



**Mannose Binding Lectin Genetic Polymorphism : Association with HIV-1
Infection in Adults and Children in Zimbabwe**

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A Thesis

Submitted to the School of Public Health, Faculty of Health Sciences University of the
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of

Doctor of Philosophy

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Supervisors

Professor Charles S. Chasela

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Candidate Declaration

I, Rutendo Beaunah Lynmarry Zinyama-Gutsire do solemnly declare, in accordance with Rule G27, that this thesis is a construction of my own original work. I am submitting this thesis for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. This thesis has not been submitted before for any degree or examination at this or any other University. I do confirm that this work being submitted for examination and assessment for the above degree is my own original work and all sources of assistance have been indicated and acknowledged.

I confirm that I have read the sections related to referencing and plagiarism in the University of the Witwatersrand Plagiarism Policy and I confirm that I have followed the required expectations in referencing the work and ideas of other researchers.



Signature: _____

Rutendo Beaunah Lynmarry Zinyama-Gutsire

15 June 2017

Dedication

I dedicate this thesis to my wonderful and supportive husband Charles Lovemore Gutsire, our sons Ronald and Tanaka, my parents Mr Rutsoka Gedion and Mrs Fiona Phiana Zinyama, my sisters Fadzisayi, Rufaro, Chipso, my brother Blessmore, my Sister-in law Longina, my brother and mentor Professor Lovemore Mhondiwa Zinyama, his wife Mrs Patricia Tsitsi Zinyama, my sisters Mrs Claris Khumalo and Mrs Julith Mahachi, Dr Prisca Mufunde, my in-laws the Gutsire family, their families, friends, relatives, co-workers, my Supervisors and mentors. Thank you all for believing in me thank you for your patience and encouragement while I pursued this programme. To my dear Husband Lovemore Charles Gutsire and our sons Ronald and Tanaka, thank you for putting up and enduring my absence from home. Thank you for wishing me success in this journey when I started and throughout. Thank you for continuously cheering me on, on this bumpy but rewarding journey. I will always cherish your unwavering support and encouragement, I will always love you.

Above all I dedicate this thesis to My Lord Jesus Christ of Nazareth all the Glory to your name, you were and you will always be my constant and ever present companion and cheer leader. I pursued this programme for your glory. Thank you for your comforting covering of Grace as I pursued this dream. Again I say Ebenezer, Glory be to God Almighty for taking us this far as a family!!!!!!

DEDICATION

A billion billion thanks to my husband, our two boys, my parents and my siblings for all the support and encouragement. Thank you all for cheering me on all the way!!!!.





Foreword

The journey of my PhD programme began in 2004 when I attended a 2 weeks research methodology course for potential PhD candidates at University of Witwatersrand, Faculty of Health Sciences. The course was organised by the Late Professor Steven Kuziwa Chandiwana, who was our former Director at the National Institute of Health Research, Ministry of Health Zimbabwe. I fell in love with the University of Witwatersrand, Faculty of Health Sciences then. Then in 2010, I applied for a PhD place through the School of Public Health. I received my acceptance letter from the University in September 2011, received funding from Letten Foundation, Oslo University in May 2012 and study permit from the South African Embassy in Zimbabwe.

I booked my first bus to take me to Johannesburg on 1 July 2012, a 20-24 hour long journey. This was to be the first of my many bus trips between Harare and Johannesburg in an effort to quench and fulfil my desire for a PhD degree from one of the most renowned Universities in Africa. Many more long bus trips followed after this first one. I registered for the Interdisciplinary programme on 3 July 2012. I am grateful to Prof Cathy Kahn and Professor Charles Simion Chasela for taking me through the rigorous requirements for this PhD programme. I liked it and felt encouraged when they both said if you work hard enough you can finish in 3-4 years. I dedicated all my energy and focus to the requirements for the programme, went through protocol development, protocol presentation, ethics clearance in Zimbabwe and at Witwatersrand University. I searched for stored samples from local researchers for my additional required laboratory work, manuscript development, submission to journals, waiting for journal comments and finally getting the required three papers published. I experienced two rejections of my manuscripts, a painful experience but I was

not discouraged, as I had always been taught, I knew perseverance pays and mostly my Supervisors kept on encouraging me. I am glad I did not give up on this dream.

I will forever be grateful to my husband Charles Lovemore Gutsire and our sons Ronald and Tanaka. They kept me company on all those long bus trips through calling, phone messages and whatsapp. Always asking where are you now mummy? It was always a huge relief when I wrote back to them saying I arrived in Johannesburg safely. Then on the return trip the messages started with me saying just finished my meeting with Professor Chasela, I have my bags packed, am on my way to Park Station. Then next message from me to Husband and sons would read 'I now on the bus we are leaving Johannesburg for Zimbabwe'. This went on for 4 years. I praise God for journey mercies and protection on all these numerous trips to and from Johannesburg. Thank you Lord Jesus, thank you Holy Spirit of God for giving me the energy to persevere, for holding my hand, guiding me and protecting me all the time.

I say Ebenezer, Glory be to God Almighty for taking me this far!!!!!!!

EBENEZER!!!!!!

I also say **EBENERZER** to my Jehovah. Thank you Lord you have taken me this far,
1 Samuel 7:12. Praise God. May I find it a joy to praise God everyday of my life.

EBENEZER!!!!!!

MY FAITHFULL LORD, JEHOVA JIREH (the Lord of mighty provisions) HAS
TAKEN ME THIS FAR!!!!!!

