P. & Bloom, B. R. 1989. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc Natl Acad Sci U S A,* 86**,** 2453-7.

Chan, J., Tanaka, K., Carroll, D., Flynn, J. & Bloom, B. R. 1995. Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. *Infect Immun,* 63**,** 736-40.

Chan, J., Xing, Y., Magliozzo, R. S. & Bloom, B. R. 1992. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med,* 175**,** 1111-22.

Chan, S. P. & Slack, F. J. 2007. And now introducing mammalian mirtrons. *Dev Cell,* 13**,** 605-7.

Chen, C. Z., Li, L., Lodish, H. F. & Bartel, D. P. 2004. MicroRNAs modulate hematopoietic lineage differentiation. *Science,* 303**,** 83-6.

Clemens, D. L. & Horwitz, M. A. 1995. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med,* 181**,** 257-70.

Clerici, M. & Shearer, G. M. 1994. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol Today,* 15**,** 575-81.

Cohn, Z. A. 1963. The fate of bacteria within phagocytic cells. I. The degradation of isotopically labeled bacteria by polymorphonuclear leucocytes and macrophages. *J Exp Med,* 117**,** 27-42.

Connor, L. M., Harvie, M. C., Rich, F. J., Quinn, K. M., Brinkmann, V., Le Gros, G. & Kirman, J. R. 2010. A key role for lung-resident memory lymphocytes in protective immune responses after BCG vaccination. *Eur J Immunol,* 40**,** 2482-92.

Cooper, A. M. 2009. Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol,* 27**,** 393-422.

Cooper, A. M., Adams, L. B., Dalton, D. K., Appelberg, R. & Ehlers, S. 2002. IFN-gamma and NO in mycobacterial disease: new jobs for old hands. *Trends Microbiol,* 10**,** 221-6.

Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G. & Orme, I. M. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med,* 178**,** 2243-7.

Cooper, A. M., Magram, J., Ferrante, J. & Orme, I. M. 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med,* 186**,** 39-45.

Cooper, A. M., Segal, B. H., Frank, A. A., Holland, S. M. & Orme, I. M. 2000. Transient loss of resistance to pulmonary tuberculosis in p47(phox-/-) mice. *Infect Immun,* 68**,** 1231-4.

Cowley, S. C. & Elkins, K. L. 2003. CD4+ T cells mediate IFN-gamma-independent control of Mycobacterium tuberculosis infection both in vitro and in vivo. *J Immunol,* 171**,** 4689-99.

Cullen, B. R. 2011. Viruses and microRNAs: RISCy interactions with serious consequences. *Genes Dev,* 25**,** 1881-94.

Dahl, K. E., Shiratsuchi, H., Hamilton, B. D., Ellner, J. J. & Toossi, Z. 1996. Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of Mycobacterium tuberculosis. *Infect Immun,* 64**,** 399-405.

Dai, R. & Ahmed, S. A. 2011. MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. *Transl Res,* 157**,** 163-79.

Dalton, D. K., Pitts-Meek, S., Keshav, S., Figari, I. S., Bradley, A. & Stewart, T. A. 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science,* 259**,** 1739-42.

Dannenberg, A. M., Jr. 1991. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. *Immunol Today,* 12**,** 228-33.

De Duve, C. & Wattiaux, R. 1966. Functions of lysosomes. *Annu Rev Physiol,* 28**,** 435-92.

De Flora, S. & Bonanni, P. 2011. The prevention of infection-associated cancers. *Carcinogenesis,* 32**,** 787-95.

Delgado, J. C., Tsai, E. Y., Thim, S., Baena, A., Boussiotis, V. A., Reynes, J. M., Sath, S., Grosjean, P., Yunis, E. J. & Goldfeld, A. E. 2002. Antigen-specific and persistent tuberculin anergy in a cohort of pulmonary tuberculosis patients from rural Cambodia. *Proc Natl Acad Sci U S A,* 99**,** 7576-81.

Denis, M. 1991. Human neutrophils, activated with cytokines or not, do not kill virulent Mycobacterium tuberculosis. *J Infect Dis,* 163**,** 919-20.

Deretic, V. & Fratti, R. A. 1999. Mycobacterium tuberculosis phagosome. *Mol Microbiol,* 31**,** 1603-9.

Desjardins, M. 1995. Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. *Trends Cell Biol,* 5**,** 183-6.

Desjardins, M., Huber, L. A., Parton, R. G. & Griffiths, G. 1994. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J Cell Biol,* 124**,** 677-88.

Dheda, K., Schwander, S. K., Zhu, B., Van Zyl-Smit, R. N. & Zhang, Y. 2010. The immunology of tuberculosis: from bench to bedside. *Respirology,* 15**,** 433-50.

Dheda, K., Van Zyl Smit, R., Badri, M. & Pai, M. 2009. T-cell interferon-gamma release assays for the rapid immunodiagnosis of tuberculosis: clinical utility in high-burden vs. low-burden settings. *Curr Opin Pulm Med,* 15**,** 188-200.

Dieu, M. C., Vanbervliet, B., Vicari, A., Bridon, J. M., Oldham, E., Ait-Yahia, S., Briere, F., Zlotnik, A., Lebecque, S. & Caux, C. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med,* 188**,** 373-86.

Ding, S. W. & Voinnet, O. 2007. Antiviral immunity directed by small RNAs. *Cell*, 130**,** 413-26.

Dorhoi, A., Iannaccone, M., Farinacci, M., Fae, K. C., Schreiber, J., Moura-Alves, P., Nouailles, G., Mollenkopf, H. J., Oberbeck-Muller, D., Jorg, S., Heinemann, E., Hahnke, K., Lowe, D., Del Nonno, F., Goletti, D., Capparelli, R. & Kaufmann, S. H. 2013. MicroRNA-223 controls susceptibility to tuberculosis by regulating lung neutrophil recruitment. *J Clin Invest*.

Dorsett, Y., Mcbride, K. M., Jankovic, M., Gazumyan, A., Thai, T. H., Robbiani, D. F., Di Virgilio, M., Reina San-Martin, B., Heidkamp, G., Schwickert, T. A., Eisenreich, T., Rajewsky, K. & Nussenzweig, M. C. 2008. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity*, 28**,** 630-8.

Downey, G. P., Botelho, R. J., Butler, J. R., Moltyaner, Y., Chien, P., Schreiber, A. D. & Grinstein, S. 1999. Phagosomal maturation, acidification, and inhibition of bacterial growth in nonphagocytic cells transfected with FcgammaRIIA receptors. *J Biol Chem*, 274**,** 28436-44.

Dubos, R. 1982. The romance of death. *Am Lung Assoc Bull*, 68**,** 5-6.

Eacker, S. M., Dawson, T. M. & Dawson, V. L. 2009. Understanding microRNAs in neurodegeneration. *Nat Rev Neurosci*, 10**,** 837-41.

Edwards, D. & Kirkpatrick, C. H. 1986. The immunology of mycobacterial diseases. *Am Rev Respir Dis*, 134**,** 1062-71.

Ehlers, S., Benini, J., Kutsch, S., Endres, R., Rietschel, E. T. & Pfeffer, K. 1999. Fatal granuloma necrosis without exacerbated mycobacterial growth in tumor necrosis factor receptor p55 gene-deficient mice intravenously infected with Mycobacterium avium. *Infection and immunity,* 67**,** 3571-3579.

Engering, A. J., Cella, M., Fluitsma, D., Brockhaus, M., Hoefsmit, E. C., Lanzavecchia, A. & Pieters, J. 1997. The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *Eur J Immunol,* 27**,** 2417-25.

Eruslanov, E. B., Lyadova, I. V., Kondratieva, T. K., Majorov, K. B., Scheglov, I. V., Orlova, M. O. & Apt, A. S. 2005. Neutrophil responses to Mycobacterium tuberculosis infection in genetically susceptible and resistant mice. *Infect Immun,* 73**,** 1744-53.

Esin, S., Batoni, G., Kallenius, G., Gaines, H., Campa, M., Svenson, S. B., Andersson, R. & Wigzell, H. 1996. Proliferation of distinct human T cell subsets in response to live, killed or soluble extracts of Mycobacterium tuberculosis and Myco. avium. *Clin Exp Immunol,* 104**,** 419-25.

Eulalio, A., Schulte, L. & Vogel, J. 2012. The mammalian microRNA response to bacterial infections. *RNA Biology,* 9**,** 742-750.

Fang, F. C. 1997. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest,* 99**,** 2818-25.

Fanger, N. A., Wardwell, K., Shen, L., Tedder, T. F. & Guyre, P. M. 1996. Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. *J Immunol,* 157**,** 541-8.

Fazi, F., Rosa, A., Fatica, A., Gelmetti, V., De Marchis, M. L., Nervi, C. & Bozzoni, I. 2005. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell,* 123**,** 819-31.

Feng, C. G., Bean, A. G., Hooi, H., Briscoe, H. & Britton, W. J. 1999. Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with Mycobacterium tuberculosis. *Infect Immun,* 67**,** 3242-7.

Fenton, M. J., Vermeulen, M. W., Kim, S., Burdick, M., Strieter, R. M. & Kornfeld, H. 1997. Induction of gamma interferon production in human alveolar macrophages by Mycobacterium tuberculosis. *Infect Immun,* 65**,** 5149-56.

Figdor, C. G., Van Kooyk, Y. & Adema, G. J. 2002. C-type lectin receptors on dendritic cells and Langerhans cells. *Nat Rev Immunol,* 2**,** 77-84.

Fleischmann, J., Golde, D. W., Weisbart, R. H. & Gasson, J. C. 1986. Granulocyte-macrophage colony-stimulating factor enhances phagocytosis of bacteria by human neutrophils. *Blood,* 68**,** 708-11.

Flynn J.L. & Chan J. 2001. immunolgy of tuberculosis. *annu rev immunol,* 19**,** 93-129.

Flynn, J. L. & Chan, J. 2001a. Immunology of tuberculosis. *Annu Rev Immunol,* 19**,** 93-129.

Flynn, J. L. & Chan, J. 2001b. Tuberculosis: latency and reactivation. *Infect Immun,* 69**,** 4195-201.

Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A. & Bloom, B. R. 1993. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med,* 178**,** 2249-54.

Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schreiber, R., Mak, T. W. & Bloom, B. R. 1995a. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity,* 2**,** 561-72.

Flynn, J. L., Goldstein, M. M., Triebold, K. J., Koller, B. & Bloom, B. R. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proc Natl Acad Sci U S A,* 89**,** 12013-7.

Flynn, J. L., Goldstein, M. M., Triebold, K. J., Sypek, J., Wolf, S. & Bloom, B. R. 1995b. IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection. *J Immunol,* 155**,** 2515-24.

Flynn, J. L., Scanga, C. A., Tanaka, K. E. & Chan, J. 1998. Effects of aminoguanidine on latent murine tuberculosis. *J Immunol,* 160**,** 1796-803.

Fontana, L., Pelosi, E., Greco, P., Racanicchi, S., Testa, U., Liuzzi, F., Croce, C. M., Brunetti, E., Grignani, F. & Peschle, C. 2007. MicroRNAs 17-5p-20a-106a control

monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol,* 9**,** 775-87.

Forson, A., Kudzawu, S., Kwara, A. & Flanigan, T. 2010. High Frequency of First-Line Anti-Tuberculosis Drug Resistance among Persons with Chronic Pulmonary Tuberculosis at a Teaching Hospital Chest Clinic. *Ghana Med J,* 44**,** 42-6.

Fu, Y., Yi, Z., Wu, X., Li, J. & Xu, F. 2011. Circulating MicroRNAs in Patients with Active Pulmonary Tuberculosis. *Journal of Clinical Microbiology,* 49**,** 4246-4251.

Fulton, S. A., Reba, S. M., Martin, T. D. & Boom, W. H. 2002. Neutrophil-mediated mycobacteriocidal immunity in the lung during Mycobacterium bovis BCG infection in C57BL/6 mice. *Infect Immun,* 70**,** 5322-7.

Furcolow, M. L., Hewell, B. & Nelson, W. E. 1942. Quantitative studies of the tuberculin reaction: tuberculin sensitivity in relation to active tuberculosis. *Am. Rev.Tuberc. ,* 45**,** 16.

Gallegos, A. M., Van Heijst, J. W., Samstein, M., Su, X., Pamer, E. G. & Glickman, M. S. 2011. A gamma interferon independent mechanism of CD4 T cell mediated control of M. tuberculosis infection in vivo. *PLoS Pathog,* 7**,** e1002052.

Gansert, J. L., Kiessler, V., Engele, M., Wittke, F., Rollinghoff, M., Krensky, A. M., Porcelli, S. A., Modlin, R. L. & Stenger, S. 2003. Human NKT cells express granulysin and exhibit antimycobacterial activity. *J Immunol,* 170**,** 3154-61.

Gantier, M. P., Sadler, A. J. & Williams, B. R. 2007. Fine-tuning of the innate immune response by microRNAs. *Immunol Cell Biol,* 85**,** 458-62.

Garcia, I., Miyazaki, Y., Marchal, G., Lesslauer, W. & Vassalli, P. 1997. High sensitivity of transgenic mice expressing soluble TNFR1 fusion protein to mycobacterial infections: synergistic action of TNF and IFN-gamma in the differentiation of protective granulomas. *Eur J Immunol,* 27**,** 3182-90.

Garrison, F. H. 1913. *An Introduction to the History of Medicine,* Philadelphia, W.B. Saunders Company.

Gatfield, J. & Pieters, J. 2000. Essential role for cholesterol in entry of mycobacteria into macrophages. *Science,* 288**,** 1647-50.

Gaynor, C. D., Mccormack, F. X., Voelker, D. R., Mcgowan, S. E. & Schlesinger, L. S. 1995. Pulmonary surfactant protein A mediates enhanced phagocytosis of Mycobacterium tuberculosis by a direct interaction with human macrophages. *J Immunol,* 155**,** 5343-51.

Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B. & Van Kooyk, Y. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med,* 197**,** 7-17.

Gndp, G. N. D. P. 2010. *Standard treatment Guideline,* Ghana, Ghana Ministry of Health.

Gordon, A. H., Hart, P. D. & Young, M. R. 1980. Ammonia inhibits phagosome-lysosome fusion in macrophages. *Nature,* 286**,** 79-80.

Goren, M. B., D'arcy Hart, P., Young, M. R. & Armstrong, J. A. 1976. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A,* 73**,** 2510-4.

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. & Shiekhattar, R. 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature,* 432**,** 235-40.

Gumperz, J. E. & Brenner, M. B. 2001. CD1-specific T cells in microbial immunity. *Curr Opin Immunol,* 13**,** 471-8.

Gunn, M. D., Tangemann, K., Tam, C., Cyster, J. G., Rosen, S. D. & Williams, L. T. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci U S A,* 95**,** 258-63.

Hackam, D., Rotstein, O.D., Zhang, W.J., Demaurex, N., Woodside, M., Tsai, O., Grinstein, S. 1997. Regulation of phagosomal acidification. Differential targeting of NaC/HC exchangers, NaC/KC-ATPases, and vacuolar-type HC-atpases. *J. Biol. Chem,* 272:**,** 10.

Hackam, D. J., Rotstein, O. D., Zhang, W. J., Demaurex, N., Woodside, M., Tsai, O. & Grinstein, S. 1997. Regulation of phagosomal acidification. Differential targeting of

Na+/H+ exchangers, Na+/K+-ATPases, and vacuolar-type H+-atpases. *J Biol Chem,* 272**,** 29810-20.

Hakimi, M.-A. & Cannella, D. 2011. Apicomplexan parasites and subversion of the host cell microRNA pathway. *Trends in parasitology,* 27**,** 481-486.

Hanekom, W. A. 2005. The immune response to BCG vaccination of newborns. *Ann N Y Acad Sci,* 1062**,** 69-78.

Hart, P. D., Young, M. R., Jordan, M. M., Perkins, W. J. & Geisow, M. J. 1983. Chemical inhibitors of phagosome-lysosome fusion in cultured macrophages also inhibit saltatory lysosomal movements. A combined microscopic and computer study. *J Exp Med,* 158**,** 477-92.

Harth, G., Clemens, D. L. & Horwitz, M. A. 1994. Glutamine synthetase of Mycobacterium tuberculosis: extracellular release and characterization of its enzymatic activity. *Proc Natl Acad Sci U S A,* 91**,** 9342-6.

Harth, G. & Horwitz, M. A. 1999. An inhibitor of exported Mycobacterium tuberculosis glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. *J. Exp. Med,* 189**,** 10.

Hasan, Z., Schlax, C., Kuhn, L., Lefkovits, I., Young, D., Thole, J. & Pieters, J. 1997. Isolation and characterization of the mycobacterial phagosome: segregation from the endosomal/lysosomal pathway. *Mol Microbiol,* 24**,** 545-53.

Henderson, R. A., Watkins, S. C. & Flynn, J. L. 1997. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. *J Immunol,* 159**,** 635-43.

Herzog, H. 1998. History of tuberculosis. *Respiration,* 65**,** 5-15.

Hirsch, C. S., Ellner, J. J., Blinkhorn, R. & Toossi, Z. 1997. In vitro restoration of T cell responses in tuberculosis and augmentation of monocyte effector function against Mycobacterium tuberculosis by natural inhibitors of transforming growth factor beta. *Proc Natl Acad Sci U S A,* 94**,** 3926-31.

Hirsch, C. S., Toossi, Z., Othieno, C., Johnson, J. L., Schwander, S. K., Robertson, S., Wallis, R. S., Edmonds, K., Okwera, A., Mugerwa, R., Peters, P. & Ellner, J. J. 1999. Depressed T-cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy. *J Infect Dis,* 180**,** 2069-73.

Hoft, D. F., Worku, S., Kampmann, B., Whalen, C. C., Ellner, J. J., Hirsch, C. S., Brown, R. B., Larkin, R., Li, Q., Yun, H. & Silver, R. F. 2002. Investigation of the relationships between immune-mediated inhibition of mycobacterial growth and other potential surrogate markers of protective Mycobacterium tuberculosis immunity. *J Infect Dis,* 186**,** 1448-57.

Hoheisel, G., Zheng, L., Teschler, H., Striz, I. & Costabel, U. 1995. Increased soluble CD14 levels in BAL fluid in pulmonary tuberculosis. *Chest,* 108**,** 1614-6.

Humphreys, I. R., Stewart, G. R., Turner, D. J., Patel, J., Karamanou, D., Snelgrove, R. J. & Young, D. B. 2006. A role for dendritic cells in the dissemination of mycobacterial infection. *Microbes Infect,* 8**,** 1339-46.