2 Corinthians 2:19

My Grace is sufficient for you, for my strength is made perfect in your weakness.

Isaiah41:10,13

Jehovah God says, Do not be afraid, for I will always be with you. Do not be discouraged, do not fear for I am your God. I will give you strength, and yes I will help you, I will always uphold you with my righteous hand. For I the Lord your God will always hold your right hand, saying to do not fear, I will always help you.

What sets you apart is the extra mile you are willing to go to make your dreams a reality!!!!

(Anonymous, posted at Wits main campus student notice board)

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Dr Frances Cowan and Dr Raluca Buzdugan I am grateful for permission to access and use stored dried blood spots for my research. The mother baby dried blood samples were collected as part of the national PMTCT national survey. My special gratitude goes to all the women and children who took part in the national PMTCT survey that was carried out in Zimbabwe in September 2012.

I am grateful for the PhD fellowship 2012-2016 provided by the Letten Research Foundation, University of Oslo, Norway/Zimbabwe Collaborative PMTCT BHAMC Programme special mention the late Professor Letten F. Saugstad which enabled my registration for PhD with University of the Witwatersrand, Johannesburg, South Africa.

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Lowence Gomo for data analysis on the main MUSH study. I thank the Mupfure Community, the Village Health Workers, the community leaders and the Environmental Health Technicians, special mention Mr Marime for the willing participation and contribution to our study; Mupfure Secondary school for accommodation. My special gratitude goes to all the men and women who agreed to participate in the main MUSH study and a special thank you goes to Bente Fredriksen and Vibeke Weirup for technical assistance on MBL assays on the MUSH samples in Denmark.

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I thank all my sponsors and financial support received, as mentioned earlier.

I am very grateful for all the spiritual support, encouragement and prayers received from Reverends and members of the Methodist Church in Zimbabwe, Marlborough Circuit, New Marlborough 3 Cell Group members. Thank you all for cheering me on.

Finally my gratitude and praise to the Almighty Jehovah through Jesus Christ of Nazareth who sustained me, encouraged me and gave me strength throughout the time I pursued my PhD studies. To Him be all the glory and honour for taking me this far. EBENEZAR!!!!

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This research was supported by:

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- HIV Research Trust UK 2006, paid for MBL analysis in Denmark, return airfares and accommodation, computer and printer [1, 2].
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- Laboratory space, reagents for *MBL2* genotyping of the PMTCT MBL sub-study and accommodation were provided by Professor Michael Christiansen at Serum State Institut Copenhagen, Denmark[3].

Abstract

Background

HIV infection has remained a major global health burden since its discovery in 1983 and Sub-Saharan Africa remains the region hardest hit by the HIV/AIDS pandemic. The HIV pandemic continues to ravage most parts of Southern African countries, current prevalence between 10-20%. Individuals worldwide differ in their degree of susceptibility to HIV infection and genetic polymorphisms play a major role. Mannose Binding Lectin (MBL) is one such immunological factor found in serum/plasma, it is a normal liver-derived protein and is a key component of the innate immune defence system. MBL deficiency, due to mutations in the *MBL2* gene and promoter region, leading to decreased plasma/serum MBL concentration, characterised by defective opsonisation activities of the innate immune system and increased susceptibility to infections including HIV-1 and schistosomiasis.

Rationale

While there is a lot of advancement in HIV prevention and treatment in Southern African countries, there is still need to investigate host genetic molecules in adults and mother-baby pairs that could be playing a role in HIV-1 transmission/acquisition, disease progression and survival. It was imperative to carry out this study because of the need to quantify the burden of MBL deficiency in this Zimbabwean adult and PMTCT study populations. Also to contribute to the knowledge gap on the role of MBL deficiency in HIV-1 transmission, disease progression and survival in African populations in adults and children. The available literature shows that the majority of studies on the association of MBL deficiency and HIV-1 infection in adults and children have been done on populations outside the African continent. There is dearth of information on the role of MBL in this era when access to ART has greatly

improved even in developing countries like Zimbabwe. This will be the second study that will assess *MBL2* genes and promoter typing in mother-infant pairs in HIV vertical transmission/acquisition. This study aimed to identify and explore potential biomarkers for susceptibility to HIV infection and disease progression.

We assessed role of MBL deficiency in HIV-1 and schistosoma infections in Zimbabwean adults enrolled in the Mupfure Schistosomiasis and HIV Cohort (MUSH Cohort) (Paper 1). We also assessed the role of MBL deficiency on HIV progression and survival in this African adult population. We hypothesized that MBL deficiency has a role to play in HIV infection by increasing HIV disease progression and decreasing survival (Paper 2). We also determined prevalence of MBL deficiency, as estimated by *MBL2* haplotypes among Zimbabwean mothers and their children aged 9-18 months old as well as its association with risk of HIV-1 infection and vertical transmission from their HIV positive mothers (Paper 3).

Main Aim

The broad objective of this study was to determine the relationship between MBL deficiency and HIV infection in an adult population of males and females and among mother-infant pairs in Zimbabwe.

Study Specific Objectives

1. To determine the prevalence of MBL deficiency among the Zimbabwean adult population.
2. To determine the relationship of MBL deficiency with HIV infection among the Zimbabwean adult population.
3. To determine the effect of MBL deficiency on disease progression and survival among the Zimbabwean adult population.
4. To determine prevalence of MBL deficiency among mothers and their infants in a Zimbabwean population.
5. To determine the relationship between MBL deficiency and HIV transmission from mother

to child in a Zimbabwean population.

Methods

DNA and plasma samples for MBL and HIV analysis were collected from the 379 adult males and females from the MUSH cohort and stored dried blood samples from 622 mother infant pairs from a national PMTCT survey.

HIV-1, *S. haematobium* and *S. mansoni* infections were determined at baseline using HIV commercial kits and parasitologically respectively. Plasma MBL concentration was measured by ELISA and *MBL2* genotypes determined by PCR. We calculated and compared the proportions of plasma MBL deficiency, *MBL2* structural variant alleles *B* (codon 54A>G), *C* (codon 57A>G), and *D* (codon 52T>C) as well as *MBL2* promoter variants - 550(*H/L*), -221(*X/Y*) and +4(*P/Q*) between HIV-1 and schistosoma co-infection and control groups using Chi Square test (Paper 1).

We also assessed the role of MBL deficiency on HIV disease progression and survival in the adult (MUSH) cohort. We analysed blood samples for MBL levels, *MBL2* genotypes, HIV-1 status, viral load and CD4⁺ T cell counts (Paper 1). Participants were followed up for 3 years wherein the endpoints were measured at baseline, 6 weeks, 3, 6, 12, 24 and 36 months.

Disease progression was measured as the rate of decline in CD4⁺ T cell counts and the rate of increase in HIV viral load (Paper 2). Generalised Estimating Equations (GEE) models were used to compare rates of change of the CD4⁺ T cell count and viral load measurements over the three-year follow-up period. The role of plasma MBL deficiency and *MBL2* genetic variants on survival over the 3-year period were estimated using the Cox proportional hazard models. Regression analysis was used to test for interaction and confounding between MBL

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deficiency, *MBL2* genetic variance, age and sex. We used the Wald Chi-square statistic to choose between full and nested models.

We also assessed *MBL2* polymorphisms in Zimbabwean HIV positive mothers and their children enrolled in a national PMTCT survey carried out in 2012. MBL deficiency was defined as presence of *A/O* and *O/O* genotypes in the mothers and their children. We extracted DNA from two dried blood spots for 622 mothers and infant pairs using the Gene Extract and Amp kit reagents. *MBL2* Exon 1 genotypes and promoter region alleles - *221(X/Y)* and *-550(H/L)* SNP were detected by pyrosequencing. Differences in distribution frequency between HIV infected and uninfected children, of the *MBL2* genotypes, promoter region variants and *MBL2* haplotypes, were determined by the Chi square test or Fisher's exact tests (Paper 3).

Key findings

For specific objective number 1, we assessed 379 adults, 80% females, median age (IQR) 30 (17-41) years. HIV-1, *S. haematobium* and *S. mansoni* prevalence were 26%, 43% and 18% respectively in the MUSH baseline survey. Median (IQR) plasma MBL concentration was 800µg/L (192-1936µg/L). Prevalence of plasma MBL deficiency was 18% with high frequency of the *C* (codon 57G>A) mutant allele (20%). For specific objective number 2, we found no significant difference in median plasma MBL levels between HIV negative (912µg/L) and HIV positive (688µg/L), $p=0.066$. However plasma MBL levels at the assay detection limit of 20µg/L were more frequent among the HIV-1 infected ($p=0.007$). *S. haematobium* and *S. mansoni* infected participants had significantly higher MBL levels than uninfected. All *MBL2* variants were not associated with HIV-1 infection but promoter variants *LY* and *LL* were significantly associated with *S. haematobium* infection (Paper 1).

For specific objective number 3, we assessed 197 HIV positive adults where 83% (164) were women with a median age of 31 years old. Prevalence of plasma MBL deficiency (less than 100µg/L) and *MBL2* deficient genetic variants (*A/O* and *O/O* genotypes) was 21% (42 out of 197) and 39% (74 out of 190), respectively. We did not observe a significant role to explain individual variation in mortality, change of CD4⁺ T cell count and viral load by MBL plasma deficiency or *MBL2* genetic variants from baseline to 3 years follow up period in this adult population (Paper 2).

For specific objective number 4, from the PMTCT study, the median age (IQR) of the mothers was 30(26 - 34) years and the children mean age (IQR) was 12 (11-15) months old at the time of enrolment. All 622 mothers were HIV-1 infected, 574 babies were HIV negative and 48 were HIV-1 positive babies. *MBL2* normal structural allele *A* and variants *B* (codon 5A>G), *C* (codon 57 A>G) and promoter region SNPs -550(*H/L*) and -221(*X/Y*) were detected. Prevalence of MBL deficiency was 34% among the mothers and 32% among the children. For specific objective number 5, we found no association between maternal *MBL2* deficiency and HIV-1 transmission to their children. We found no difference in the distribution of HIV-1 infected and uninfected children between the *MBL2* genotypes of the mothers and those of the children (Paper 3).

Conclusions

The results from our study indicate high prevalence of MBL deficiency but we found no evidence of association between MBL deficiency and HIV-1 infection. However, lower plasma MBL levels were associated with reduced prevalence of both *S. haematobium* and *S.*

mansoni infections and *MBL2* promoter and variants *LY* and *LL* were associated with increased susceptibility to *S. haematobium* infection (Paper 1).

Our findings attest to the large between-population variability in a host of factors that can predispose individuals susceptible to HIV progression and mortality. We therefore cannot recommend at this time the use of plasma MBL levels or *MBL2* genetic variants as a prognostic marker in HIV infection, disease progression and survival in this adult population in Africa (Paper 2). MBL deficiency was not associated with HIV-1 infection among the children nor was it associated with HIV-1 vertical transmission in this study population (Paper 3).