Hussey, G., Hawkridge, T. & Hanekom, W. 2007. Childhood tuberculosis: old and new vaccines. *Paediatr Respir Rev,* 8**,** 148-54.

Ilangumaran, S., Arni, S., Poincelet, M., Theler, J. M., Brennan, P. J., Nasir Ud, D. & Hoessli, D. C. 1995. Integration of mycobacterial lipoarabinomannans into glycosylphosphatidylinositol-rich domains of lymphomonocytic cell plasma membranes. *J Immunol,* 155**,** 1334-42.

Izzo, A. A. & North, R. J. 1992. Evidence for an alpha/beta T cell-independent mechanism of resistance to mycobacteria. Bacillus-Calmette-Guerin causes progressive infection in severe combined immunodeficient mice, but not in nude mice or in mice depleted of CD4+ and CD8+ T cells. *J Exp Med,* 176**,** 581-6.

Jackson, D. B. 2009. Serum-based microRNAs: are we blinded by potential? *Proc Natl Acad Sci U S A,* 106**,** E5.

Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. & Lanzavecchia, A. 2001. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur J Immunol,* 31**,** 3388-93.

Jayasankar, K. & Ramanathan, V. D. 1999. Biochemical & histochemical changes relating to fibrosis following infection with Mycobacterium tuberculosis in the guinea pig. *Indian J Med Res,* 110**,** 91-7.

Jiang, Q., Wang, Y., Hao, Y., Juan, L., Teng, M., Zhang, X., Li, M., Wang, G. & Liu, Y. 2009. miR2Disease: a manually curated database for microRNA deregulation in human disease. *Nucleic Acids Res,* 37**,** D98-104.

Jiang, W., Swiggard, W. J., Heufler, C., Peng, M., Mirza, A., Steinman, R. M. & Nussenzweig, M. C. 1995. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature,* 375**,** 151-5.

Jo, E. K., Park, J. K. & Dockrell, H. M. 2003. Dynamics of cytokine generation in patients with active pulmonary tuberculosis. *Curr Opin Infect Dis,* 16**,** 205-10.

Johnnidis, J. B., Harris, M. H., Wheeler, R. T., Stehling-Sun, S., Lam, M. H., Kirak, O., Brummelkamp, T. R., Fleming, M. D. & Camargo, F. D. 2008. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature,* 451**,** 1125-9.

Jones, G. S., Amirault, H. J. & Andersen, B. R. 1990. Killing of Mycobacterium tuberculosis by neutrophils: a nonoxidative process. *J Infect Dis,* 162**,** 700-4.

Jonsdottir, K., Janssen, S. R., Da Rosa, F. C., Gudlaugsson, E., Skaland, I., Baak, J. P. & Janssen, E. A. 2012. Validation of expression patterns for nine miRNAs in 204 lymph-node negative breast cancers. *PLoS One,* 7**,** e48692.

Jouanguy, E., Doffinger, R., Dupuis, S., Pallier, A., Altare, F. & Casanova, J. L. 1999. IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Curr Opin Immunol,* 11**,** 346-51.

Junqueira-Kipnis, A. P., Kipnis, A., Jamieson, A., Juarrero, M. G., Diefenbach, A., Raulet, D. H., Turner, J. & Orme, I. M. 2003. NK cells respond to pulmonary infection with Mycobacterium tuberculosis, but play a minimal role in protection. *J Immunol,* 171**,** 6039-45.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. & Liu, Y. J. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med,* 194**,** 863-9.

Kaiser, F., Cook, D., Papoutsopoulou, S., Rajsbaum, R., Wu, X., Yang, H. T., Grant, S., Ricciardi-Castagnoli, P., Tsichlis, P. N., Ley, S. C. & O'garra, A. 2009. TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. *J Exp Med,* 206**,** 1863-71.

Kalinski, P., Schuitemaker, J. H., Hilkens, C. M., Wierenga, E. A. & Kapsenberg, M. L. 1999. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. *J Immunol,* 162**,** 3231-6.

Kaufmann, S. H. 2001. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol,* 1**,** 20-30.

Kim, V. N. 2005. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol,* 6**,** 376-85.

Kim, V. N., Han, J. & Siomi, M. C. 2009. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol,* 10**,** 126-39.

Kim, V. N. & Nam, J. W. 2006. Genomics of microRNA. *Trends Genet,* 22**,** 165-73.

Kindler, V., Sappino, A. P., Grau, G. E., Piguet, P. F. & Vassalli, P. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell,* 56**,** 731-40.

Kleinsteuber, K., Heesch, K., Schattling, S., Kohns, M., Sander-Julch, C., Walzl, G., Hesseling, A., Mayatepek, E., Fleischer, B., Marx, F. M. & Jacobsen, M. 2013. Decreased expression of miR-21, miR-26a, miR-29a, and miR-142-3p in CD4(+) T cells and peripheral blood from tuberculosis patients. *PLoS One,* 8**,** e61609.

Kogo, R., Mimori, K., Tanaka, F., Komune, S. & Mori, M. 2011. Clinical significance of miR-146a in gastric cancer cases. *Clin Cancer Res,* 17**,** 4277-84.

Kohlhaas, S., Garden, O. A., Scudamore, C., Turner, M., Okkenhaug, K. & Vigorito, E. 2009. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol,* 182**,** 2578-82.

Kornfeld, S. 1987. Trafficking of lysosomal enzymes. *FASEB J,* 1**,** 462-8.

Kriehuber, E., Breiteneder-Geleff, S., Groeger, M., Soleiman, A., Schoppmann, S. F., Stingl, G., Kerjaschki, D. & Maurer, D. 2001. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med,* 194**,** 797-808.

Krutzfeldt, J. & Stoffel, M. 2006. MicroRNAs: a new class of regulatory genes affecting metabolism. *Cell Metab,* 4**,** 9-12.

Ladel, C. H., Hess, J., Daugelat, S., Mombaerts, P., Tonegawa, S. & Kaufmann, S. H. 1995. Contribution of alpha/beta and gamma/delta T lymphocytes to immunity against Mycobacterium bovis bacillus Calmette Guerin: studies with T cell receptor-deficient mutant mice. *Eur J Immunol,* 25**,** 838-46.

Ladel, C. H., Szalay, G., Riedel, D. & Kaufmann, S. H. 1997. Interleukin-12 secretion by Mycobacterium tuberculosis-infected macrophages. *Infect Immun,* 65**,** 1936-8.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. 2001. Identification of novel genes coding for small expressed RNAs. *Science,* 294**,** 853-8.

Lalvani, A., Brookes, R., Wilkinson, R. J., Malin, A. S., Pathan, A. A., Andersen, P., Dockrell, H., Pasvol, G. & Hill, A. V. 1998. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A,* 95**,** 270-5.

Lalvani, A., Pathan, A. A., Mcshane, H., Wilkinson, R. J., Latif, M., Conlon, C. P., Pasvol, G. & Hill, A. V. 2001. Rapid detection of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells. *Am J Respir Crit Care Med,* 163**,** 824-8.

Lau, N. C., Lim, L. P., Weinstein, E. G. & Bartel, D. P. 2001. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science,* 294**,** 858-62.

Le Cabec, V., Cols, C. & Maridonneau-Parini, I. 2000. Nonopsonic phagocytosis of zymosan and Mycobacterium kansasii by CR3 (CD11b/CD18) involves distinct molecular determinants and is or is not coupled with NADPH oxidase activation. *Infect Immun,* 68**,** 4736-45.

Lee, R. C. & Ambros, V. 2001. An extensive class of small RNAs in Caenorhabditis elegans. *Science,* 294**,** 862-4.

Lee, R. C., Feinbaum, R. L. & Ambros, V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell,* 75**,** 843-54.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. & Kim, V. N. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature,* 425**,** 415-9.

Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H. & Kim, V. N. 2004. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J,* 23**,** 4051-60.

Li, Q. J., Chau, J., Ebert, P. J., Sylvester, G., Min, H., Liu, G., Braich, R., Manoharan, M., Soutschek, J., Skare, P., Klein, L. O., Davis, M. M. & Chen, C. Z. 2007. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell,* 129**,** 147-61.

Lin, Y., Zhang, M., Hofman, F. M., Gong, J. & Barnes, P. F. 1996. Absence of a prominent Th2 cytokine response in human tuberculosis. *Infect Immun,* 64**,** 1351-6.

Liu, G., Friggeri, A., Yang, Y., Park, Y. J., Tsuruta, Y. & Abraham, E. 2009. miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. *Proc Natl Acad Sci U S A,* 106**,** 15819-24.

Liu, Z., Xiao, B., Tang, B., Li, B., Li, N., Zhu, E., Guo, G., Gu, J., Zhuang, Y., Liu, X., Ding, H., Zhao, X., Guo, H., Mao, X. & Zou, Q. 2010. Up-regulated microRNA-146a negatively modulate Helicobacter pylori-induced inflammatory response in human gastric epithelial cells. *Microbes Infect,* 12**,** 854-63.

Lowrie, D. B., Tascon, R. E., Bonato, V. L., Lima, V. M., Faccioli, L. H., Stavropoulos, E., Colston, M. J., Hewinson, R. G., Moelling, K. & Silva, C. L. 1999. Therapy of tuberculosis in mice by DNA vaccination. *Nature,* 400**,** 269-71.

Lyadova, I., Yeremeev, V., Majorov, K., Nikonenko, B., Khaidukov, S., Kondratieva, T., Kobets, N. & Apt, A. 1998. An ex vivo study of T lymphocytes recovered from the lungs of I/St mice infected with and susceptible to Mycobacterium tuberculosis. *Infect Immun,* 66**,** 4981-8.

Ma, F., Xu, S., Li, X., Zhang, Q., Xu, X., Liu, M., Hua, M., Li, N., Yao, H. & Cao, X. 2011a. **The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon**-g. *nature immunology***,** 1-10.

Ma, F., Xu, S., Liu, X., Zhang, Q., Xu, X., Liu, M., Hua, M., Li, N., Yao, H. & Cao, X. 2011b. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. *Nat Immunol*, 12**,** 861-9.

Macmicking, J., Xie, Q. W. & Nathan, C. 1997. Nitric oxide and macrophage function. *Annu Rev Immunol*, 15**,** 323-50.

Majeed, M., Perskvist, N., Ernst, J. D., Orselius, K. & Stendahl, O. 1998. Roles of calcium and annexins in phagocytosis and elimination of an attenuated strain of Mycobacterium tuberculosis in human neutrophils. *Microb Pathog*, 24**,** 309-20.

Mcshane, H. 2011. Tuberculosis vaccines: beyond bacille Calmette-Guerin. *Philos Trans R Soc Lond B Biol Sci*, 366**,** 2782-9.

Mellman, I., Fuchs, R. & Helenius, A. 1986. Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem*, 55**,** 663-700.

Mittrucker, H. W., Steinhoff, U., Kohler, A., Krause, M., Lazar, D., Mex, P., Miekley, D. & Kaufmann, S. H. 2007. Poor correlation between BCG vaccination-induced T cell

responses and protection against tuberculosis. *Proc Natl Acad Sci U S A,* 104**,** 12434-9.

Mogues, T., Goodrich, M. E., Ryan, L., Lacourse, R. & North, R. J. 2001. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *J Exp Med,* 193**,** 271-80.

Monticelli, S., Ansel, K. M., Xiao, C., Socci, N. D., Krichevsky, A. M., Thai, T. H., Rajewsky, N., Marks, D. S., Sander, C., Rajewsky, K., Rao, A. & Kosik, K. S. 2005. MicroRNA profiling of the murine hematopoietic system. *Genome Biol,* 6**,** R71.

Moreira, A. L., Tsenova-Berkova, L., Wang, J., Laochumroonvorapong, P., Freeman, S., Freedman, V. H. & Kaplan, G. 1997. Effect of cytokine modulation by thalidomide on the granulomatous response in murine tuberculosis. *Tuber Lung Dis,* 78**,** 47-55.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol,* 136**,** 2348-57.

Muller, I., Cobbold, S. P., Waldmann, H. & Kaufmann, S. H. 1987. Impaired resistance to Mycobacterium tuberculosis infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. *Infect Immun,* 55**,** 2037-41.

Munk, M. E., Gatrill, A. J. & Kaufmann, S. H. 1990. Target cell lysis and IL-2 secretion by gamma/delta T lymphocytes after activation with bacteria. *J Immunol,* 145**,** 2434-9.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O. & Jones, J. D. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science,* 312**,** 436-9.

Newport, M., Levin, M., Blackwell, J., Shaw, M. A., Williamson, R. & Huxley, C. 1995. Evidence for exclusion of a mutation in NRAMP as the cause of familial disseminated atypical mycobacterial infection in a Maltese kindred. *J Med Genet,* 32**,** 904-6.

Nicholson, S., Bonecini-Almeida Mda, G., Lapa E Silva, J. R., Nathan, C., Xie, Q. W., Mumford, R., Weidner, J. R., Calaycay, J., Geng, J., Boechat, N., Linhares, C., Rom, W. & Ho, J. L. 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med,* 183**,** 2293-302.

Nielsen, L. B., Wang, C., Sorensen, K., Bang-Berthelsen, C. H., Hansen, L., Andersen, M. L., Hougaard, P., Juul, A., Zhang, C. Y., Pociot, F. & Mortensen, H. B. 2012. Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. *Exp Diabetes Res,* 2012**,** 896362.

North, R. J. & Jung, Y.-J. 2004. Immunity to Tuberculosis. *Annu. Rev. Immunol.,* 22**,** 599-623.

O'connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G. & Baltimore, D. 2007. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci U S A,* 104**,** 1604-9.

Oddo, M., Renno, T., Attinger, A., Bakker, T., Macdonald, H. R. & Meylan, P. R. 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. *J Immunol,* 160**,** 5448-54.

Ogata, K., Linzer, B. A., Zuberi, R. I., Ganz, T., Lehrer, R. I. & Catanzaro, A. 1992. Activity of defensins from human neutrophilic granulocytes against Mycobacterium avium-Mycobacterium intracellulare. *Infect Immun,* 60**,** 4720-5.

Okamura, K., Hagen, J. W., Duan, H., Tyler, D. M. & Lai, E. C. 2007. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. *Cell,* 130**,** 89-100.

Orme, I. M. 1987. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with Mycobacterium tuberculosis. *J Immunol,* 138**,** 293-8.

Orme, I. M. & Collins, F. M. 1983. Protection against Mycobacterium tuberculosis infection by adoptive immunotherapy. Requirement for T cell-deficient recipients. *J Exp Med,* 158**,** 74-83.

Orme, I. M. & Collins, F. M. 1984. Adoptive protection of the Mycobacterium tuberculosis-infected lung. Dissociation between cells that passively transfer protective immunity and those that transfer delayed-type hypersensitivity to tuberculin. *Cell Immunol,* 84**,** 113-20.

Orme, I. M., Miller, E. S., Roberts, A. D., Furney, S. K., Griffin, J. P., Dobos, K. M., Chi, D., Rivoire, B. & Brennan, P. J. 1992. T lymphocytes mediating protection and cellular cytolysis during the course of Mycobacterium tuberculosis infection. Evidence for

different kinetics and recognition of a wide spectrum of protein antigens. *J Immunol,* 148**,** 189-96.

Orme, I. M., Roberts, A. D., Griffin, J. P. & Abrams, J. S. 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection. *J Immunol,* 151**,** 518-25.

Ottenhoff, T. H., Kumararatne, D. & Casanova, J. L. 1998. Novel human immunodeficiencies reveal the essential role of type-I cytokines in immunity to intracellular bacteria. *Immunol Today,* 19**,** 491-4.

Owusu-Dabo, E., Adjei, O., Meyer, C. G., Horstmann, R. D., Enimil, A., Kruppa, T. F., Bonsu, F., Browne, E. N., Chinbuah, M. A., Osei, I., Gyapong, J., Berberich, C., Kubica, T., Niemann, S. & Ruesch-Gerdes, S. 2006. Mycobacterium tuberculosis drug resistance, Ghana. *Emerg Infect Dis,* 12**,** 1171-2.

Palomino, J. C., Leão, S. C. & Ritacco, V. 2007. *Tuberculosis 2007 ;From basic science to patient care*.

Pauley, K. M., Cha, S. & Chan, E. K. 2009. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun,* 32**,** 189-94.

Pedroza-Gonzalez, A., Garcia-Romo, G. S., Aguilar-Leon, D., Calderon-Amador, J., Hurtado-Ortiz, R., Orozco-Estevez, H., Lambrecht, B. N., Estrada-Garcia, I., Hernandez-Pando, R. & Flores-Romo, L. 2004. In situ analysis of lung antigen-

presenting cells during murine pulmonary infection with virulent Mycobacterium tuberculosis. *Int J Exp Pathol,* 85**,** 135-45.

Peterson, P. K., Gekker, G., Hu, S., Sheng, W. S., Anderson, W. R., Ulevitch, R. J., Tobias, P. S., Gustafson, K. V., Molitor, T. W. & Chao, C. C. 1995. CD14 receptor-mediated uptake of nonopsonized Mycobacterium tuberculosis by human microglia. *Infect Immun,* 63**,** 1598-602.

Pfyffer, G. E., Auckenthaler, R., Van Embden, J. D. & Van Soolingen, D. 1998. Mycobacterium canettii, the smooth variant of M. tuberculosis, isolated from a Swiss patient exposed in Africa. *Emerg Infect Dis,* 4**,** 631-4.

Pieters, J. 2001. Entry and survival of pathogenic mycobacteria in macrophages. *Microbes Infect,* 3**,** 249-55.

Pulikkan, J. A., Dengler, V., Peramangalam, P. S., Peer Zada, A. A., Muller-Tidow, C., Bohlander, S. K., Tenen, D. G. & Behre, G. 2010. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood,* 115**,** 1768-78.

Qi, Y., Cui, L., Ge, Y., Shi, Z., Zhao, K., Guo, X., Yang, D., Yu, H., Cui, L., Shan, Y., Zhou, M., Wang, H. & Lu, Z. 2012. Altered serum microRNAs as biomarkers for the early diagnosis of pulmonary tuberculosis infection. *BMC Infect Dis,* 12**,** 384.

Raja, A. 2004. Immunology of tuberculosis. *Indian J Med Res,* 120**,** 213-32.

Rajaram, M. V., Ni, B., Morris, J. D., Brooks, M. N., Carlson, T. K., Bakthavachalu, B., Schoenberg, D. R., Torrelles, J. B. & Schlesinger, L. S. 2011. Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. *Proc Natl Acad Sci U S A,* 108**,** 17408-13.

Randhawa, A. K., Ziltener, H. J., Merzaban, J. S. & Stokes, R. W. 2005. CD43 is required for optimal growth inhibition of Mycobacterium tuberculosis in macrophages and in mice. *J Immunol,* 175**,** 1805-12.

Randhawa, P. S. 1990. Lymphocyte subsets in granulomas of human tuberculosis: an in situ immunofluorescence study using monoclonal antibodies. *Pathology,* 22**,** 153-5.

Redford, P. S., Murray, P. J. & O'garra, A. 2011. The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunol,* 4**,** 261-70.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. & Ruvkun, G. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature,* 403**,** 901-6.

Ren, C., Chen, H., Han, C., Wang, D. & Fu, D. 2012. Increased plasma microRNA and CD133/CK18-positive cancer cells in the pleural fluid of a pancreatic cancer patient with liver and pleural metastases and correlation with chemoresistance. *Oncol Lett,* 4**,** 691-694.

Reyrat, J. M., Berthet, F. X. & Gicquel, B. 1995. The urease locus of Mycobacterium tuberculosis and its utilization for the demonstration of allelic exchange in Mycobacterium bovis bacillus Calmette-Guerin. *Proc Natl Acad Sci U S A,* 92**,** 8768-72.

Reyrat, J. M., Lopez-Ramirez, G., Ofredo, C., Gicquel, B. & Winter, N. 1996. Urease activity does not contribute dramatically to persistence of Mycobacterium bovis bacillus Calmette-Guerin. *Infect Immun,* 64**,** 3934-6.

Rich, A. R. 1944. The pathogenesis of tuberculosis. *J Am Med Assoc,* 126.

Riedel, D. D. & Kaufmann, S. H. 1997. Chemokine secretion by human polymorphonuclear granulocytes after stimulation with Mycobacterium tuberculosis and lipoarabinomannan. *Infect Immun,* 65**,** 4620-3.

Roche, P. W., Triccas, J. A. & Winter, N. 1995. BCG vaccination against tuberculosis: past disappointments and future hopes. *Trends Microbiol,* 3**,** 397-401.

Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L. & Bradley, A. 2004. Identification of mammalian microRNA host genes and transcription units. *Genome Res,* 14**,** 1902-10.

Rodriguez, A., Vigorito, E., Clare, S., Warren, M. V., Couttet, P., Soond, D. R., Van Dongen, S., Grocock, R. J., Das, P. P., Miska, E. A., Vetrie, D., Okkenhaug, K., Enright, A. J., Dougan, G., Turner, M. & Bradley, A. 2007. Requirement of bic/microRNA-155 for normal immune function. *Science,* 316**,** 608-11.

Rogers, N. C., Slack, E. C., Edwards, A. D., Nolte, M. A., Schulz, O., Schweighoffer, E., Williams, D. L., Gordon, S., Tybulewicz, V. L., Brown, G. D. & Reis E Sousa, C. 2005. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity,* 22**,** 507-17.

Rook G.A.W. & Hernandez-Pando R. 1996. The Pathogenesis of Tuberculosis. *Annu Rev. Microbiol.,* 50**,** 259-284.

Rook, G. A. 1990. Mycobacteria, cytokines and antibiotics. *Pathol Biol (Paris),* 38**,** 276-80.

Rook, G. A., Taverne, J., Leveton, C. & Steele, J. 1987. The role of gamma-interferon, vitamin D3 metabolites and tumour necrosis factor in the pathogenesis of tuberculosis. *Immunology,* 62**,** 229-34.

Rosa, A., Ballarino, M., Sorrentino, A., Sthandier, O., De Angelis, F. G., Marchioni, M., Masella, B., Guarini, A., Fatica, A., Peschle, C. & Bozzoni, I. 2007. The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage differentiation. *Proc Natl Acad Sci U S A,* 104**,** 19849-54.

Ruby, J. G., Jan, C. H. & Bartel, D. P. 2007. Intronic microRNA precursors that bypass Drosha processing. *Nature,* 448**,** 83-6.

Russell, D. G. 1995. Mycobacterium and Leishmania: stowaways in the endosomal network. *Trends Cell Biol,* 5**,** 125-8.

Russell, D. G. 2007. Who puts the tubercle in tuberculosis? *Nat Rev Microbiol,* 5**,** 39-47.

Sakula, A. 1979. Robert Koch (1843--1910): founder of the science of bacteriology and discoverer of the tubercle bacillus. A study of his life and work. *Br J Dis Chest,* 73**,** 389-94.

Salgame, P., Abrams, J. S., Clayberger, C., Goldstein, H., Convit, J., Modlin, R. L. & Bloom, B. R. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science,* 254**,** 279-82.

Saunders, B. M., Frank, A. A., Orme, I. M. & Cooper, A. M. 2002. CD4 is required for the development of a protective granulomatous response to pulmonary tuberculosis. *Cell Immunol,* 216**,** 65-72.

Scanga, C. A., Mohan, V. P., Yu, K., Joseph, H., Tanaka, K., Chan, J. & Flynn, J. L. 2000. Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. *J Exp Med,* 192**,** 347-58.

Schetter, A. J., Heegaard, N. H. & Harris, C. C. 2010. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis,* 31**,** 37-49.

Schlesinger, L. S. 1993. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. *J Immunol,* 150**,** 2920-30.

Schlesinger, L. S. 1996. Role of mononuclear phagocytes in M tuberculosis pathogenesis. *J Investig Med,* 44**,** 312-23.

Schlesinger, L. S., Bellinger-Kawahara, C. G., Payne, N. R. & Horwitz, M. A. 1990. Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. *J Immunol,* 144**,** 2771-80.

Schnitger, A. K., Machova, A., Mueller, R. U., Androulidaki, A., Schermer, B., Pasparakis, M., Kronke, M. & Papadopoulou, N. 2011. Listeria monocytogenes infection in macrophages induces vacuolar-dependent host miRNA response. *PLoS One,* 6**,** e27435.

Schulte, L. N., Eulalio, A., Mollenkopf, H. J., Reinhardt, R. & Vogel, J. 2011. Analysis of the host microRNA response to Salmonella uncovers the control of major cytokines by the let-7 family. *EMBO J,* 30**,** 1977-89.

Selwyn, P. A., Hartel, D., Lewis, V. A., Schoenbaum, E. E., Vermund, S. H., Klein, R. S., Walker, A. T. & Friedland, G. H. 1989. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. *N Engl J Med,* 320**,** 545-50.

Serbina, N. V. & Flynn, J. L. 1999. Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of Mycobacterium tuberculosis-infected mice. *Infect Immun,* 67**,** 3980-8.

Serbina, N. V., Lazarevic, V. & Flynn, J. L. 2001. CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection. *J Immunol,* 167**,** 6991-7000.

Sharbati, J., Lewin, A., Kutz-Lohroff, B., Kamal, E., Einspanier, R. & Sharbati, S. 2011. Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection. *PLoS One,* 6**,** e20258.

Shiloh, M. U. & Nathan, C. F. 2000. Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. *Curr Opin Microbiol,* 3**,** 35-42.

Silva, M. V., Massaro Junior, V., Machado, J. R., Castellano, L. R., Rodrigues, D. B. R. J. & Rodrigues, V. 2012. Time-dependent IFN-{gamma} and CXCL-10 production in tuberculosis patients after the establishment of clinical cure. *Journal of Immunology,* 188.

Singh, P. K., Singh, A. V. & Chauhan, D. S. 2013a. Current understanding on micro RNAs and its regulation in response to Mycobacterial infections. *Journal of biomedical science,* 20**,** 1-9.

Singh, Y., Kaul, V., Mehra, A., Chatterjee, S., Tousif, S., Dwivedi, V. P., Suar, M., Van Kaer, L., Bishai, W. R. & Das, G. 2013b. Mycobacterium tuberculosis controls microRNA-99b (miR-99b) expression in infected murine dendritic cells to modulate host immunity. *J Biol Chem,* 288**,** 5056-61.

Steiner, D. F., Thomas, M. F., Hu, J. K., Yang, Z., Babiarz, J. E., Allen, C. D., Matloubian, M., Blelloch, R. & Ansel, K. M. 2011. MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells. *Immunity,* 35**,** 169-81.

Steinman, R. M. 2001. Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. *Mt Sinai J Med,* 68**,** 160-6.

Stenger, S., Hanson, D. A., Teitelbaum, R., Dewan, P., Niazi, K. R., Froelich, C. J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S. A., Bloom, B. R., Krensky, A. M. & Modlin, R. L. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science,* 282**,** 121-5.

Stenger, S., Mazzaccaro, R. J., Uyemura, K., Cho, S., Barnes, P. F., Rosat, J. P., Sette, A., Brenner, M. B., Porcelli, S. A., Bloom, B. R. & Modlin, R. L. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science,* 276**,** 1684-7.

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. & Russell, D. G. 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science,* 263**,** 678-81.

Styblo, K. 1980. Recent advances in epidemiological research in tuberculosis. *Adv Tuberc Res,* 20**,** 1-63.

Sugawara, I., Yamada, H., Mizuno, S., Li, C. Y., Nakayama, T. & Taniguchi, M. 2002. Mycobacterial infection in natural killer T cell knockout mice. *Tuberculosis (Edinb),* 82**,** 97-104.

Taganov, K. D., Boldin, M. P. & Baltimore, D. 2007. MicroRNAs and immunity: tiny players in a big field. *Immunity,* 26**,** 133-7.

Taganov, K. D., Boldin, M. P., Chang, K. J. & Baltimore, D. 2006. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A,* 103**,** 12481-6.

Tailleux, L., Schwartz, O., Herrmann, J. L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L. P., Gluckman, J. C., Lagrange, P. H., Gicquel, B. & Neyrolles, O. 2003. DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. *J Exp Med,* 197**,** 121-7.

Tan, B. H., Meinken, C., Bastian, M., Bruns, H., Legaspi, A., Ochoa, M. T., Krutzik, S. R., Bloom, B. R., Ganz, T., Modlin, R. L. & Stenger, S. 2006. Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol,* 177**,** 1864-71.

Thai, T. H., Calado, D. P., Casola, S., Ansel, K. M., Xiao, C., Xue, Y., Murphy, A., Frendewey, D., Valenzuela, D., Kutok, J. L., Schmidt-Supprian, M., Rajewsky, N., Yancopoulos, G., Rao, A. & Rajewsky, K. 2007. Regulation of the germinal center response by microRNA-155. *Science,* 316**,** 604-8.

Thurnher, M., Ramoner, R., Gastl, G., Radmayr, C., Bock, G., Herold, M., Klocker, H. & Bartsch, G. 1997. Bacillus Calmette-Guerin mycobacteria stimulate human blood dendritic cells. *Int J Cancer,* 70**,** 128-34.

Tili, E., Michaille, J. J., Cimino, A., Costinean, S., Dumitru, C. D., Adair, B., Fabbri, M., Alder, H., Liu, C. G., Calin, G. A. & Croce, C. M. 2007. Modulation of miR-155 and

miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol,* 179**,** 5082-9.

Ting, L. M., Kim, A. C., Cattamanchi, A. & Ernst, J. D. 1999. Mycobacterium tuberculosis inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. *J Immunol,* 163**,** 3898-906.

Tiruviluamala, P. & Reichman, L. B. 2002. Tuberculosis. *Annu Rev Public Health,* 23**,** 403-26.

Toossi, Z., Gogate, P., Shiratsuchi, H., Young, T. & Ellner, J. J. 1995. Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. *J Immunol,* 154**,** 465-73.

Turcatel, G., Rubin, N., El-Hashash, A. & Warburton, D. 2012. MIR-99a and MIR-99b modulate TGF-beta induced epithelial to mesenchymal plasticity in normal murine mammary gland cells. *PLoS One,* 7**,** e31032.

Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Unternaehrer, J., Steinman, R. M. & Mellman, I. 2000. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science,* 288**,** 522-7.

Ulrichs, T., Anding, P., Porcelli, S., Kaufmann, S. H. & Munk, M. E. 2000. Increased numbers of ESAT-6-and purified protein derivative-specific gamma interferon-producing cells in subclinical and active tuberculosis infection. *Infection and immunity,* 68**,** 6073-6076.

Urban, C. F., Lourido, S. & Zychlinsky, A. 2006. How do microbes evade neutrophil killing? *Cell Microbiol,* 8**,** 1687-96.

Vanheyningen, T. K., Collins, H. L. & Russell, D. G. 1997. IL-6 produced by macrophages infected with Mycobacterium species suppresses T cell responses. *J Immunol,* 158**,** 330-7.

Vankayalapati, R., Klucar, P., Wizel, B., Weis, S. E., Samten, B., Safi, H., Shams, H. & Barnes, P. F. 2004. NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen. *J Immunol,* 172**,** 130-7.

Vankayalapati, R., Wizel, B., Weis, S. E., Safi, H., Lakey, D. L., Mandelboim, O., Samten, B., Porgador, A. & Barnes, P. F. 2002. The NKp46 receptor contributes to NK cell lysis of mononuclear phagocytes infected with an intracellular bacterium. *J Immunol,* 168**,** 3451-7.

Verbon, A., Juffermans, N., Van Deventer, S. J., Speelman, P., Van Deutekom, H. & Van Der Poll, T. 1999. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol,* 115**,** 110-3.

Vigorito, E., Perks, K. L., Abreu-Goodger, C., Bunting, S., Xiang, Z., Kohlhaas, S., Das, P. P., Miska, E. A., Rodriguez, A., Bradley, A., Smith, K. G., Rada, C., Enright, A. J., Toellner, K. M., Maclennan, I. C. & Turner, M. 2007. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity,* 27**,** 847-59.

Voskuil, M. I., Bartek, I. L., Visconti, K. & Schoolnik, G. K. 2011. The response of mycobacterium tuberculosis to reactive oxygen and nitrogen species. *Front Microbiol,* 2**,** 105.

Walker, L. & Lowrie, D. B. 1981. Killing of Mycobacterium microti by immunologically activated macrophages. *Nature,* 293**,** 69-71.

Wang, C. H., Liu, C. Y., Lin, H. C., Yu, C. T., Chung, K. F. & Kuo, H. P. 1998. Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages. *Eur Respir J,* 11**,** 809-15.

Wang, J., Wakeham, J., Harkness, R. & Xing, Z. 1999. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J Clin Invest,* 103**,** 1023-9.

Wang, R., Wang, H. B., Hao, C. J., Cui, Y., Han, X. C., Hu, Y., Li, F. F., Xia, H. F. & Ma, X. 2012. MiR-101 is involved in human breast carcinogenesis by targeting Stathmin1. *PLoS One,* 7**,** e46173.

Who 2010. *Global tuberculosis control: WHO report,* Global Tuberculosis Programme, World Health Organization.

Who 2011. *Global tuberculosis control :WHO Report*, Global Tuberculosis Programme, World Health Organization.

Who 2012. *Global tuberculosis control :WHO Report,* France.

Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol,* 11**,** 228-34.

Wolf, A. J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K. & Ernst, J. D. 2008. Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J Exp Med,* 205**,** 105-15.

Wozniak, T. M., Ryan, A. A., Triccas, J. A. & Britton, W. J. 2006. Plasmid interleukin-23 (IL-23), but not plasmid IL-27, enhances the protective efficacy of a DNA vaccine against Mycobacterium tuberculosis infection. *Infect Immun,* 74**,** 557-65.

Www.Unaids.Org/En/Dataanalysis/Epidemiology. [Accessed 27th June, 2011 2011].

Xiao, B., Liu, Z., Li, B. S., Tang, B., Li, W., Guo, G., Shi, Y., Wang, F., Wu, Y., Tong, W. D., Guo, H., Mao, X. H. & Zou, Q. M. 2009. Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response. *J Infect Dis,* 200**,** 916-25.

Xiao, C., Calado, D. P., Galler, G., Thai, T. H., Patterson, H. C., Wang, J., Rajewsky, N., Bender, T. P. & Rajewsky, K. 2007. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell,* 131**,** 146-59.

Xiao, C. & Rajewsky, K. 2009. MicroRNA control in the immune system: basic principles. *Cell,* 136**,** 26-36.

Yi, Z., Fu, Y., Ji, R., Li, R. & Guan, Z. 2012. Altered microRNA signatures in sputum of patients with active pulmonary tuberculosis. *PLoS One,* 7**,** e43184.

Yu, Y., Zhang , Y., Hu , S., Jin, D., Chen, X., Jin, Q. & Liu, H. 2012. Different Patterns of Cytokines and Chemokines Combined with IFN-c Production Reflect Mycobacterium tuberculosis Infection and Disease. *PLoS ONE,* 7.

Zhang, B. & Farwell, M. A. 2008. microRNAs: a new emerging class of players for disease diagnostics and gene therapy. *J Cell Mol Med,* 12**,** 3-21.

Zhang, B., Liu, X. X., He, J. R., Zhou, C. X., Guo, M., He, M., Li, M. F., Chen, G. Q. & Zhao, Q. 2011. Pathologically decreased miR-26a antagonizes apoptosis and facilitates carcinogenesis by targeting MTDH and EZH2 in breast cancer. *Carcinogenesis,* 32**,** 2-9.

Zhang, B., Pan, X., Cobb, G. P. & Anderson, T. A. 2007. microRNAs as oncogenes and tumor suppressors. *Dev Biol,* 302**,** 1-12.

Zhang, M., Gong, J., Iyer, D. V., Jones, B. E., Modlin, R. L. & Barnes, P. F. 1994. T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection. *J Clin Invest,* 94**,** 2435-42.

Zhang, M., Lin, Y., Iyer, D. V., Gong, J., Abrams, J. S. & Barnes, P. F. 1995. T-cell cytokine responses in human infection with Mycobacterium tuberculosis. *Infect Immun,* 63**,** 3231-4.

Zhang, Y., Zhang, B., Zhang, A., Li, X., Liu, J., Zhao, J., Zhao, Y., Gao, J., Fang, D. & Rao, Z. 2013. IL-6 upregulation contributes to the reduction of miR-26a expression in hepatocellular carcinoma cells. *Braz J Med Biol Res,* 46**,** 32-8.

Zhou, B., Wang, S., Mayr, C., Bartel, D. P. & Lodish, H. F. 2007. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci U S A,* 104**,** 7080-5.

Zimmerli, S., Edwards, S. & Ernst, J. D. 1996. Selective receptor blockade during phagocytosis does not alter the survival and growth of Mycobacterium tuberculosis in human macrophages. *Am J Respir Cell Mol Biol,* 15**,** 760-70.