Key words

Mannose Binding Lectin (MBL), plasma MBL levels, *MBL2* genetic polymorphism, single-nucleotide polymorphisms (SNPs), MBL deficiency, HIV infection, *Schistosoma haematobium*, *Schistosoma mansoni*, Zimbabwean adults, plasma MBL levels, *MBL2* genetic polymorphism, *MBL2* exon 1 region, *MBL2* promoter region, single-nucleotide polymorphisms (SNPs), MBL deficiency, viral load, CD4⁺ T cell counts, disease progression, survival, longitudinal study, Zimbabwean adults, pyrosequencing, mothers, children, HIV-1 vertical transmission, prevention of mother to child transmission (PMTCT).

Presentations arising from this thesis

Paper 1

1. Abstract Title: Association of Mannose Binding Lectin Genetic Polymorphism With HIV-1 Infection, Disease Progression And Survival In A Rural Adult Cohort In Zimbabwe.

Rutendo B.L Zinyama-Gutsire, Charles Chasela, Hans O. Madsen, Simbarashe Rusakaniko, Per Kallestrup, Michael Christiansen, Exnevia Gomo, Henrik Ullum, Christian Erikstrup, Shungu Munyati, Edith N. Kurewa, Babill Stray-Pedersen, Peter Garred, Takafira Mduluz

An abstract based on this paper was submitted, accepted and presented in Durban at the 6th South Africa AIDS conference as a poster, 18–21 June 2013, funded by the SA AIDS conference, abstract number A2288739.

2. Abstract Title: Association of Mannose Binding Lectin Genetic Polymorphism With HIV-1 Infection, Disease Progression And Survival In A Rural Adult Cohort In Zimbabwe.

Rutendo B.L Zinyama-Gutsire, Charles Chasela, Hans O. Madsen, Simbarashe Rusakaniko, Per Kallestrup, Michael Christiansen, Exnevia Gomo, Henrik Ullum, Christian Erikstrup, Shungu Munyati, Edith N. Kurewa, Babill Stray-Pedersen, Peter Garred, Takafira Mduluz

An abstract based on this paper was submitted, accepted for presentation as an oral presentation at the Letten Research Symposium held at the Letten House, Harare, Zimbabwe on 14-15 August 2016.

Paper 2

1. Abstract Title: Absence of an association between Mannose Binding Lectin deficiency and HIV-1 disease progression in an adult population in Zimbabwe

Rutendo B.L Zinyama-Gutsire, Charles Chasela, Per Kallestrup, Simbarashe Rusakaniko, Michael Christiansen, Bernard Ngara, Exnevia Gomo⁷, Henrik Ullum, Christian Erikstrup, Hans O. Madsen, Babill Stray-Pedersen, Peter Garred, Takafira Mduluza

An abstract based on this paper was submitted, accepted for presentation as a poster at the IAS Conference, 19-22 July 2015 in Canada, abstract number S20417.

2. Abstract Title: Absence of an association between Mannose Binding Lectin deficiency, HIV-1 disease progression and survival in an adult population in Zimbabwe

Rutendo B.L Zinyama-Gutsire, Charles Chasela, Per Kallestrup, Simbarashe Rusakaniko, Michael Christiansen, Bernard Ngara, Exnevia Gomo, Henrik Ullum, Christian Erikstrup, Hans O. Madsen, Babill Stray-Pedersen, Peter Garred, Takafira Mduluza

An abstract based on this paper was accepted for presentation as a poster at the Letten Research Symposium held at the Letten House, Harare, Zimbabwe on 14-15 August 2016.

Paper 3

1. Abstract Title: Mannose Binding Lectin Genetic Polymorphism: Absence of an Association with HIV-1 Vertical Transmission in a PMTCT Cross-Sectional Survey in Zimbabwe.

Rutendo B.L. Zinyama-Gutsire, Michael Christiansen, Paula L. Hedley, Simbarashe Rusakaniko, Christian Hagen, Babill Stray-Pedersen, Raluca Buzdugan, Frances Cowan and Charles Chasela

An abstract on this study was submitted and accepted for presentation as a poster at the AIDS 2016: 21st International AIDS Conference, 18–22 July 2016, Durban, South Africa funded by the IAS, the 21st International AIDS Conference abstract number TUPEA016, University of the Witwatersrand travel grant 2016, the Letten Research Foundation, University of Oslo Norway/Zimbabwe Collaborative PMTCT BHAMC Programme, the Medical Research Council of Zimbabwe.

2. Abstract Title: HIV-1 Vertical Transmission in Zimbabwe in 622 Mother and Infant Pairs: Rethinking the Contribution of Mannose Binding Lectin Deficiency in Africa

Rutendo B.L. Zinyama-Gutsire, Michael Christiansen, Paula L. Hedley, Simbarashe Rusakaniko, Christian Hagen, Babill Stray-Pedersen, Raluca Buzdugan, Frances Cowan and Charles Chasela

An abstract based on this paper was also accepted for presentation as a poster at the Letten Research Symposium held at the Letten House, Harare, Zimbabwe on 14-15 August 2016.

PhD Programme Interim Seminar Presentation Title:

Mannose Binding Lectin Genetic Polymorphism : Association with HIV-1 Infection in Adults and Children in Zimbabwe

Rutendo B.L. Zinyama-Gutsire, Michael Christiansen, Simbarashe Rusakaniko, Charles Chasela, et al

Oral presentation at the University of the Witwatersrand, Faculty of Health Sciences, School of Public Health academic meeting for the PhD Interim Seminar, progress assessment, 1

April 2016, Johannesburg, South Africa.

Thesis overview and structure

This PhD was undertaken using the route of thesis with publications. This thesis therefore consists of two parts: an integrating narrative which provides the introduction, rationale, the research methodology and findings of the research, which are summarised, the research papers produced as part of this PhD (Appendix A, B and C) and a methodology paper on pyrosequencing still under review (Appendix D). This research comprised two related studies: the adult MBL study which explored MBL polymorphism and its possible association with susceptibility to HIV infection and the PMTCT study which also explored MBL polymorphism among mothers and their babies and possible association with HIV infection and vertical transmission. The adult MBL study was a sub-study of the MUSH cohort implemented in Zimbabwe to investigate immunological mechanisms in HIV and schistosomiasis co-infections. The PMTCT study used stored dried blood samples for mothers and their infants collected during a national PMTCT survey carried out in 2012 in Zimbabwe.

Integrating Narrative

The integrating narrative comprises eight chapters. **Chapter one** includes the introduction and background to the study and has the following sections: introduction, origination of the research idea, role of the candidate in this research thesis, brief background on role of MBL in HIV disease, study rationale, broad objective, study specific objectives, study hypothesis and the conceptual framework. **Chapter two** provides a detailed profile of Zimbabwe, the country where the research was undertaken, putting this research into context.

Chapter three is literature review, divided into the following sections: collectins, MBL discovery, MBL gene structure, MBL pseudogene, MBL genetic variants, role of MBL in

diseases, then final sections reviewing available literature on studies done exploring role of MBL in HIV infection in adults and children worldwide, in Africa and in Zimbabwe.

Chapter four is a synthesis of all the methods that were used in the two studies reported in this thesis and includes the following sections: study design, setting, adult study sample size and its calculation, PMTCT study sample size and its calculation, research participants, inclusion criteria, exclusion criteria, laboratory methods, data collection and statistical analysis methods). Detailed methods for each individual study are described in the appended Papers 1-3. **Chapter five** provides a synthesis and summary of the main findings of the adult MBL and PMTCT studies (detailed results are presented in the individual papers attached). The key cross-cutting themes that emerge from this work are discussed in **Chapter six**, while highlighting the significance of the findings in light of available literature, and the implications of our results to public health focusing on role of MBL genetic polymorphism in HIV infection and susceptibility. This chapter also discusses the contributions of this thesis to available literature and knowledge and also gives the conclusions made based on the results from our two studies, highlights the limitations of our research, the public health implications of our results and recommendations for future studies that can be explored. The three published papers, the manuscript under review and approval/permission letters are attached as (Appendices A-R) and other papers on the MUSH study co-authored by the candidate, related to my PhD work, but not included in this thesis (Appendix R).

List of papers included in this PhD thesis

This thesis is submitted in the integrating narrative format, approved by the Faculty of Health Sciences, of published work. This thesis consists of three academic papers and one manuscript under review, listed below:

1. **Rutendo B.L Zinyama-Gutsire**, Charles Chasela, Hans O. Madsen, Simbarashe Rusakaniko, Per Kallestrup, Michael Christiansen, Exnevia Gomo, Henrik Ullum, Christian Erikstrup, Shungu Munyati, Edith N. Kurewa, Babill Stray-Pedersen, Peter Garred, Takafira Mduluza.

Role of Mannose-Binding Lectin Deficiency in HIV-1 and *schistosoma* Infections in a Rural Adult Population in Zimbabwe

PLoS One. 2015 Apr 1;10(4):e0122659. doi: 10.1371/journal.pone.0122659. eCollection 2015.

Student's contribution to the paper

Design of the study, seeking funding for the project grant, project management (including training fieldworkers and supervision of data collection), data management including data entry, cleaning and coding, data analysis and writing of the manuscript.

2. **Rutendo B.L Zinyama-Gutsire**, Charles Chasela, Per Kallestrup, Simbarashe Rusakaniko, Michael Christiansen, Bernard Ngara, Exnevia Gomo, Henrik Ullum, Christian Erikstrup, Hans O. Madsen, Babill Stray-Pedersen, Peter Garred, Takafira Mduluza.

HIV-1 Disease Progression and Survival in an Adult Population in Zimbabwe: Is There an Effect of the Mannose Binding Lectin Deficiency?

Student's contribution to the paper

Design of the study, seeking funding for the project grant, project management (including training fieldworkers and supervision of data collection), data management including data entry, cleaning and coding, data analysis and writing of the manuscript.

3. **Rutendo B.L. Zinyama-Gutsire**, Michael Christiansen, Christian Hagen, Simbarashe Rusakaniko, Paula L. Hedley, Babill Stray-Pedersen, Raluca Buzdugan R, Frances Cowan F, Charles Chasela.

HIV-1 Vertical Transmission in Zimbabwe in 622 Mother and Infant Pairs: Rethinking the Contribution of Mannose Binding Lectin Deficiency in Africa.

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Student's contribution to the paper

Design of the sub-study, sought permission to access and use stored dried blood samples, seeking funding for the project grant, project management, data management including data entry, cleaning and coding, data analysis and writing of the manuscript.

4. **Rutendo B.L. Zinyama-Gutsire**, Michael Christiansen, Christian Hagen, Simbarashe Rusakaniko, Paula L. Hedley, Babill Stray-Pedersen, Raluca Buzdugan R, Frances Cowan F, Charles Chasela.