APPENDICES

APPENDIX A

PATIENT DEMOGRAPHICS AND LABORATORY CODING

Table 13 PATIENT DEMOGRAPHICS AND LABORATORY CODING

PATIENT	AGE	SEX	BCG SCAR	CLASSIFICATION	DIAGNOSIS	CONCOMITANT DISEASE	THERAPY	TP0	TP1	TP6
001								TB001		
002	24	M	Y	NEW CASE	PTB	NONE	HRZE	TB002	TB010	TB067
003	30	F	Y	NEW CASE	PTB	NONE	HRZE	TB003	TB009	TB088
004								TB004		
005	41	M	Y	NEW CASE	PTB	NONE	HRZE	TB005	TB011	TB081
006	27	M	Y	NEW CASE	PTB	NONE	HRZE	TB006	TB012	TB107
007	33	F	Y	NEW CASE	PTB	NONE	HRZE	TB007	TB016	
008	30	F	Y	NEW CASE	PTB	NONE	HRZE	TB008	TB019	
009	26	F	Y	NEW CASE	PTB	NONE	HRZE	TB014	TB020	TB068
010	40	M	Y	NEW CASE	PTB	NONE	HRZE	TB013		
011	40	F	Y	NEW CASE	PTB	NONE	HRZE	TB015	TB025	TB070
012	31	M	Y	NEW CASE	PTB	NONE	HRZE	TB017	TB026	TB077
013	32	M	N	NEW CASE	PTB	NONE	HRZE	TB021	TB029	TB079
014	44	M	N	NEW CASE	PTB	NONE	HRZE	TB022		
015	35	F	N	NEW CASE	PTB	NONE	HRZE	TB023	TB033	TB078
016	32	M	Y	NEW CASE	PTB	NONE	HRZE	TB024	TB034	TB086
017	28	F	N	NEW CASE	PTB	NONE	HRZE	TB027	TB039	TB091
018	46	M	N	NEW CASE	PTB	NONE	HRZE	TB028	TB040	TB087
019	57	F	N	NEW CASE	PTB	NONE	HRZE	TB030	TB042	TB089

020	39	M	N	NEW CASE	PTB	NONE	HRZE	TB031	TB043	TB093
021	60	M	N	NEW CASE	PTB	NONE	HRZE	TB032		
022	38	M	Y	NEW CASE	PTB	NONE	HRZE	TB035	TB045	TB092
023	32	F	Y	NEW CASE	PTB	NONE	HRZE	TB036	TB046	TB106
024	50	M	Y	NEW CASE	PTB	NONE	HRZE	TB037	TB064	TB102
025	54	F	Y	NEW CASE	PTB	NONE	HRZE	TB038		
026	19	M	Y	NEW CASE	PTB	NONE	HRZE	TB041	TB066	TB114
027	28	M	N	NEW CASE	PTB	HYPERTENSION	HRZE	TB044	TB059	TB101
028	44	M	Y	NEW CASE	PTB	HYPERTENSION	HRZE	TB047	TB063	TB105
029	29	M	Y	NEW CASE	PTB	NONE	HRZE	TB048	TB085	TB130
030	18	F	Y	NEW CASE	PTB	NONE	HRZE	TB049	TB060	TB125
031	42	M	Y	NEW CASE	PTB	NONE	HRZE	TB050	TB061	TB099
032	48	F	N	NEW CASE	PTB	NONE	HRZE	TB051	TB062	TB104
033	26	M	Y	NEW CASE	PTB	NONE	HRZE	TB052	TB071	TB109
034	33	M		NEW CASE	PTB	NONE	HRZE	TB053		
035	45	M	Y	NEW CASE	PTB	NONE	HRZE	TB054	TB065	TB111
036	71	M		NEW CASE	PTB	HYPERTENSION	HRZE	TB055	TB069	
037	61	M	N	NEW CASE	PTB	NONE	HRZE	TB056	TB072	TB143
038	28	M	Y	NEW CASE	PTB	NONE	HRZE	TB057	TB074	TB113
039	32	F		NEW CASE	PTB	NONE	HRZE	TB058	TB073	TB112
040								TB075		
041	33	M						TB076		
042	28	M	N	NEW CASE	PTB	NONE	HRZE	TB080	TB090	TB144
043	60	M	N	NEW CASE	PTB	NONE	HRZE	TB082	TB097	TB138
044	27	M	N	NEW CASE	PTB	NONE	HRZE	TB083	TB098	
045	59	F	N	NEW CASE	PTB	HYPERTENSION	HRZE	TB084	TB108	TB142
046	41	M	N	NEW CASE	PTB	NONE	HRZE	TB094	TB100	TB141
047	28	F	N	NEW CASE	PTB	DYSMENORRHOEA	HRZE	TB095	TB103	
048	32	F	N	NEW CASE	PTB	HIV	HRZE	TB096	TB110	TB145

049	44	M	N	NEW CASE	PTB	NONE	HRZE	TB115	TB122	
050	50	M	N	NEW CASE	PTB	NONE	HRZE	TB116	TB123	TB146
051	29	M	N	NEW CASE	PTB	NONE	HRZE	TB117	TB135	TB148
052	43	M	N	NEW CASE	PTB	SCROTAL HERNIA	HRZE	TB118	TB124	TB147
053	38	M	N	NEW CASE	PTB	NONE	HRZE	TB119	TB127	TB152
054	41	M	N	NEW CASE	PTB	NONE	HRZE	TB120	TB128	TB149
055	26	M	Y	NEW CASE	PTB	NONE	HRZE	TB121	TB129	TB150
056	20	M	Y	NEW CASE	PTB	NONE	HRZE	TB126	TB136	TB151
057	55	M	Y	NEW CASE	PTB	NONE	HRZE	TB131		
058	27	M	Y	NEW CASE	PTB	NONE	HRZE	TB132	TB137	TB153
059	30	F	N	NEW CASE	PTB	NONE	HRZE	TB133	TB139	TB155
060	27	M	N	NEW CASE	PTB	NONE	HRZE	TB134	TB140	TB154

APPENDIX B

CELL COUNTS OF PATIENTS AT VARIOUS TIME POINTS

Table 14 CELL COUNTS OF PATIENTS AT VARIOUS TIME POINTS

PATIENTS	PMBCs			CD4+ T CELLS		
	Time point 0	Time point 1	Time point 2	Time point 0	Time point 1	Time point 6
KNUST042	2.84×10^7	8.2×10^7	6.48×10^7	1.8×10^6	6×10^5	6×10^5
KNUST043	3.36×10^7	4.16×10^7	3.2×10^7	2.4×10^6	4.2×10^6	5.8×10^6
KNUST044	6.96×10^7	4×10^7		5×10^6	1.6×10^6	
KNUST045	1.96×10^7	2.16×10^7	4.6×10^7	1.4×10^6	1.6×10^6	2.6×10^6
KNUST046	3.52×10^7	7.32×10^7	2.8×10^7	2.4×10^6	2×10^5	2.0×10^6
KNUST047	6.44×10^7	2.8×10^7		8×10^5	4×10^5	
KNUST0481	2.48×10^7	4.8×10^6		6×10^5	4×10^5	
KNUST049	5.32×10^7	7.28×10^7		1×10^6	1.2×10^6	
KNUST050	7.2×10^7	9.72×10^7		1×10^6	6×10^5	
KNUST051	3.4×10^7	N/A		4×10^5	N/A	
KNUST052	4.24×10^7	3.68×10^7		4×10^5	8×10^5	
KATH053	4.96×10^7	4.24×10^7		5.2×10^6	3×10^6	
KATH054	4.08×10^7	5.04×10^7		2.8×10^6	2.6×10^6	
KNUST055	2.64×10^7	5.2×10^7		1.2×10^6	1.4×10^6	
KNUST056	2.6×10^7	5.24×10^7		3×10^6	1.8×10^6	
KNUST057	3.48×10^7			1.6×10^6		
KNUST058	7.24×10^7	4.12×10^7		6×10^5	2.4×10^6	
KNUST059	2.56×10^7	6.68×10^7		1.6×10^6	1.4×10^6	
KNUST060	3.36×10^7	3.96×10^7		1.4×10^6	2.2×10^6	

APPENDIX C

PATIENT INTERFERON-GAMMA RESPONSE FOR FIRST TIME POINT

Table 15 PATIENT INTERFERON-GAMMA RESPONSES FOR FIRST TIME POINT

TP0	UNST	PPD	ESAT6	P/I	PHA	SEB	PPD	ESAT6	P/I	PHA	SEB
TB001											
TB002											
TB003											
TB004											
TB005											
TB006											
TB007											
TB008											
TB014											
TB013											
TB015											
TB017											
TB021											
TB022											
TB023											
TB024											
TB027											
TB028											
TB030											
TB031											
TB032											

TB035												
TB036												
TB037												
TB038												
TB041												
TB044												
TB047												
TB048												
TB049												
TB050												
TB051												
TB052												
TB053												
TB054												
TB055												
TB056												
TB057												
TB058												
TB075												
TB076												
TB080	0	1073.864	1953.396	65176.72	46717.63	87534.56		1073.864	1953.396	65176.72	46717.63	87534.56
TB082	2202.805	2518.345	2486.54	50551.02	26847.89	47872.7		315.54	283.735	48348.22	24645.09	45669.9
TB083	3287	3729.246	-549.042	65740	64785.37	94729.49		442.2455	-3836.04	62453	61498.37	91442.49
TB084	3966.959	3905.348	4092.308	87901.1	72880.57	88304.77		-61.6115	125.3485	83934.14	68913.61	84337.81
TB094	0	1212.146	783.5065	41932.4	19166.59	73840.8		1212.146	783.5065	41932.4	19166.59	73840.8
TB095	0	1017.678	-38.0618	4083.595	17280.34	21148.97		1017.678	-38.0618	4083.595	17280.34	21148.97
TB096	0	274.6829	-83.2561	30910.94	-1628.97	-652.769		274.6829	-83.2561	30910.94	-1628.97	-652.769
TB115	7.120415	2820.286	211.9831	67599.25	75117.92	54631.65		2813.166	204.8627	67592.13	75110.8	54624.53
TB116	9.2324	91.59985	5.00843	98603.21	73132.65	108022.7		82.36745	-4.22397	98593.98	73123.42	108013.5

TB117	21.90432	165.5194	7.120415	944.9631	24514.73	17333.98		143.6151	-14.7839	923.0588	24492.83	17312.08
TB118	0	2667.741	585.7835	19339.16	59859.65	65245.8		2667.741	585.7835	19339.16	59859.65	65245.8
TB119	0	4581.9	3807.121	10597.01	23772.38	74568.01		4581.9	3807.121	10597.01	23772.38	74568.01
TB120	0	4064	1406.137	21286.46	11798.54	84677.41		4064	1406.137	21286.46	11798.54	84677.41
TB121	0	3301.651	3668.324	33260.31	34337.54	82854.41		3301.651	3668.324	33260.31	34337.54	82854.41
TB126	25.50663	3523.962	-35.8698	-1197.73	50358.44	138100		3498.455	-61.3764	-1223.24	50332.93	138074.5
TB131	0	6675.57	1513.924	117796.7	55382.5	117203.7		6675.57	1513.924	117796.7	55382.5	117203.7
TB132	3391.785	6295.305	4441.905	115469.1	82216.14	122787.8		2903.521	1050.121	112077.3	78824.36	119396
TB133	0	6003.245	95.64405	121799.5	25628.29	98227.39		6003.245	95.64405	121799.5	25628.29	98227.39
TB134	0	2509.684	3903.255	96552.14	95262.34	7842.968		2509.684	3903.255	96552.14	95262.34	7842.968

APPENDIX D

PATIENT INTERFERON-GAMMA RESPONSES FOR SECOND TIME POINT

Table 16 PATIENT INTERFERON-GAMMA RESPONSES FOR SECOND TIME POINT

TP1	UNST	**PPD**	ESAT6	**P/I**	PHA	**SEB**		**PPD**	ESAT6	**P/I**	PHA	**SEB**
TB010												
TB009												
TB011												
TB012												
TB016												
TB019												
TB020												
TB025												
TB026												
TB029												
TB033												
TB034												
TB039												
TB040												
TB042												
TB043												
TB045												
TB046												

TB064												
TB066												
TB059												
TB063												
TB085	0	4963.89	-93.3086	2980.43	48590.42	86613.17		4963.89	-93.3086	2980.43	48590.42	86613.17
TB060												
TB061												
TB062												
TB071												
TB065												
TB069												
TB072												
TB074												
TB073												
TB090	0	455.8757	196.8789	596.5199	14919.04	59932.67		455.8757	196.8789	596.5199	14919.04	59932.67
TB097	0	1634.128	1357.539	108211.3	51628.02	84240.23		1634.128	1357.539	108211.3	51628.02	84240.23
TB098	0	1519.434	-86.8605	20739.65	15676.03	20598.99		1519.434	-86.8605	20739.65	15676.03	20598.99
TB108	58.5215	114.2838	8.787575	17251.07	8540.095	39510.77		55.7623	-49.7339	17192.55	8481.574	39452.25
TB100	0	205.7044	4.566038	41191.06	44229.24	35789.87		205.7044	4.566038	41191.06	44229.24	35789.87
TB103	0	-103.503	-97.1094	-2197.94	257.2917	5218.909		-103.503	-97.1094	-2197.94	257.2917	5218.909
TB110	0	833.2	-129.32	-216.383	11198.43	347.9089		833.2	-129.32	-216.383	11198.43	347.9089
TB122	0	577.6295	-75.1293	-1121.1	34399.15	58305.37		577.6295	-75.1293	-1121.1	34399.15	58305.37
TB123	0	-0.95212	-111.158	-612.46	49616.05	111755.3		-0.95212	-111.158	-612.46	49616.05	111755.3
TB135												
TB124	0	1257.94	85.9411	-1841.68	63179.86	114934.3		1257.94	85.9411	-1841.68	63179.86	114934.3

TB127	0	7672.935	7068.515	145829.6	62285.5	133794.9		7672.935	7068.515	145829.6	62285.5	133794.9
TB128	0	1714.711	911.5055	46436.3	41762.14	64703.17		1714.711	911.5055	46436.3	41762.14	64703.17
TB129	0	6732.725	5319.73	339.3224	61103.52	128744.7		6732.725	5319.73	339.3224	61103.52	128744.7
TB136	30.72379	844.4305	71.7824	22562.16	5802.789	28944.91		813.7067	41.05861	22531.44	5772.065	28914.19
								0	0	0	0	0
TB137	83.5598	18877.04	73.79185	191754.9	64185.88	459200.6		18793.48	-9.76795	191671.3	64102.32	459117
TB139	69.79825	17546.82	37.11058	435706.5	456626.6	455319.1		17477.02	-32.6877	435636.7	456556.8	455249.3
TB140	34.72017	3443.725	1441.301	345697.3	303.687	307407.1		3409.005	1406.581	345662.6	268.9668	307372.4

APPENDIX E

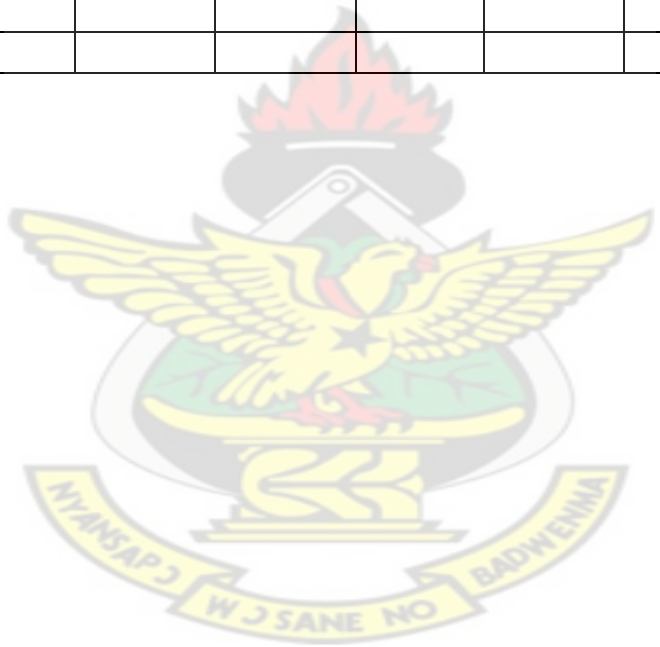
PATIENT INTERFERON-GAMMA RESPONSES FOR THIRD TIME POINT

Table 17 PATIENT INTERFERON-GAMMA RESPONSES FOR THIRD TIME POINT

TP2	UNST	**PPD**	ESAT6	**P/I**	PHA	**SEB**		**PPD**	ESAT6	**P/I**	PHA	**SEB**
TB067												
TB088	0	896.206	469.2435	44349.64	32896.66	69275.03		896.206	469.2435	44349.64	32896.66	69275.03
TB081	0	3417.743	3976.689	77427.33	66211.46	82453.23		3417.743	3976.689	77427.33	66211.46	82453.23
TB107	0	617.6515	-53.7566	-1060.06	3295.425	47016.08		617.6515	-53.7566	-1060.06	3295.425	47016.08
TB068												
TB070												
TB077	0	1981.113	-23.703	68077.69	57748.73	84023.82		1981.113	-23.703	68077.69	57748.73	84023.82
TB079												
TB078	14.17597	804.0915	1834.216	83561.88	63864.8	73953.55		789.9155	1820.04	83547.7	63850.62	73939.37
TB086	0	6563.56	-117.83	34541.05	54590.98	72390.7		6563.56	-117.83	34541.05	54590.98	72390.7
TB091	0	2555.045	-143.702	46387.14	36389.87	66019.09		2555.045	-143.702	46387.14	36389.87	66019.09
TB087	0	1825.138	-117.83	32665.87	13712.19	61168.51		1825.138	-117.83	32665.87	13712.19	61168.51
TB089	0	56.0711	666.299	63134.57	31589.13	81718.13		56.0711	666.299	63134.57	31589.13	81718.13
TB093	0	1212.146	783.5065	41932.4	19166.59	73840.8		1212.146	783.5065	41932.4	19166.59	73840.8