Optimization of *MBL2* genotyping assay using dried blood spots: Experiences from the PMTCT Survey in Zimbabwe.

(manuscript under review)

Student's contribution to the paper

Design of the study, seeking funding for the project grant, project management data management including cleaning and coding, data analysis and writing of the manuscript.

Policy Brief

Role of Mannose Binding Lectin Genetic Polymorphism in HIV-1 Infection among Adults and Children in Zimbabwe

This policy brief can be found at:

<http://www.chp.ac.az/PolicyBriefs/Documents>

List of Abbreviations

AIDS	-	Acquired Immunodeficiency Syndrome
CD4 ⁺ T cell count	-	Cluster of Differentiation 4 T cell count
CD8 ⁺ T cell count	-	Cluster of Differentiation 8 T cell count
CDC	-	Centres for Disease Control
CI	-	Confidence interval
DHS		Demographic and Health Survey
DNA	-	Deoxyribonucleic Acid
EIA	-	Enzyme Immuno Assay
<i>e.g.</i>	-	for example
<i>et al</i>	-	and others
ENHR	-	Essential National Health Research
°C	-	Degrees Celcius
Fig	-	Figure
HIV-1	-	Human Immunodeficiency Virus
hr	-	hour
MASP-1	-	MBL-associated serine protease-1
MASP-2	-	MBL-associated serine protease-2
MBL	-	Mannose Binding Lectin
MDGs -		Millenium Development Goals
MoHCC		Ministry of Health and Child Care
MRCZ		Medical Research Council of Zimbabwe
MUSH Cohort	-	Mupfure Schistosomiasis and HIV cohort
PCR	-	Polymerase Chain Reaction

PMTCT	-	Prevention of Mother to Child Transmission
RNA	-	Ribonucleic Acid
μl	-	microlitres
ml	-	millilitres
%	-	percentage
UNAIDS	-	Joint United Nations Programme on HIV/AIDS
WHO	-	World Health Organization
ZDHS	-	Zimbabwe Demographic and Health Survey
ZIMSTAT	-	Zimbabwe National Statistics Agency

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CHAPTER 1 BACKGROUND TO THE STUDY

1.0 Introduction

This chapter describes what inspired me to undertake this study on the role of MBL deficiency in HIV infection among Zimbabwean adults and children. Zimbabwe is one of the countries in the world worst affected by the HIV pandemic and the prevalence declined from a high of 33% in the 1990s and has now declined to 13% among the 15-49 age group [4]. However, HIV related diseases still remain the main causes of morbidity and mortality among both adults and children in Zimbabwe.

With my background as a laboratory scientist, I was very keen to know about and followed developments with keen interest on the immunological and genetic factors that were being reported from other countries that increased susceptibility to HIV infection among sexually active age groups and vertical transmission from mothers to their children. For this research I decided to focus on Mannose Binding Lectin as there was conflicting evidence available in literature about its role in HIV infection. We therefore determined the role of MBL deficiency in HIV infection in a rural adult study population from the MUSH cohort and from a PMTCT national survey which enrolled mothers and their infants. Some of the adults in the MUSH cohort were also co-infected with schistosomiasis, a common infection in rural Zimbabwe.

1.1 The Idea

The role of MBL deficiency in diseases has been a topic of interest over the last two decades. We decided to investigate the role of MBL deficiency in the Zimbabwean adults and children because of the high HIV prevalence in our population. An

understanding and appreciation of the immunological factors fuelling the HIV-1 epidemic and increasing susceptibility to the infection in African populations is very important in an effort to curb the HIV-1 scourge.

Over the last decades there has been increased interest in investigating the host genetic factors that could lead to increased susceptibility to infections including HIV. Mannose Binding Lectin (MBL) is one such molecule that has been reported on. Mannose-Binding Lectin is a key component of the innate immune system and polymorphism in the *MBL2* gene and promoter region lead to MBL deficiency [5-7]. My hope and expectation is that the results from this research will result in improvement in the health of the population of my country as we understand the role of host genetic factors in infections focussing on HIV and schistosomiasis.

1.2 Originality of the thesis and role of the candidate

The candidate conceived the MBL adult sub-study in 2001. Then in 2013 to give a more complete picture of the role of MBL in HIV infection the candidate developed the PMTCT MBL sub-study. The candidate carried out MBL analysis on the samples from the adult and from the PMTCT samples. The results of MBL analysis in the adult MUSH cohort and the PMTCT samples form the basis of this thesis.

1.3 Role of the PhD candidate in the MUSH study

The adult cohort addressing the role of MBL in disease progression was a sub-study that was nested into the MUSH cohort in Zimbabwe, conceived and led by the PhD candidate. The sub-study assessed the relationship between MBL plasma

concentration, *MBL2* genotypes, HIV infection, HIV disease progression and survival in these adults. The PhD candidate carried out HIV testing, MBL ELISA and *MBL2* genotyping by PCR in an established laboratory using established primers in Dr Garred's MBL laboratory in Denmark in 2006.

1.4 Role of the PhD candidate in the national PMTCT national survey

The candidate did not take part in the national PMTCT survey and did not participate in sample collection. Stored dried blood samples from mothers and baby pairs were obtained from the CESHAAAR Research Centre in Zimbabwe that had stored whole blood samples for mother –infant pairs from a national PMTCT survey carried out in 2012. Permission to access and use stored dried blood spots for this research project was sought and granted by the CESHAAAR study team and University of California. Ethical and scientific clearance was granted by University of the Witwatersrand and the Medical research Council of Zimbabwe

1.5 The Candidate's Role in Relation to the Thesis

In relation to this thesis the candidate's role can be outlined as follows:

- 1 Conception of the adult and PMTCT MBL sub-studies and design of the protocol.
- 2 Supervision of the collection of all MUSH related data which included variables used for this analysis.

- 3 Participation in follow up of MUSH research participants for sample collection over the three year follow up period and for schistosomiasis treatment.
- 4 Preparation of research ethics submissions to University of the Witwatersrand and the Medical research Council of Zimbabwe.
- 5 Preparation and submission of application to ship dried blood samples to Denmark in 2014.
- 6 Securing a Letten PhD fellowship for registration at University of the Witwatersrand.
- 7 Conduct of detailed literature review for the study protocol and the thesis.
- 8 Negotiation with Professor Michael Christiansen for permission to do MBL assays on the PMTCT samples at serum State Institute in Denmark.
- 9 Optimisation of the DNA extraction, PCR and pyrosequencing assays for the PMTCT samples.
- 10 Data cleaning, recoding of variables, exploration analysis, tracking of missing data, not only for data related to this analysis but from data collected for the main MUSH cohort. The tracking of missing data was done on site from the study results laboratory sheets.
- 11 Conduct of statistical analysis using STATA.
- 12 Interpretation of results and generation of all tables and figures included in this thesis.
- 13 Scientific presentations of results from this thesis at:
 - (a) 6th South Africa AIDS, conference, Durban as a poster, 18–21 June 2013
 - (b) IAS Conference 2015 in Vancouver, Canada, July 2015

- (c) Letten Research Symposium held at the Letten House, Harare, Zimbabwe on 14-15 August 2016.
- (d) AIDS 2016, 21st International AIDS Conference, 18–22 July 2016, Durban, South Africa

1.6 Expected Contributions

The potential contributions of this study to public health research and to public health policy in Zimbabwe are identified as follows:

1.7 Public Health Research

1. Establish the prevalence of MBL deficiency among adults in the Zimbabwe MUSH cohort.
2. Establish the prevalence of MBL deficiency among adults in the Zimbabwe MUSH cohort and its role in HIV infection.
3. Determine the role of MBL deficiency in HIV disease progression and survival among the MUSH cohort adults
4. Establish the prevalence of MBL deficiency among mothers and their infants a subset of samples collected during a PMTCT national survey.
5. Establish the prevalence of MBL deficiency among Zimbabwean mothers and their infants and its role in HIV vertical transmission.

1.8 Policy Makers (Implementation)

1. Provide evidence on the role of MBL deficiency in HIV infection and vertical transmission

2. Explore the use of dried blood spots for MBL genotyping using the PMTCT samples
3. This study aimed to identify and explore potential biomarkers for susceptibility to HIV infection and disease progression since Zimbabwe has a high burden of HIV infection it is important to understand the immunological correlates.

1.9 Mannose Binding Lectin (MBL)

Human MBL is a normal serum protein discovered in the 1980s, produced in the liver and derived from a single gene on chromosome 10 [8]. It is a multichain calcium dependent lectin that belongs to the collectin family, a group of carbohydrate binding proteins [8, 9]. It is a key first line defence molecule, acts as an opsonin in innate immunity and activates the complement system by binding to the sugar groups found on the surfaces of various infectious agents like bacteria, viruses and parasites [8, 10]. MBL deficiency which is an inherited condition, is due to point mutations in the *MBL2* gene and promoter region and this is common in both black and white populations[7]. The *MBL2* gene has well documented promoter region positions, -550, -221 and +4 [7]. Point mutations in the *MBL2* gene and *MBL2* promoter region lead to genetic polymorphism, defined as the appearance of different forms of the *MBL2* gene and promoter region genetic variants [6, 7]. *MBL2* genetic polymorphism negatively affects gene expression and results in impaired protein function [8]. The normal MBL concentration in plasma/serum is 1000µg/L and above and MBL deficiency is below 100µg/L [5-7, 10-13]. The presence of *MBL2* genetic and promoter region variants are strongly associated with low plasma/serum MBL

levels defined as MBL deficiency [6, 7]. MBL deficiency has been reported to be associated with increased susceptibility to several infections like TB [14], malaria [15], cystic fibrosis [16], cytomegalovirus infection [17], cryptosporidiosis [18], schistosomiasis [19], early childhood infections like recurrent respiratory infections [20] and HIV [10, 21, 22].

1.10 Polymorphism in the Mannose Binding Lectin Gene and Promoter Region

Polymorphism in the *MBL2* gene due to genetic point mutations result in presence of variant *MBL2* alleles. The normal *MBL2* allele is known as A [8]. The variant *MBL2* alleles encode three different structural variants B, C, and D, due to mutations at codons 54, 57 and 52 respectively of the MBL polypeptide, which affect the structural integrity of MBL protein, resulting in 2-fold to 100-fold decrease in circulating MBL serum/plasma levels [6]. Mutations and variations in the *MBL2* gene are the basis why there is such a wide range of circulating MBL levels in different populations [6]. These mutations lead to a reduction of functional MBL to 10% in individuals that are heterozygous for defective alleles compared with the functional MBL found in individuals with two functional alleles [6, 8]. Mutations in the *MBL2* gene cannot explain all the large interracial and inter-individual variations in MBL concentrations reported so far. In addition to the mutations in the *MBL2* gene, there are also point mutations in the *MBL2* promoter region at positions -550 (*H/L*), -221 (*X/Y*) and in the 5' untranslated region of exon 1 at position +4 (*P/Q*), which also affect plasma/serum MBL levels which have been well documented [6].