TB092	0	1473.733	-132.047	18389.6	23828.53	73063.8		1473.733	-132.047	18389.6	23828.53	73063.8
TB106	0	3452.486	2771.282	-1029.92	3702.34	54913.22		3452.486	2771.282	-1029.92	3702.34	54913.22
TB102	0	1239.203	888.8205	-1865.46	16983.57	42661.22		1239.203	888.8205	-1865.46	16983.57	42661.22
TB114												
TB101	0	833.031	537.653	-1062.06	23890.35	33061.13		833.031	537.653	-1062.06	23890.35	33061.13
TB105	0	69.13035	-95.8307	-944.75	1868.539	4860.855		69.13035	-95.8307	-944.75	1868.539	4860.855
TB130	0	7640.7	1811.418	103976.9	79692.75	144056.6		7640.7	1811.418	103976.9	79692.75	144056.6
TB125	58.9024	37.11058	-6.47301	1613.884	1395.965	960.13		-21.7918	-65.3754	1554.982	1337.063	901.2276
TB099	0	-29.1915	-130.464	30135.49	10162.3	44594.94		-29.1915	-130.464	30135.49	10162.3	44594.94
TB104	321.0474	3115.154	947.6435	-1942.19	7955.472	31970.72		2794.107	626.5961	-2263.24	7634.425	31649.67
TB109	49.47898	3989.763	28.37974	10047.18	3476.276	3054.291		3940.284	-21.0992	9997.701	3426.797	3004.812
TB111	0	2396.288	-213.964	-2941.11	-700.061	63822.66		2396.288	-213.964	-2941.11	-700.061	63822.66
TB143												
TB113	0	1506.32	1043.6	56680.34	53746.03	56341.77		1506.32	1043.6	56680.34	53746.03	56341.77
TB112	427.7161	2453.524	-26.9418	13471.72	31367.83	67418.01		2025.807	-454.658	13044	30940.11	66990.29
TB144												
TB138	30.72379	1193.429	43.78789	87136.15	28459.67	61530.51		1162.705	13.0641	87105.43	28428.95	61499.79
TB142												
TB141	-66.1801	3078.425	1270.277	72941.47	-1480.83	58816.96		3144.605	1336.457	73007.65	-1414.65	58883.14
TB145	0	1204.204	-34.8046	8618.757	7370.835	19270.67		1204.204	-34.8046	8618.757	7370.835	19270.67
TB146	0	-34.8046	-32.5762	-696.092	-740.661	-696.092		-34.8046	-32.5762	-696.092	-740.661	-696.092

TB148	43.78789	109.1084	40.05529	838.4317	1211.692	1137.04		65.32051	-3.7326	794.6438	1167.904	1093.252
TB147	0	-14.7487	-32.5762	-740.661	-651.523	-606.955		-14.7487	-32.5762	-740.661	-651.523	-606.955
TB152	64.4094	24199.61	15450.19	225251.3	22638.98	345212		24135.2	15385.78	225186.9	22574.57	345147.6
TB149	34.72017	15790.38	2164.127	211876.8	108.3282	297248.4		15755.66	2129.407	211842.1	73.60803	297213.7
TB150	14.8844	58.90665	9.38161	16695.97	48722.16	6460.802		44.02225	-5.50279	16681.09	48707.28	6445.918
TB151	31.39275	15978.45	6200.01	279178.6	-252.59	244621.1		15947.06	6168.617	279147.2	-283.983	244589.7
TB153												
TB155												
TB154												



APPENDIX F

LTBIS INTERFERON-GAMMA RESPONSES

Table 18 LTBIs INTERFERON-GAMMA RESPONSES

SAMPLE	UNST	**PPD**	ESAT6	**P/I**	PHA	**SEB**		**SAMPLE**	**PPD**	ESAT6	**P/I**	PHA	**SEB**
C73	0	2844.765	-84.6478	62184.97	27366.82	60962.99		**C73**	2844.765	-84.6478	62184.97	27366.82	60962.99
C74	0	-139.051	-154.954	44608.5	42850.85	49730.79		**C74**	-139.051	-154.954	44608.5	42850.85	49730.79
C75	2824.677	2733.447	2950.224	57230.08	30697.97	57581.61		**C75**	-91.2305	125.5465	54405.4	27873.29	54756.93
C76	0	2802.868	-275.317	79552.86	39166.31	90657.71		**C76**	2802.868	-275.317	79552.86	39166.31	90657.71
C77	4601.073	4757.905	4686.795	92878.68	30029.17	102074.3		**C77**	156.8315	85.7215	88277.61	25428.1	97473.23
C78	0	403.6374	-593.851	70922.26	33243.73	81091.96		**C78**	403.6374	-593.851	70922.26	33243.73	81091.96
C79	0	4174.103	185.2668	62172.85	70054.92	90004.41		**C79**	4174.103	185.2668	62172.85	70054.92	90004.41
C80	59.9186	4542.711	362.6665	70841	44517.87	89282.06		**C80**	4482.792	302.7479	70781.08	44457.95	89222.14
C81	17.42768	50.35815	34.42405	77830.76	66443.18	65593.37		**C81**	32.93047	16.99637	77813.33	66425.75	65575.94
C82	0	69.1786	-72.0914	42482.94	22384.74	37764.23		**C82**	69.1786	-72.0914	42482.94	22384.74	37764.23
C83	0	5553.95	1122.15	53988.43	33307.67	86582.47		**C83**	5553.95	1122.15	53988.43	33307.67	86582.47
C84	0	4889.835	1136.714	29346.29	7063.499	71610.77		**C84**	4889.835	1136.714	29346.29	7063.499	71610.77

C87	0	746.967	29.1465	37375.57	51662.62	92408.48		**C87**	746.967	29.1465	37375.57	51662.62	92408.48
C88	13.54176	1681.521	51.6868	4362.759	24128.83	51940.05		**C88**	1667.979	38.14504	4349.217	24115.29	51926.51
C86	142.7155	-76.025	-92.295	19232.73	2565.066	84963.34		**C86**	-218.741	-235.01	19090.01	2422.351	84820.62
C90	19.79234	66.25605	-37.2313	184.648	564.8056	-68.7903		**C90**	46.46371	-57.0236	164.8557	545.0133	-88.5826
C99	0	4066.074	-104.8	97131.8	19606.11	58729.24		**C99**	4066.074	-104.8	97131.8	19606.11	58729.24
C107	0	2283.091	-54.5496	19563.5	78271.36	144504.5		**C107**	2283.091	-54.5496	19563.5	78271.36	144504.5
C110	0	3814.832	-62.5552	54414.62	41178.67	144931.5		**C110**	3814.832	-62.5552	54414.62	41178.67	144931.5
C113	0	391.0965	-46.5439	107945.5	56816.31	111841.6		**C113**	391.0965	-46.5439	107945.5	56816.31	111841.6
C115	0	5828.73	1020.254	75640.01	63037.86	126808.3		**C115**	5828.73	1020.254	75640.01	63037.86	126808.3
C119	3.591027	5370.675	6221.99	80466.36	64914.78	128685.2		**C119**	5367.084	6218.399	80462.77	64911.19	128681.6
C104	0	3587.606	21.46642	122071.3	23309.82	130785.6		**C104**	3587.606	21.46642	122071.3	23309.82	130785.6
C105	0	1572.156	108.6089	21477.59	39844.55	140125.5		**C105**	1572.156	108.6089	21477.59	39844.55	140125.5
C101	0	7718.155	6537.64	122673	25473.72	132397.8		**C101**	7718.155	6537.64	122673	25473.72	132397.8
C97	18.17506	7096.45	-10.8541	32876.15	41584.9	117302.6		**C97**	7078.275	-29.0292	32857.97	41566.72	117284.4
C98	6.079575	8170.535	7345.62	69888.33	45455.46	151073.3		**C98**	8164.455	7339.54	69882.25	45449.38	151067.2
C102	0	279.4375	-6.01591	24.8276	53438.48	153347.1		**C102**	279.4375	-6.01591	24.8276	53438.48	153347.1

C94	2.631434	7605.77	351.3766	43089.26	82910.51	129261.5		**C94**	7603.139	348.7452	43086.63	82907.88	129258.9
C95	0	5275.855	534.406	74995.73	74204.24	134109.3		**C95**	5275.855	534.406	74995.73	74204.24	134109.3
C96	0	717.435	74.35915	92012.51	36905.83	130696		**C96**	717.435	74.35915	92012.51	36905.83	130696
C100	22.41839	6836.55	-7.26205	-194.708	49272.68	133367.2		**C100**	6814.132	-29.6804	-217.127	49250.26	133344.8
C89	13.95088	4011.923	9674.69	339061	157901.6	296087.9		**C89**	3997.972	9660.739	339047	157887.6	296073.9
C106	0	9600.88	13.95088	186933	98854.64	250490.5		**C106**	9600.88	13.95088	186933	98854.64	250490.5
C108	0	485.5065	1.649431	141827.7	38085.47	276323.6		**C108**	485.5065	1.649431	141827.7	38085.47	276323.6
C109	9.8504	354.291	-2.45105	64000.53	40791.8	258363.5		**C109**	344.4406	-12.3014	63990.68	40781.95	258353.6
C111	0	5324.475	-14.3381	965.6094	7134.698	137335		**C111**	5324.475	-14.3381	965.6094	7134.698	137335
C91	0	6516.545	1342.398	109411.7	141277.6	163263.7		**C91**	6516.545	1342.398	109411.7	141277.6	163263.7
C92	0	7462.785	13.49237	177225.3	69846.04	144153.4		**C92**	7462.785	13.49237	177225.3	69846.04	144153.4
C93	0	8040.265	6430.735	41319.8	95496.47	136592.8		**C93**	8040.265	6430.735	41319.8	95496.47	136592.8
C103	0	3567.734	1151.79	-296.207	19975.75	46339.27		**C103**	3567.734	1151.79	-296.207	19975.75	46339.27
C112	0	7157.765	1201.721	91227.18	24918.91	81540.58		**C112**	7157.765	1201.721	91227.18	24918.91	81540.58
C114	0	7979.13	174611.8	102960.9	18328.03	117440.9		**C114**	7979.13	174611.8	102960.9	18328.03	117440.9
C116	0	5110.6	8841.152	133269	80042.65	175011.3		**C116**	5110.6	8841.152	133269	80042.65	175011.3

C117	91.5901	6868.84	189.6532	226069.4	211251	225633.6		**C117**	6777.25	98.0631	225977.8	211159.4	225542
C118	48.0065	18810.75	6247.775	235875.7	120161.3	372510.3		**C118**	18762.74	6199.769	235827.7	120113.3	372462.3
C137	0	16972.3	122.6315	96615.38	499.0452	160692.9		**C137**	16972.3	122.6315	96615.38	499.0452	160692.9
C138	55.99445	11569.35	17291.34	240403.4	72037.96	155613.8		**C138**	11513.36	17235.35	240347.4	71981.97	155557.8
C139	1859.291	15730.8	2223.417	198788.9	12910.67	98220.43		**C139**	13871.51	364.126	196929.6	11051.38	96361.14
C140	1772.594	7572.615	2249.427	27822.54	22447.33	93885.59		**C140**	5800.021	476.833	26049.95	20674.74	92113
C131	90.67325	402.7822	-48.0418	1813.465	-93.8675	426.3141		**C131**	312.109	-138.715	1722.792	-184.541	335.6409
C128	0	18021.12	51.654	128863.2	216288.5	155563.4		**C128**	18021.12	51.654	128863.2	216288.5	155563.4
C129	0	28169.55	25286.88	505973.8	483054.1	464623.9		**C129**	28169.55	25286.88	505973.8	483054.1	464623.9
C132	51.654	122.5394	-7.4172	324.2252	-148.344	1269.365		**C132**	70.8854	-59.0712	272.5712	-199.998	1217.711
C133	16.21126	16.21126	39.83975	1269.365	-620.914	87.94056		**C133**	0	23.62849	1253.154	-637.125	71.7293
C136	41.92159	43.78789	40.05529	1771.582	689.1277	1622.278		**C136**	1.8663	-1.8663	1729.66	647.2061	1580.356
C130	279.4044	3617.597	718.4055	27293.02	20072.9	20652.29		**C130**	3338.193	439.0011	27013.62	19793.5	20372.89

APPENDIX G

COMPILED PATIENT CT VALUES FOR SINGLE ASSAY

Table 19 COMPILED PATIENT Ct VALUES FOR SINGLE ASSAY

SAMPLE	Ct(mir29a)	Ct(RNU48)	Rel Ct
TB133	24.12538	24.017204	0.1081753
TB152	25.325129	25.639769	-0.31464
TB116	27.984797	28.055918	-0.071121
TB120	22.179274	22.555109	-0.375835
TB126	24.093409	23.985394	0.1080151
TB131	22.935226	23.940027	-1.004801
TB140	25.416269	26.441133	-1.024864
TB134	23.859316	24.669838	-0.810522
TB138	22.034151	22.892294	-0.858143
TB139	24.690557	25.680014	-0.989456
TB145	30.271013	27.65037	2.6206436
TB145*	27.733957	27.26161	0.4723473
TB129	25.231539	29.782867	-4.551329
TB123	24.08782	24.303688	-0.215868
TB121	24.434212	24.213274	0.2209377
TB128	24.643179	25.671316	-1.028137
TB117B	28.454313	26.96698	1.4873333
TB 84	25.714048	25.938873	-0.224825
TB118	28.084814	25.413668	2.6711464
TB82	26.276087	26.182032	0.0940552
TB83	32.641441	24.475702	8.1657391
TB151	26.697893	27.383066	-0.685173

TB148	25.816536	24.893852	0.9226837
TB108	24.786917	25.386938	-0.600021
TB152	33.707027	27.051428	6.6555996
TB122	25.849197	25.012405	0.836792
TB110	26.876352	27.857704	-0.981352
TB136	27.43219		#VALUE!
TB149	26.849508	25.991018	0.85849
TB153	24.617096	25.029327	-0.412231
TB146	28.61725	27.989496	0.6277542
TB137	22.925173	25.056028	-2.130856
TB119	22.988958	23.947992	-0.959034
TB80	29.454592	26.709208	2.7453842
TB115	30.607985	27.696787	2.9111977
TB150	28.371758	26.626226	1.7455311
TB127	24.264784	24.049397	0.2153873
TB103	30.313276	29.764318	0.5489578
TB147	30.210062	28.525688	1.6843739
TB155	26.534363	24.731119	1.8032436

APPENDIX H

COMPILED LTBI CT VALUES FOR ASSAY

Table 20 COMPILED LTBI Ct VALUES FOR PLATE ASSAY

TARGET	SAMPLE(ΔCt)										
	C004	C006	C027	C02	C012	C009	C008	C003	C029	C030	C032
Let-7a	9.19467	9.559535	8.984927	10.34876	9.125514	9.250487	11.66947	10.07229	14.58772	12.5385	13.0042
miR-15a	10.67949	11.37199	11.12366	11.68357	11.50446	15.05349	13.33979	13.93487	11.93951	12.19824	Undetermined
miR-17	3.87297	3.478476	4.341018	3.079893	3.212617	4.375333	4.043393	4.1896	3.627535	2.90769	2.18474
miR-21	3.55976	4.107495	3.942056	3.174492	2.678504	4.0509	3.429109	4.615513	4.416374	4.03702	4.643742
miR-23a	12.02071	14.01549	11.2042	14.33733	11.86124	12.83102	Undetermined	14.82268	Undetermined	14.30048	Undetermined
miR-25	8.749292	8.518805	8.573827	8.999518	8.318233	9.42177	9.066705	9.42698	9.094955	7.877207	8.622837
miR26a	3.746196	3.969872	4.157	3.770972	3.203159	3.916312	4.485766	4.317947	3.919174	2.863478	2.841251
miR-27a	9.64595	10.48297	9.373922	9.961752	8.95733	10.88541	9.92676	11.34617	9.720695	8.89743	10.95231
miR-29a	3.752574	4.5965	2.431077	4.246113	3.048413	4.464757	4.913014	5.248547	3.918792	3.432009	3.96232
miR-98	14.71287	13.53671	11.90191	Undetermined	12.35546	Undetermined	15.16001	14.22572	Undetermined	12.72408	15.23971
miR-99b	11.7267	11.81439	10.78295	11.70502	10.62751	11.9207	11.75445	12.96235	11.7976	11.511	11.42043
miR-101	10.29533	10.86014	9.878158	11.29492	9.147044	10.74734	10.28909	11.17863	10.83904	9.777893	9.98997
miR-106a	3.834546	3.491864	4.000352	2.867707	2.987577	4.254708	4.133092	4.230345	3.335716	3.043947	2.206097
miR-106b	8.654483	9.00786	6.210588	7.99236	7.522916	8.991504	8.742665	9.11788	8.67017	7.579786	7.731453

miR-122	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-126	11.97411	11.3605	11.89354	9.51007	12.08984	12.80949	10.94721	12.78393	8.242084	8.74016	10.42616
miR-142-3p	0.361709	0.59139	-0.02279	-0.12521	-0.88178	0.895898	0.712216	1.82501	0.68683	0.029356	0.911474
miR-143	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-146a	1.534465	1.995494	0.460717	0.865721	1.10227	3.15549	1.722835	2.938156	0.933082	1.668083	1.48794
miR-150	-1.66661	-1.17864	-3.11332	-1.84728	-2.07777	-0.93755	-1.4856	-0.73641	-1.75206	-1.61642	-1.81521
miR-181a	9.91215	9.229552	8.6535	9.20492	9.92763	9.855487	9.59942	10.31689	10.58572	8.956386	9.94151
miR-191	4.679845	4.457826	2.852424	4.034191	3.898914	4.980071	5.000966	4.795618	3.670925	3.694937	3.552046
miR-203	14.93963	Undetermined	14.33966	Undetermined	14.96341	Undetermined	15.80721	17.78216	Undetermined	Undetermined	Undetermined
miR218	Undetermined	Undetermined	15.81478	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-223	4.834108	4.524407	1.182998	4.699246	5.967134	6.567927	4.908137	4.393057	0.637321	1.405006	3.098095
miR455	Undetermined	Undetermined	Undetermined	Undetermined	16.36309	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
u6 snRNA	-0.46683	-1.01384	0.37521	-2.00686	-1.10564	-0.22384	-0.38541	0.814283	-1.7299	-1.52335	-2.16076
RNU48	-5E-06	3E-06	3E-06	4E-06	3E-06	0	-5E-06	0	-4E-06	2E-06	4E-06
RNU44	2.930598	3.699351	1.6955	3.215168	1.704885	2.775884	2.887597	3.736256	3.279634	2.686	3.783419
miR-30a*	7.100373	7.698562	6.224284	7.41942	6.007373	7.779552	8.129031	8.280977	7.813621	6.360597	7.384981
miR-144*	Undetermined	Undetermined	15.7518	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	12.39029	14.51795	14.77888
miR-155*	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
H2O	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined

APPENDIX I

FIRST PART OF COMPILED PATIENT CT VALUES FOR PLATE ASSAY

Table 21 FIRST PART OF COMPILED PATIENT Ct VALUES FOR PLATE ASSAY

TARGET	SAMPLE(ΔCt)														
	TB002	TB010	TB067	TB005	TB011	TB081	TB024	TB034	TB086	TB027	TB039	TB091	TB028	TB040	TB087
Let-7a	11.78046	9.27549	12.65732	11.00894	8.551108	11.78001	7.889204	10.47659	7.408346	8.379966	12.32732	9.56215	13.11806	11.29506	8.439786
miR-15a	12.43496	10.66512	15.85201	11.78144	11.96026	15.09303	10.11464	9.119773	11.11323	12.10863	13.22824	10.31619	13.59933	12.44162	12.75265
miR-17	3.195727	1.868467	3.146784	3.808341	3.404778	2.97461	3.308761	3.549159	2.309717	2.706475	2.505923	1.376003	3.421707	3.273964	2.598598
miR-21	3.915038	2.436155	5.754522	4.254716	2.8424	4.48209	2.530313	3.173682	2.29052	2.949271	3.731822	2.808094	4.833213	3.66008	4.02174
miR-23a	Undetermined	13.73674	16.36051	12.16273	11.64398	Undetermined	11.2943	12.71514	12.06054	13.55507	14.82436	12.8104	Undetermined	Undetermined	12.03004
miR-25	8.77474	7.086997	8.707006	8.94925	8.331295	8.29292	7.75399	8.21327	6.926098	8.133947	8.250704	6.728735	9.452093	8.881756	7.440439
miR26a	3.818925	2.332976	3.732218	3.905595	3.008309	3.4317	3.500809	2.544978	2.088491	2.893718	2.899075	1.528858	4.145645	3.58957	2.289388

miR-27a	9.55136	7.261535	9.899225	10.06722	8.936623	9.57358	8.883045	7.480796	8.49292	9.260614	10.06796	8.175234	10.55148	9.35308	8.905515
miR-29a	4.369376	2.773202	4.187416	4.091905	3.06632	3.36177	3.129926	2.558259	2.538345	3.480094	3.15265	2.198103	4.313695	3.785112	3.072778
miR-98	15.97402	12.22569	Undetermined	Undetermined	12.76809	Undetermined	12.3003	13.11731	12.29859	12.62391	Undetermined	Undetermined	16.7284	15.05102	12.30799
miR-99b	11.57083	10.77486	10.9904	11.90594	11.52448	11.51669	11.49926	10.23405	10.98886	10.9255	11.94084	10.45026	11.83297	11.42653	9.949003
miR-101	10.67501	9.391696	10.01346	11.10052	8.965645	9.63362	9.434082	8.343062	8.59939	9.42206	9.98626	7.528406	10.60974	9.976505	9.086828
miR-106a	3.203199	1.868444	3.172794	3.893594	3.303854	3.03106	2.705211	2.666726	2.328033	2.722396	2.250476	1.358978	3.423005	3.418909	2.603683
miR-106b	8.141646	6.183139	8.52136	8.74401	7.387299	7.88598	7.002512	6.569745	6.298107	7.80612	7.888137	6.622183	8.919523	8.08502	7.543624
miR-122	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-126	7.153245	8.95263	9.683054	13.59397	11.22096	12.1774	10.01404	11.34747	10.76119	9.668603	9.988517	7.851656	10.35243	10.70446	10.83251
miR-142-3p	0.973483	-0.62031	1.116312	0.53007	-0.65922	0.26059	-1.09466	0.73187	-1.71731	-0.48344	-0.25537	-1.7192	1.166059	0.08678	-0.39098
miR-143	Undetermined	13.54019	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-146a	1.750426	0.51755	3.054542	1.686462	1.603691	1.47359	1.798647	0.766097	1.446552	2.324424	1.202883	0.765339	1.942777	1.7969	1.366924
miR-150	-1.3627	-3.0595	-1.84545	-0.87541	1.3297	-1.75828	-1.95212	-2.5335	-2.52979	-1.68429	-2.56832	-3.36508	-0.83298	-0.88755	-2.50384
miR-181a	10.19237	8.187904	10.47254	11.16497	9.680575	10.839	8.777803	10.44288	8.443373	8.632142	8.620063	8.321413	10.80399	10.72601	8.977178
miR-191	3.98685	2.656137	4.749729	4.693864	4.442065	4.1206	3.155117	5.024337	3.227333	3.559733	2.615306	2.091185	4.280576	4.141312	3.452318

miR-203	Undetermined	14.43516	Undetermined	Undetermined	15.95058	14.6374	13.90804	Undetermined	13.06488	Undetermined	Undetermined	12.52728	14.75303	13.60219	Undetermined
miR218	Undetermined	Undetermined	Undetermined	Undetermined	18.58923	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-223	1.489137	-0.49362	3.547966	6.455207	3.0448	6.08945	1.891567	3.036519	3.545887	0.55943	3.177222	3.110807	2.882379	2.754448	4.082824
miR455	Undetermined	Undetermined	Undetermined	Undetermined	17.38241	15.14769	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	13.88673
u6 snRNA	-1.29498	-0.4237	-0.82659	-1.07738	0.345772	-1.34865	-0.08207	-1.41489	-0.48449	-0.31592	-2.12942	-2.89769	-0.46786	-0.53158	-0.34321
RNU48	0	1E-06	4E-06	-3E-06	-2E-06	0	4E-06	0	-4E-06	-3E-06	-5E-06	4E-06	1E-06	0	-3E-06
RNU44	3.550881	3.755176	4.157146	3.446575	2.675999	2.58584	2.258045	3.007794	4.035293	2.321458	2.618485	4.584448	3.78142	2.971296	4.068763
miR-30a*	7.586019	5.876407	7.3533	8.078159	6.717297	6.10738	6.743091	7.17886	7.54225	6.737767	6.441918	7.99466	7.785404	7.247593	7.127458
miR-144*	Undetermined	Undetermined	Undetermined	Undetermined	16.63401	Undetermined	17.31852	Undetermined	Undetermined	13.03878	12.58715	Undetermined	15.9639	15.5641	Undetermined
miR-155*	Undetermined	Undetermined	Undetermined	Undetermined	16.05627	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	15.14365	Undetermined	Undetermined	Undetermined	Undetermined
H2O	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined

APPENDIX J

SECOND PART OF COMPILED PATIENT CT VALUES FOR PLATE ASSAY

Table 22 SECOND PART OF COMPILED PATIENT Ct VALUES FOR PLATE ASSAY

TARGET	SAMPLE(ΔCt)															
	TB006	TB012	TB007	TB016	TB014	TB068	TB015	TB070	TB52	TB71	TB30	TB42	TB41	TB66	TB008	TB018
Let-7a	9.72088	10.00218	9.21593	9.46014	13.95185	12.31875	12.34355	12.0979	Undetermined	10.06852	10.15428	13.15879	14.17204	Undetermined	9.965113	10.5896
miR-15a	11.29192	13.35848	11.15776	11.74833	12.79805	12.6621	15.60636	11.47391	Undetermined	9.829537	11.73348	12.99548	12.869	12.08043	12.36001	14.05977
miR-17	3.204748	3.64094	3.88515	3.877388	2.058886	2.225595	3.234109	2.427552	2.291098	1.565436	2.883529	2.992938	2.638055	2.57561	3.636452	2.540322
miR-21	3.571839	4.12895	2.828266	4.072967	4.327153	5.236549	4.111869	2.411794	3.632263	2.20984	3.834033	5.47935	5.12236	4.51023	3.255953	3.84649
miR-23a	Undetermined	14.21678	13.607	13.32984	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	12.02862	Undetermined
miR-25	8.793593	8.98387	8.917305	8.785555	8.8524	8.01489	8.396623	6.883567	8.276545	7.27724	7.97428	9.2724	8.39804	8.012207	8.74181	8.4827
miR26a	3.515438	3.632072	4.193804	4.54476	3.102782	2.314393	4.16388	2.711945	2.687426	2.565836	2.881375	4.100066	3.815102	3.020054	3.391297	3.26039
miR-	9.563	10.586	9.2185	10.173	10.351	8.8938	10.427	7.7853	9.1878	8.1526	9.4302	9.4739	11.005	9.0840	9.9184	10.358

27a	612	32	9	96	01	96	72		8	53	26	37	52	7	96	88
miR-29a	4.223 54	3.8167 07	3.6762 77	4.6373 16	3.7713 15	3.3701 99	4.5404 65	2.2690 95	3.0138	2.3519 79	3.5693 96	5.1977 9	4.8491 27	3.6937 77	3.5529 3	3.4656 05
miR-98	14.47 593	13.782 57	15.479 58	Undet ermin ed	14.457 45	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	16.332 15	Undet ermin ed	Undet ermin ed	Undet ermin ed	13.935 31	12.664 12
miR-99b	11.76 894	11.265 97	12.640 61	11.011 02	12.049 62	9.5372 8	11.980 61	11.111 11	11.627 95	9.3248 52	12.069 07	11.480 42	11.168 23	11.143 33	12.216 19	12.809 38
miR-101	10.49 119	10.193 42	10.230 26	11.677 63	10.093 1	8.5393 92	10.369 54	8.4354 92	8.3419 63	9.3988 8	9.3054 97	11.844 7	10.856 87	9.7282 68	10.023 9	8.8390 35
miR-106a	3.355 606	3.3888 03	3.7907 62	3.7735 35	2.124	2.4575 38	3.2448 66	2.1692 57	1.8772 05	1.4921 4	2.8727 9	3.4486 91	2.7343 32	2.4489 23	3.4252 88	2.1086 64
miR-106b	8.290 74	8.0719 4	7.7895 05	8.9329 7	8.0098 63	7.8422 87	8.2413 38	6.1998 05	7.0126 86	6.0940 48	7.8459 9	9.5594 6	8.3931 57	7.5775 23	8.2791 52	6.8527 53
miR-122	Unde termi ned	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed
miR-126	10.86 377	9.7094 2	11.882 77	10.818 74	9.1626 04	11.340 66	11.131 38	9.7604 66	11.043 88	10.768 16	11.200 5	9.8546 7	8.6811 2	10.467 11	13.207 52	11.230 62
miR-142-3p	0.167 847	0.3585 4	-0.2457 6	1.3003 56	0.4463 5	0.4087 2	0.4422 31	-1.2652 9	-0.9407 2	-1.3320 7	-0.3731	1.8780 43	1.6375 38	0.2918 44	-0.3361 9	-0.6441
miR-143	Unde termi ned	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed	Undet ermin ed
miR-146a	2.111 939	1.8296 87	2.3096 62	2.1886	2.2685 72	1.7907 29	3.7371 78	1.2151 65	1.2721 25	0.7830 45	1.3666 96	2.1061 64	2.8118 33	1.9081 12	2.0523 88	1.0467 74
miR-	-	-	-	-	-1.785	-	-	-	-	-	-	-	-	-	-	-

150	1.54044	1.65398	1.13324	1.46564		3.05886	1.39577	2.34573	3.1187	2.57213	1.92205	1.48381	1.88159	2.53749	1.59352	2.72824
miR-181a	8.993124	9.250643	9.640755	9.825996	10.3857	8.88747	9.450093	9.16738	8.984156	8.933274	9.22058	10.36694	11.27514	10.18868	9.472033	9.094338
miR-191	4.240713	3.84376	4.762798	4.123232	3.76804	2.68272	4.972274	3.441577	2.724358	2.955666	3.93364	3.804764	3.577532	3.067576	4.439864	3.029602
miR-203	Undetermined	15.19498	Undetermined	Undetermined	Undetermined	Undetermined	15.03998	Undetermined	Undetermined	Undetermined	15.54305	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR218	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-223	4.516073	2.05437	5.330633	4.68226	3.606427	4.323306	6.61572	4.71641	3.483288	2.871666	4.646304	1.003997	3.188374	4.145731	6.695454	2.16924
miR455	Undetermined	14.31286	14.70746	Undetermined	Undetermined	Undetermined	15.02666	12.3443	12.04632	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
u6 snRNA	-1.48287	0.28311	-0.22571	-0.5099	-2.1827	-3.08363	-1.43404	-1.18414	-3.02214	-0.49518	-0.95898	-2.26756	-2.49533	-2.89897	-0.11679	-3.55034
RNU48	-2E-06	2E-06	4E-06	0	-1E-06	-2E-06	-3E-06	3E-06	-4E-06	-5E-06	-5E-06	4E-06	3E-06	4E-06	4E-06	4E-06
RNU44	3.486574	3.154754	2.438448	2.60259	3.24698	2.365047	2.120828	1.944669	2.313614	3.407963	2.589976	3.558573	2.424662	1.998864	2.87468	3.052885
miR-30a*	7.20584	7.192327	7.525917	7.921062	7.409735	6.45911	7.090575	5.38252	6.297506	6.34016	7.068519	7.92402	6.728259	6.339344	7.10173	8.33229
miR-144*	Undetermi	17.69796	Undetermin	15.56608	14.27921	Undetermin	Undetermin	Undetermin	Undetermin	11.93124	15.38136	13.17916	Undetermin	Undetermin	Undetermin	Undetermin

	ned		ed			ed	ed	ed	ed				ed	ed	ed	ed
miR-155*	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	14.91824	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
H2O	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined

APPENDIX K

RAW DATA DISPLAY FROM THE APPLIED BIOSYSTEM'S REAL TIME PCR

Table 23 RAW DATA DISPLAY FROM THE APPLIED BIOSYSTEM'S REAL TIME PCR

Block Type	96fast																			
Chemistry	TAQMAN																			
Experiment File Name	D:\My Projects\2012_1206\Anthony\TB119.eds																			
Experiment Run End Time	2012-12-12 13:24:27 PM CET																			
Instrument Type	sds7500fast																			
Passive Reference	ROX																			
Well	Sample Name	Target Name	Task	Reporter	Quencher	Ст	Ст Mean	Ст SD	Quantity	Quantity Mean	Quantity SD	Automatic Ct Threshold	Ct Threshold	Automatic Baseline	Baseline Start	Baseline End	Comments	HIGHSD	NOAMP	OUTLIERRG	EXPFAIL
A1	TB119	Let-7a	UNKNOWN	FAM	NFQ-MGB	29.88091	29.845335	0.2267794				TRUE	0.03	TRUE	3	27		N	N	N	N
A2	TB119	miR-15a	UNKNOWN	FAM	NFQ-MGB	32.9892	33.34318	0.6707799				TRUE	0.03	TRUE	3	31		Y	N	N	N
A3	TB1	miR-	UN	FA	NF	23.8	24.	1.0				TRUE	0.03	TRUE	3	21		Y	N	N	N

	19	17	KNOWN	M	Q-MGB	7753	64947	37904													
A4	TB119	miR-21	UNKNOWN	FAM	NFQ-MGB	22.97044	23.456692	0.8292722				TRUE	0.03	TRUE	3	20		Y	N	N	N
A5	TB119	Let-7a	UNKNOWN	FAM	NFQ-MGB	29.60288	29.845535	0.2267944				TRUE	0.03	TRUE	3	27		N	N	N	N
A6	TB119	miR-15a	UNKNOWN	FAM	NFQ-MGB	32.92083	33.3418	0.6707999				TRUE	0.03	TRUE	3	30		Y	N	N	N
A7	TB119	miR-17	UNKNOWN	FAM	NFQ-MGB	24.2415	24.649477	1.0379044				TRUE	0.03	TRUE	3	22		Y	N	N	N
A8	TB119	miR-21	UNKNOWN	FAM	NFQ-MGB	22.98587	23.456692	0.8292722				TRUE	0.03	TRUE	3	21		Y	N	N	N
A9	TB119	Let-7a	UNKNOWN	FAM	NFQ-MGB	30.05227	29.845535	0.2267944				TRUE	0.03	TRUE	3	28		N	N	N	N
A10	TB119	miR-15a	UNKNOWN	FAM	NFQ-MGB	34.11536	33.3418	0.6707999				TRUE	0.03	TRUE	3	31		Y	N	N	N
A11	TB119	miR-17	UNKNOWN	FAM	NFQ-MGB	25.82937	24.649477	1.0379044				TRUE	0.03	TRUE	3	22		Y	N	N	N
A12	TB119	miR-21	UNKNOW	FAM	NFQ-MG	24.41444	23.45692	0.8292722				TRUE	0.03	TRUE	3	22		Y	N	Y	N

			N		B		2	2													
B1	TB119	miR-23a	UNKNOWN	FAM	NFQ-MGB	31.38325	31.696657	0.430476				TRUE	0.03	TRUE	3	29		N	N	N	N
B2	TB119	miR-25	UNKNOWN	FAM	NFQ-MGB	28.04004	28.571288	0.683774				TRUE	0.03	TRUE	3	26		Y	N	N	N
B3	TB119	miR 26a	UNKNOWN	FAM	NFQ-MGB	23.21441	23.989926	0.912455				TRUE	0.03	TRUE	3	21		Y	N	N	N
B4	TB119	miR-27a	UNKNOWN	FAM	NFQ-MGB	28.76923	29.444409	1.277573				TRUE	0.03	TRUE	3	26		Y	N	N	N
B5	TB119	miR-23a	UNKNOWN	FAM	NFQ-MGB	32.18742	31.696657	0.430476				TRUE	0.03	TRUE	3	30		N	N	N	N
B6	TB119	miR-25	UNKNOWN	FAM	NFQ-MGB	28.33106	28.571288	0.683774				TRUE	0.03	TRUE	3	26		Y	N	N	N
B7	TB119	miR 26a	UNKNOWN	FAM	NFQ-MGB	23.75839	23.989926	0.912455				TRUE	0.03	TRUE	3	21		Y	N	N	N
B8	TB119	miR-27a	UNKNOWN	FAM	NFQ-MGB	28.64546	29.444409	1.277573				TRUE	0.03	TRUE	3	26		Y	N	N	N
B9	TB119	miR-23a	UNKNOWN	FAM	NFQ-MGB	31.51904	31.696657	0.430476				TRUE	0.03	TRUE	3	29		N	N	N	N
B10	TB1	miR-	UN	FA	NF	29.3	28.	0.6				TRUE	0.03	TRUE	3	27		Y	N	N	N

	19	25	KNOWN	M	Q-MGB	4275	57128	83774													
B11	TB119	miR26a	UNKNOWN	FAM	NFQ-MGB	24.99497	23.989926	0.9124555				TRUE	0.03	TRUE	3	22		Y	N	N	N
B12	TB119	miR-27a	UNKNOWN	FAM	NFQ-MGB	30.91757	29.444409	1.277573				TRUE	0.03	TRUE	3	28		Y	N	N	N
C1	TB119	miR-29a	UNKNOWN	FAM	NFQ-MGB	21.95804	21.785547	0.178215				TRUE	0.03	TRUE	3	20		N	N	N	N
C2	TB119	miR-98	UNKNOWN	FAM	NFQ-MGB	31.8117	33.668144	1.61689				TRUE	0.03	TRUE	3	29		Y	N	N	N
C3	TB119	miR-99b	UNKNOWN	FAM	NFQ-MGB	29.37023	29.092556	0.2437559				TRUE	0.03	TRUE	3	27		N	N	N	N
C4	TB119	miR-101	UNKNOWN	FAM	NFQ-MGB	27.91705	28.657555	0.928899				TRUE	0.03	TRUE	3	25		Y	N	N	N
C5	TB119	miR-29a	UNKNOWN	FAM	NFQ-MGB	21.6021	21.785547	0.178215				TRUE	0.03	TRUE	3	18		N	N	N	N
C6	TB119	miR-98	UNKNOWN	FAM	NFQ-MGB	34.76831	33.668144	1.61689				TRUE	0.03	TRUE	3	32		Y	N	N	N
C7	TB119	miR-99b	UNKNOW	FAM	NFQ-MG	28.91381	29.09225	0.243775				TRUE	0.03	TRUE	3	26		N	N	N	N

			N		B		6	9													
C8	TB119	miR-101	UNKNOWN	FAM	NFQ-MGB	28.35579	28.657555	0.9288989				TRUE	0.03	TRUE	3	26		Y	N	N	N
C9	TB119	miR-29a	UNKNOWN	FAM	NFQ-MGB	21.79626	21.787547	0.1782155				TRUE	0.03	TRUE	3	19		N	N	N	N
C10	TB119	miR-98	UNKNOWN	FAM	NFQ-MGB	34.42442	33.668144	1.61699				TRUE	0.03	TRUE	3	31		Y	N	N	N
C11	TB119	miR-99b	UNKNOWN	FAM	NFQ-MGB	28.99363	29.092556	0.2437599				TRUE	0.03	TRUE	3	27		N	N	N	N
C12	TB119	miR-101	UNKNOWN	FAM	NFQ-MGB	29.69981	28.657555	0.9288989				TRUE	0.03	TRUE	3	28		Y	N	N	N
D1	TB119	miR-106a	UNKNOWN	FAM	NFQ-MGB	22.90804	22.884435	0.2040845				TRUE	0.03	TRUE	3	20		N	N	N	N
D2	TB119	miR-106b	UNKNOWN	FAM	NFQ-MGB	26.39225	26.680222	0.4346344				TRUE	0.03	TRUE	3	24		N	N	N	N
D3	TB119	miR-122	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	Y	N	Y
D4	TB119	miR-126	UNKNOWN	FAM	NFQ-MGB	30.64623	31.227556	0.5102006				TRUE	0.03	TRUE	3	28		Y	N	N	N
D5	TB1	miR-	UN	FA	NF	22.6	22.	0.2				TRUE	0.03	TRUE	3	20		N	N	N	N

	19	106a	KNOWN	M	Q-MGB	6946	8843 5	0408 5													
D6	TB119	miR-106b	UNKNOWN	FAM	NFQ-MGB	26.46824	26.6802 2	0.4346 34				TRUE	0.03	TRUE	3	24		N	N	N	N
D7	TB119	miR-122	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	N	N	Y
D8	TB119	miR-126	UNKNOWN	FAM	NFQ-MGB	31.43546	31.2275 6	0.5102 06				TRUE	0.03	TRUE	3	29		Y	N	N	N
D9	TB119	miR-106a	UNKNOWN	FAM	NFQ-MGB	23.07556	22.8843 5	0.2040 85				TRUE	0.03	TRUE	3	21		N	N	N	N
D10	TB119	miR-106b	UNKNOWN	FAM	NFQ-MGB	27.18017	26.6802 2	0.4346 34				TRUE	0.03	TRUE	3	24		N	N	N	N
D11	TB119	miR-122	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	N	N	Y
D12	TB119	miR-126	UNKNOWN	FAM	NFQ-MGB	31.601	31.2275 6	0.5102 06				TRUE	0.03	TRUE	3	29		Y	N	N	N
E1	TB119	miR-142-3p	UNKNOWN	FAM	NFQ-MGB	20.71163	20.5503 4	0.1398 05				TRUE	0.03	TRUE	3	18		N	N	N	N
E2	TB119	miR-143	UNKNOW	FAM	NFQ-MG	33.8969	33.8969					TRUE	0.03	TRUE	3	32		N	N	N	N

			N		B																
E3	TB119	miR-146a	UNKNOWN	FAM	NFQ-MGB	22.41477	22.464425	0.051532				TRUE	0.03	TRUE	3	20		N	N	N	N
E4	TB119	miR-150	UNKNOWN	FAM	NFQ-MGB	18.67625	18.862188	0.185229				TRUE	0.03	TRUE	3	16		N	N	N	N
E5	TB119	miR-142-3p	UNKNOWN	FAM	NFQ-MGB	20.47545	20.550334	0.139805				TRUE	0.03	TRUE	3	17		N	N	N	N
E6	TB119	miR-143	UNKNOWN	FAM	NFQ-MGB	Undetermined	33.8969					TRUE	0.03	TRUE	3	39		N	N	N	Y
E7	TB119	miR-146a	UNKNOWN	FAM	NFQ-MGB	22.46038	22.464425	0.051532				TRUE	0.03	TRUE	3	20		N	N	N	N
E8	TB119	miR-150	UNKNOWN	FAM	NFQ-MGB	18.8636	18.862188	0.185229				TRUE	0.03	TRUE	3	17		N	N	N	N
E9	TB119	miR-142-3p	UNKNOWN	FAM	NFQ-MGB	20.46392	20.550334	0.139805				TRUE	0.03	TRUE	3	18		N	N	N	N
E10	TB119	miR-143	UNKNOWN	FAM	NFQ-MGB	Undetermined	33.8969					TRUE	0.03	TRUE	3	39		N	N	N	Y
E11	TB119	miR-146a	UNKNOWN	FAM	NFQ-MGB	22.51761	22.464425	0.051532				TRUE	0.03	TRUE	3	20		N	N	N	N
E12	TB1	miR-	UN	FA	NF	19.0	18.	0.1				TRUE	0.03	TRUE	3	16		N	N	N	N

	19	150	KNOWN	M	Q-MGB	467	86218	85229													
F1	TB119	miR-181a	UNKNOWN	FAM	NFQ-MGB	30.79262	30.585631	0.1789772				TRUE	0.03	TRUE	3	28		N	N	N	N
F2	TB119	miR-191	UNKNOWN	FAM	NFQ-MGB	25.41672	25.280499	0.122804				TRUE	0.03	TRUE	3	23		N	N	N	N
F3	TB119	miR-203	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	Y	N	Y
F4	TB119	miR218	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	Y	N	Y
F5	TB119	miR-181a	UNKNOWN	FAM	NFQ-MGB	30.49363	30.585631	0.1789772				TRUE	0.03	TRUE	3	28		N	N	N	N
F6	TB119	miR-191	UNKNOWN	FAM	NFQ-MGB	25.17827	25.280499	0.122804				TRUE	0.03	TRUE	3	22		N	N	N	N
F7	TB119	miR-203	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	Y	N	Y
F8	TB119	miR218	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	Y	N	Y
F9	TB119	miR-181a	UNKNOW	FAM	NFQ-MG	30.47269	30.58563	0.178997				TRUE	0.03	TRUE	3	28		N	N	N	N

			N		B		1	2													
F10	TB119	miR-191	UNKNOWN	FAM	NFQ-MGB	25.24649	25.2804	0.122804				TRUE	0.03	TRUE	3	22		N	N	N	N
F11	TB119	miR-203	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	Y	N	Y
F12	TB119	miR 218	UNKNOWN	FAM	NFQ-MGB	Undetermined						TRUE	0.03	TRUE	3	39		N	Y	N	Y
G1	TB119	miR-223	UNKNOWN	FAM	NFQ-MGB	23.26587	23.33878	0.100995				TRUE	0.03	TRUE	3	21		N	N	N	N
G2	TB119	miR 455	UNKNOWN	FAM	NFQ-MGB	36.26877	35.2942	1.041654				TRUE	0.03	TRUE	3	34		Y	N	N	N
G3	TB119	u6 snRNA	UNKNOWN	FAM	NFQ-MGB	21.86148	23.45284	3.107315				TRUE	0.03	TRUE	3	19		Y	N	N	N
G4	TB119	RNU48	UNKNOWN	FAM	NFQ-MGB	20.33549	21.149928	1.195022				TRUE	0.03	TRUE	3	18		Y	N	N	N
G5	TB119	miR-223	UNKNOWN	FAM	NFQ-MGB	23.29642	23.33878	0.100995				TRUE	0.03	TRUE	3	21		N	N	N	N
G6	TB119	miR 455	UNKNOWN	FAM	NFQ-MGB	34.19642	35.2942	1.041654				TRUE	0.03	TRUE	3	31		Y	N	N	N
G7	TB1	u6	UN	FA	NF	21.4	23.	3.1				TRUE	0.03	TRUE	3	19		Y	N	N	N

	19	snRNA	KNOWN	M	Q-MGB	6354	45284	07315													
G8	TB119	RNU48	UNKNOWN	FAM	NFQ-MGB	20.5911	21.1492	1.1950	28			TRUE	0.03	TRUE	3	18		Y	N	N	N
G9	TB119	miR-223	UNKNOWN	FAM	NFQ-MGB	23.45406	23.33878	0.1009	95			TRUE	0.03	TRUE	3	21		N	N	N	N
G10	TB119	miR455	UNKNOWN	FAM	NFQ-MGB	35.41742	35.2942	1.0416	54			TRUE	0.03	TRUE	3	33		Y	N	N	N
G11	TB119	u6 snRNA	UNKNOWN	FAM	NFQ-MGB	27.03349	23.4528	3.1073	15			TRUE	0.03	TRUE	3	24		Y	N	N	N
G12	TB119	RNU48	UNKNOWN	FAM	NFQ-MGB	22.52126	21.1492	1.1950	28			TRUE	0.03	TRUE	3	20		Y	N	N	N
H1	TB119	RNU44	UNKNOWN	FAM	NFQ-MGB	21.76852	25.1132	5.9402	95			TRUE	0.03	TRUE	3	19		Y	N	N	N
H2	TB119	miR-30a*	UNKNOWN	FAM	NFQ-MGB	25.39412	25.2984	0.1353	39			TRUE	0.03	TRUE	3	23		N	N	N	N
H3	TB119	miR-144*	UNKNOWN	FAM	NFQ-MGB	35.07868	36.9452	2.6396	55			TRUE	0.03	TRUE	3	33		Y	N	N	N
H4	TB119	miR-155*	UNKNOW	FAM	NFQ-MG	35.68552	35.6855					TRUE	0.03	TRUE	3	33		N	N	N	N

			N		B		2														
H5	TB119	RNU44	UNKNOWN	FAM	NFQ-MGB	21.59939	25.113324	5.9402955				TRUE	0.03	TRUE	3	19		Y	N	N	N
H6	TB119	miR-30a*	UNKNOWN	FAM	NFQ-MGB	25.20272	25.298422	0.1353339				TRUE	0.03	TRUE	3	23		N	N	N	N
H7	TB119	miR-144*	UNKNOWN	FAM	NFQ-MGB	38.81171	36.94552	2.6396555				TRUE	0.03	TRUE	3	35		Y	N	N	N
H8	TB119	miR-155*	UNKNOWN	FAM	NFQ-MGB	Undetermined	35.685552					TRUE	0.03	TRUE	3	39		N	N	N	Y
H9	TB119	RNU44	UNKNOWN	FAM	NFQ-MGB	31.9718	25.113324	5.9402955				TRUE	0.03	TRUE	3	28		Y	N	Y	N
H10	TB119	miR-30a*	UNKNOWN	FAM	NFQ-MGB	Undetermined	25.298422	0.1353339				TRUE	0.03	TRUE	3	39		N	N	N	Y
H11	TB119	miR-144*	UNKNOWN	FAM	NFQ-MGB	Undetermined	36.94552	2.6396555				TRUE	0.03	TRUE	3	39		N	Y	N	Y
H12	TB119	miR-155*	UNKNOWN	FAM	NFQ-MGB	Undetermined	35.685552					TRUE	0.03	TRUE	3	39		N	Y	N	Y

APPENDIX L

CONVERTED RT-PCR RESULTS FROM RAW DATA

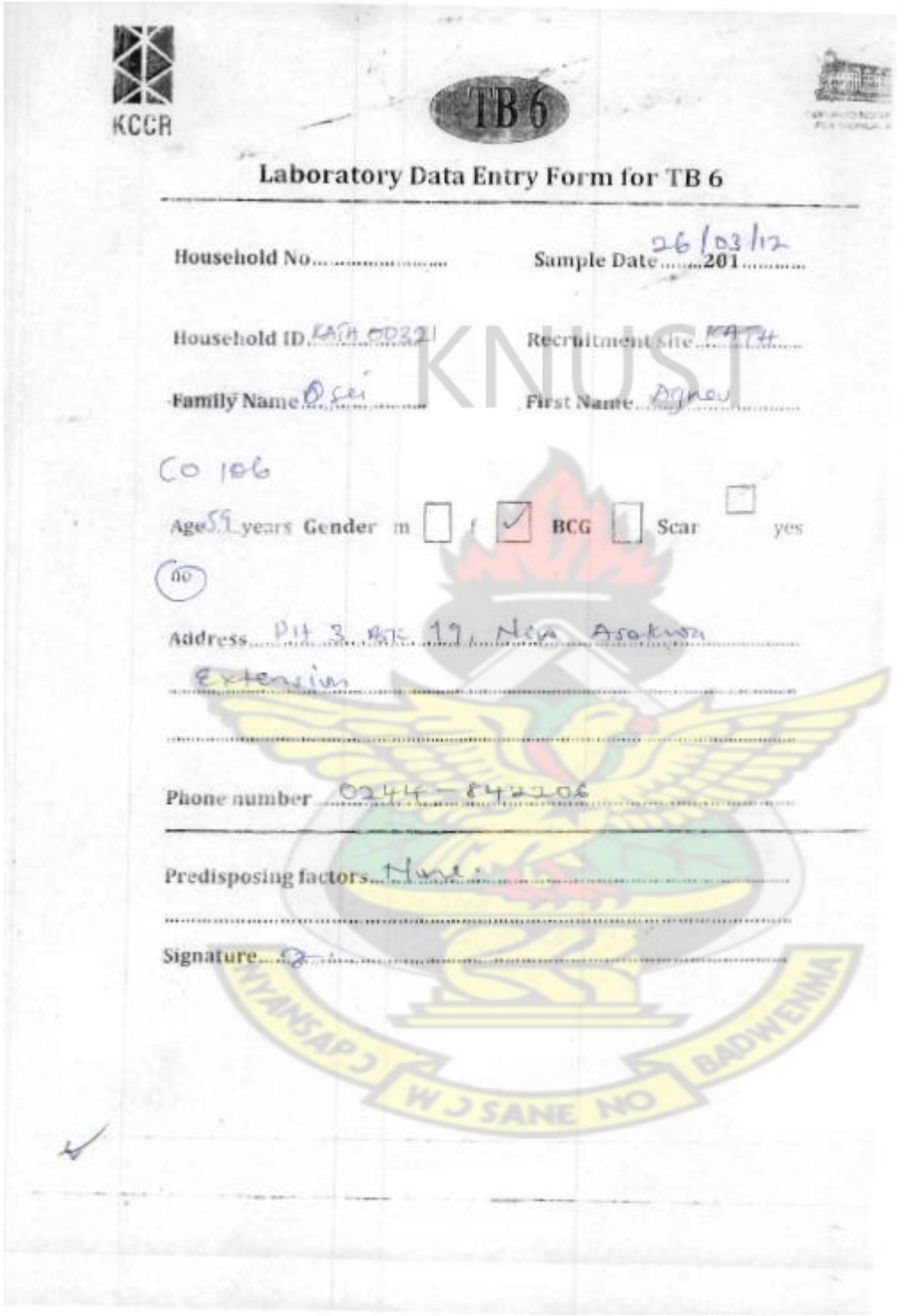
Table 24 CONVERTED RT-PCR RESULTS FROM RAW DATA

TARGET	Ct1	Ct2	Ct3	MEAN Ct	Rel CT
Let-7a	33.35	33.54	32.92	33.27	8.31333
miR-15a	36.13	33.92	35.04	35.03	10.07333
miR-17	27.2	27.01	27.04	27.08333	2.126663
miR-21	26.22	26.21	26.43	26.28667	1.329997
miR-23a	37.08	35.4	33.55	35.34333	10.38666
miR-25	32.1	31.05	31.86	31.67	6.71333
miR26a	27.63	28.07	27.5	27.73333	2.776663
miR-27a	32.17	32.09	33.07	32.44333	7.486663
miR-29a	27.85	27.63	27.54	27.67333	2.716663
miR-98	34.02	34.37	Undetermined	34.195	9.23833
miR-99b	34.65	33.34	34.03	34.00667	9.049997
miR-101	35.2	33.03	32.64	33.62333	8.666663
miR-106a	27.01	27	26.8	26.93667	1.979997
miR-106b	31.19	31.33	31.47	31.33	6.37333
miR-122	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-126	29.15	29.47	29.16	29.26	4.30333
miR-142-3p	25.47	25.46	25.08	25.33667	0.379997
miR-143	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-146a	26	26	25.81	25.93667	0.979997
miR-150	21.81	22.09	21.49	21.79667	-3.16

miR-181a	32.01	33.05	32.57	32.54333	7.586663
miR-191	28.06	27.37	28.11	27.84667	2.889997
miR-203	Undetermined	35.92	Undetermined	35.92	10.96333
miR218	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
miR-223	23.49	23.91	23.67	23.69	-1.26667
miR455	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
u6 snRNA	22.8	22.96	24.28	23.34667	-1.61
RNU48	24.28	24.27	26.32	24.95667	-3.3E-06
RNU44	32.42	27.41	27.07	28.96667	4.009997
miR-30a*	32.66	30.41		21.02333	-3.93334
miR-144*	Undetermined	34.41		34.41	9.45333
miR-155*	Undetermined	Undetermined		Undetermined	Undetermined
H2O	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined

APPENDIX M

DATA ENTRY FORM FOR CONTROLS


KCCR

TB 6

Laboratory Data Entry Form for TB 6

Household No..... Sample Date 26/03/12 ....201.....

Household ID EACH 00321 Recruitment site KATH

Family Name Osei First Name Agnes

CO 106

Age 59 years Gender m ☐ f ☑ BCG ☐ Scar ☐ yes

no

Address Plt 3, Blk 19, New Asokwa Extension

Phone number 0244 - 842206

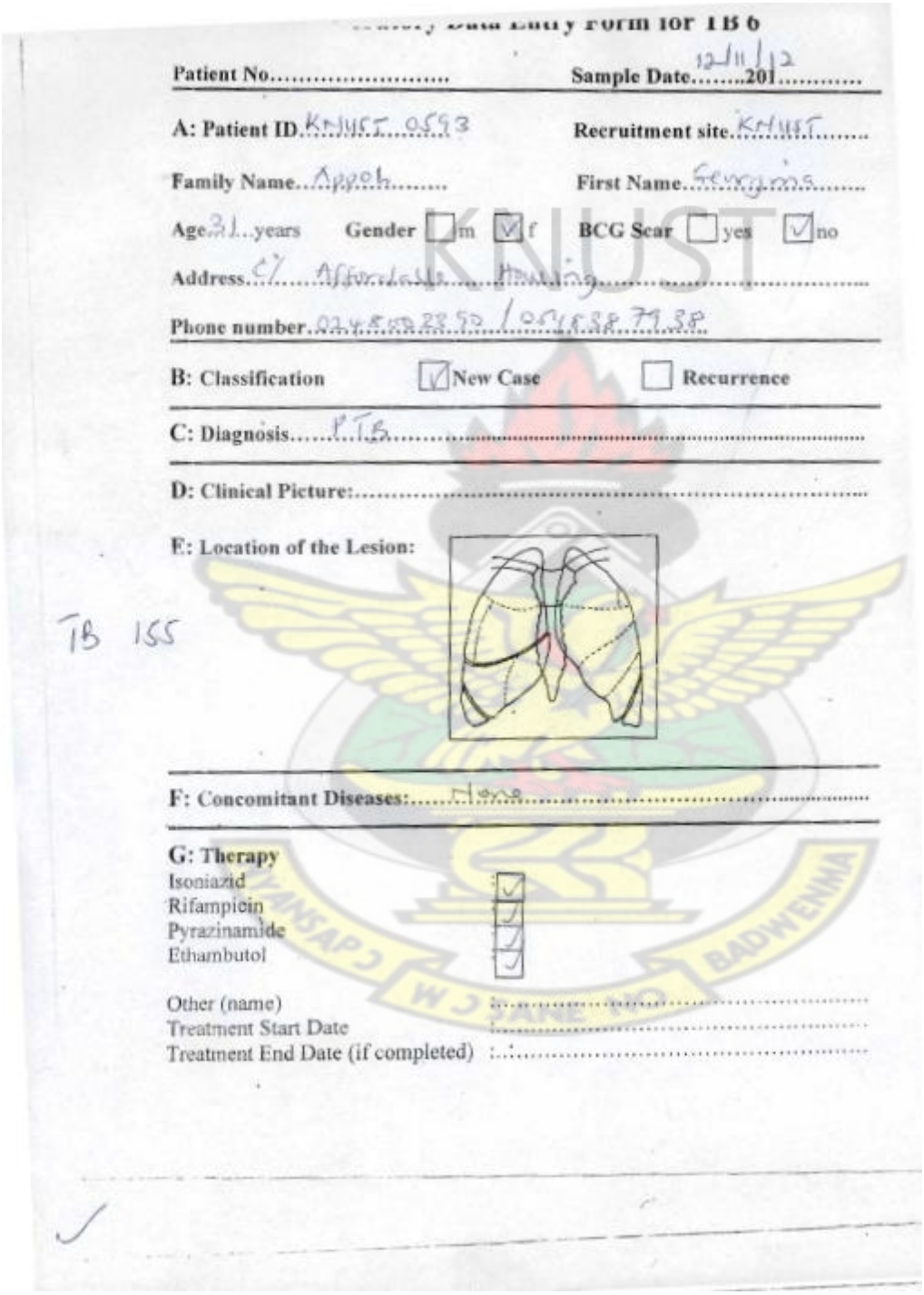
Predisposing factors Hwt.

Signature

Figure 42 A typical entered data sheet for household contacts of tuberculosis patients enrolled for this study

APPENDIX N

DATA ENTRY FORM FOR TB PATIENTS



Data Entry Form for TB 6

Patient No..... Sample Date 12/11/12 ..201.....

A: Patient ID. KNUST 0593 Recruitment site. KNUST

Family Name. Appoh First Name. Georgina

Age. 31 years Gender ☐ m ☑ f BCG Scar ☐ yes ☑ no

Address. C/ Affordable Housing

Phone number. 0245528252 / 0548387938

B: Classification ☑ New Case ☐ Recurrence

C: Diagnosis. PTB

D: Clinical Picture:

E: Location of the Lesion:

TB 155

F: Concomitant Diseases: None

G: Therapy

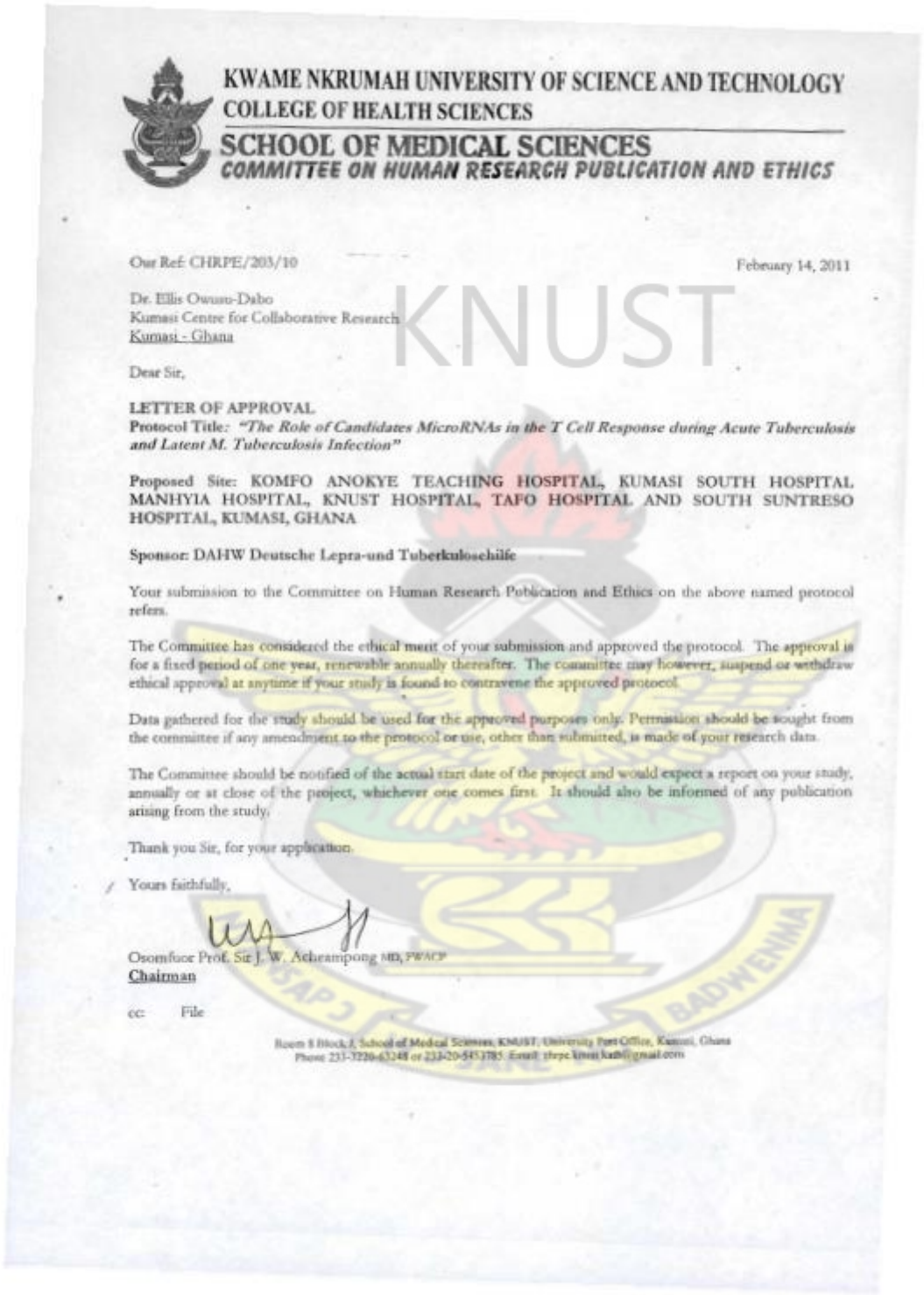
Isoniazid ☑
Rifampicin ☑
Pyrazinamide ☑
Ethambutol ☑

Other (name) :
Treatment Start Date :
Treatment End Date (if completed) :

Figure 43 A typical entered data sheet for tuberculosis patients enrolled for this study

APPENDIX O

ETHICS APPROVAL LETTER



KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICAL SCIENCES
COMMITTEE ON HUMAN RESEARCH PUBLICATION AND ETHICS

Our Ref: CHRPE/203/10 — Febeuary 14, 2011

Dr. Ellis Owusu-Dabo
Kumasi Centre for Collaborative Research
Kumasi - Ghana

Dear Sir,

LETTER OF APPROVAL
Protocol Title: ***"The Role of Candidates MicroRNAs in the T Cell Response during Acute Tuberculosis and Latent M. Tuberculosis Infection"***

Proposed Site: KOMFO ANOKYE TEACHING HOSPITAL, KUMASI SOUTH HOSPITAL MANHYIA HOSPITAL, KNUST HOSPITAL, TAFO HOSPITAL AND SOUTH SUNTRESO HOSPITAL, KUMASI, GHANA

Sponsor: DAHW Deutsche Lepra-und Tuberkulosehilfe

Your submission to the Committee on Human Research Publication and Ethics on the above named protocol refers.

The Committee has considered the ethical merit of your submission and approved the protocol. The approval is for a fixed period of one year, renewable annually thereafter. The committee may however, suspend or withdraw ethical approval at anytime if your study is found to contravene the approved protocol.

Data gathered for the study should be used for the approved purposes only. Permission should be sought from the committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee should be notified of the actual start date of the project and would expect a report on your study, annually or at close of the project, whichever one comes first. It should also be informed of any publication arising from the study.

Thank you Sir, for your application.

Yours faithfully,

Osomfuor Prof. Sir J. W. Acheampong MD, FWACP
Chairman

cc: File

Room 8 Block J, School of Medical Sciences, KNUST, University Post Office, Kumasi, Ghana
Phone 233-7220-63248 or 233-20-545-1785. Email: chrpe.knust.kath@gmail.com

Figure 44 Ethics Approval letter from Local Ethics committee

APPENDIX P

DATA ENTRY FORM TEMPLATE FOR TB PATIENTS

KCCR

TB 6

BERNHARD NOCHT INSTITUTE FOR TROPICAL MEDICINE

Laboratory Data Entry Form for TB 6

Patient No……. **Sample Date**……..201……

A: Patient ID…… **Recruitment site**……..

Family Name…… **First Name**……..

Age……years **Gender** ☐ m ☐ f **BCG Scar** ☐ yes ☐ no

B: Classification ☐ **New Case** ☐ **Recurrence**

C: Diagnosis……

D: Clinical Picture:……..

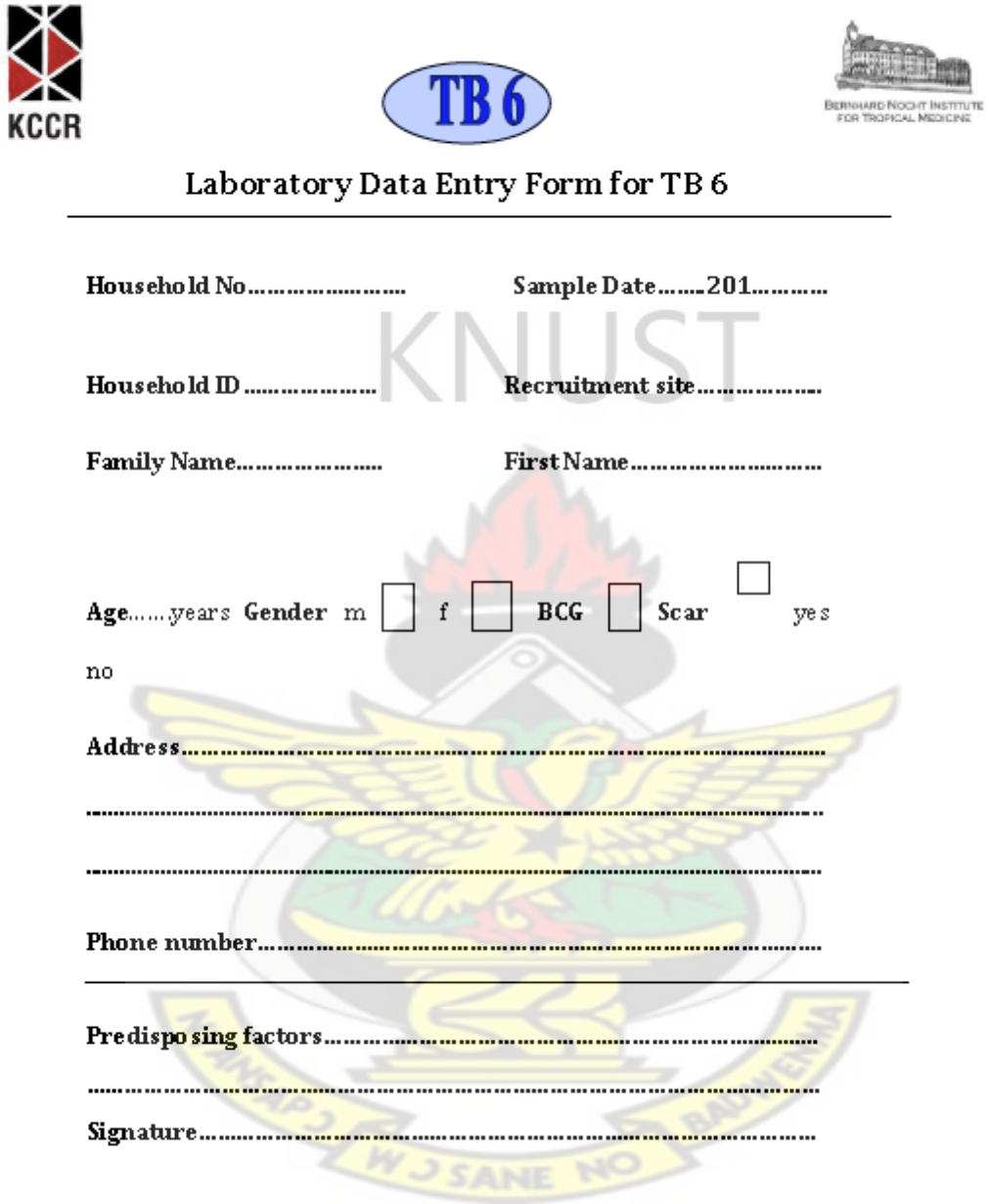
E: Location of the Lesion:

F: Concomitant Diseases:……

G: Therapy

Isoniazid	☐
Rifampicin	☐
Pyraziamide	☐
Ethambutol	☐
Other (name)	……
Treatment Start Date	……
Treatment End Date (if completed)	……

Figure 45 Data entry form for tuberculosis patients



TB 6

Laboratory Data Entry Form for TB 6

Household No..... Sample Date.....201.....

Household ID..... Recruitment site.....

Family Name..... First Name.....

Age.....years Gender m ☐ f ☐ BCG ☐ Scar ☐ yes

no

Address.....

.....

.....

Phone number.....

Predisposing factors.....

.....

Signature.....

Figure 46 Data entry form for household contacts of tuberculosis patients

APPENDIX Q

PICTURES OF STUDY FROM FIELD TO THE LABORATORY

Figure 47 Study team arrives at the house of the patients to take samples from patients and their contacts

Figure 48 Taking blood samples from study participants.

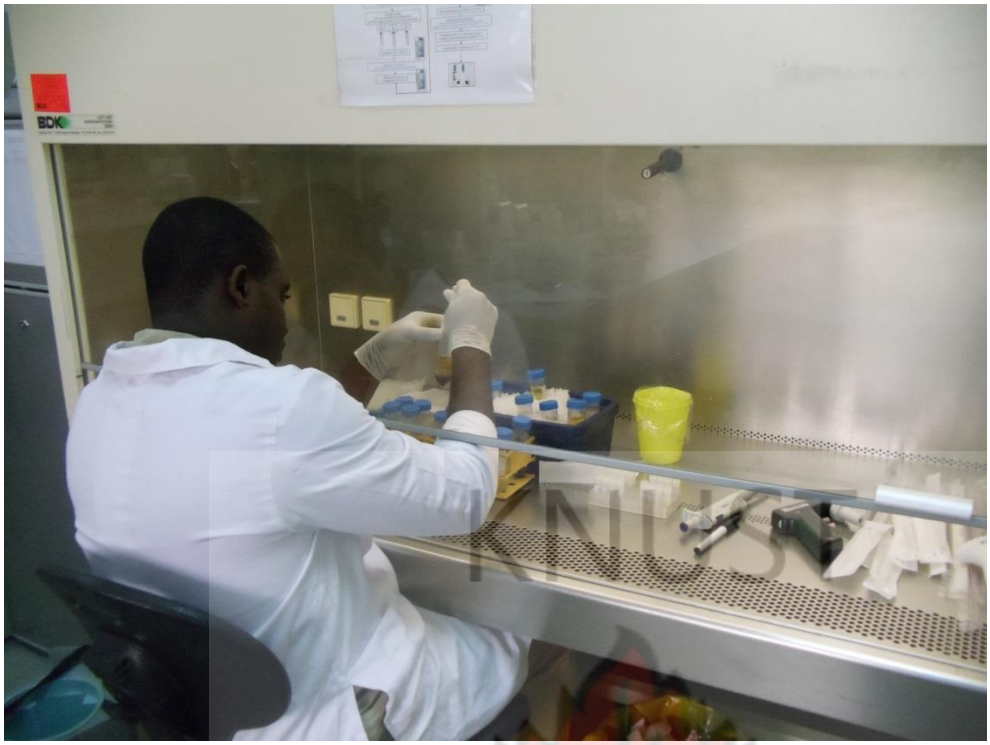


Figure 49 Laboratory processing of samples