1.11 Mannose Binding Lectin and disease

The ability to respond effectively to infectious agents is a prerequisite for the survival of multi-cellular organisms. MBL is a key constituent of the innate immune defence system where it acts as an opsonin and it belongs to a group of host-defence associated protein domains involved in recognition of infectious agents [23]. MBL recognises a broad range of sugar molecular patterns on a broad range of infectious agents and is able to distinguish these from normal host cells. MBL activates the complement system by binding to sugar groups, which include high mannose and N-acetylglucosamine oligosaccharides which are found on the surface of various gram positive and gram negative bacteria, viruses, yeasts, mycobacteria and parasites [8, 9].

Upon binding to the micro-organism, MBL activates the complement system through co-opting the action of MBL-associated serine proteases (MASPs) called MASP1 and MASP2 [24, 25]. MBL, recognises sugar molecules on the surface of an infecting pathogen, binds to both pathogen and MASPs, MASP2 binds and cleaves C3 component of the complement system and this indirectly activate the complement system cascade in an antibody independent manner, leads to deposition of opsonins on the surface on infecting pathogens and clearance of the infection [24, 26]. MBL deficiency therefore results in an impaired first line host defence system therefore leading to increased susceptibility to various infections.

1.12 Mannose Binding Lectin and HIV infection

MBL has been shown to play a defensive role in HIV infection as MBL and HIV both compete for binding to the CCR5 receptor, the major receptor for HIV-1 [27]. MBL activates the complement system by binding to carbohydrate molecules which are also present on the HIV-1 virion surface thus contributing to HIV-1 clearance [28]. MBL binds and opsonizes the HIV-1 virus hence affecting HIV-1 virus trafficking and antigen presentation during HIV infection [29]. In vitro studies suggest a link between MBL levels and HIV pathogenesis, purified MBL has been shown to bind to HIV infected cell lines and can inhibit infection of lymphoblast by HIV, MBL can bind to and activate gp120 complement and MBL binds to both CCR5 and CXCR4-tropic HIV primary strains[29]. Individuals with MBL deficiency have dysfunctional MBL protein and are more at risk of acquiring HIV infection and HIV disease progression, amongst infected populations [10, 21].

1.13 Mannose Binding Lectin and HIV infection in adults and children

Zimbabwe is one of the countries that are worst affected by the HIV pandemic with a prevalence of 13.6% among adults [4]. HIV MTCT is the second major route of HIV transmission and the national MTCT rate in Zimbabwe has declined from a high level of 26-29% in 2009 [30] to 6.6% in 2015 [31, 32]. Several studies have been done worldwide on MBL because of its role in innate immunity and the possible association of MBL deficiency with HIV infection and disease progression in adults and children, reviewed in [33, 34]. Results from studies in adults on the role of MBL in HIV infection are still conflicting, there are reports of a detrimental effect of the promoter region variants XA [22] and increased susceptibility to HIV transmission [21]. There is conflicting information on role of MBL deficiency on HIV infection

and disease progression among infected adults[35]. While other studies have reported association of MBL deficiency with increased risk of HIV infection [21], others have reported no association between MBL gene polymorphism and also promoter region polymorphism and HIV infection [36]. Similarly, while some studies have reported increased rate of HIV disease progression to AIDS, others have shown either no effect or delayed rate of disease progression [37]. Most of these studies have been done in European populations, only two studies have been done on African adults, in Tanzania [10] and Gabon [38], which showed increased risk of HIV infection among those with MBL deficiency, but no such study has been reported for any adult population from Southern African countries. Only 3 studies have reported investigations on MBL deficiency and HIV in children in Southern Africa [39-41] and only one such study reported on Zimbabwean children [39]. The results from these three studies are conflicting, Mhandire et al [39] and Zupin et al [41] both reported no association between presence of MBL2 exon 1 variants and HIV infection in the Zimbabwean children investigated, in contrast to results showing statistically significant association between MBL deficiency, MBL variants among HIV infected children [40]. Our research study aimed to address the research gap that while there have been many studies on MBL and HIV among adults and children, the available results are conflicting.

1.14 Role of MBL deficiency in HIV disease progression and survival

Several studies have investigated the role of MBL deficiency in HIV disease progression [21, 42-50]and survival [21, 44]. HIV disease progression is characterized by decline in CD4⁺ T cell count and increase in HIV viral load [51-

57]eventually leading to death in the absence of treatment [51-57]. Available literature on the association between MBL deficiency and disease progression is conflicting, some report faster HIV disease progression due to MBL deficiency [21, 46, 49, 50] but others found no association [42, 47, 58, 59]. Others report association between MBL deficiency and decreased survival in HIV infected people [21]but conflictingly others reported increased survival in those with variant *MBL2* genotypes and MBL deficient individuals [44]. We hypothesized that MBL deficiency had a role to play in HIV infection by increasing HIV disease progression and decreasing survival. We therefore assessed the role of plasma MBL deficiency and *MBL2* genetic variants, on HIV-1 disease progression and survival in a Zimbabwean adult population enrolled in the Mupfure Schistosomiasis and HIV cohort (MUSH) cohort.

There is therefore need to carry out more such investigations in Sub-Saharan Africa where there are high HIV prevalence rates and where the predominant HIV is subtype C, unlike in European populations with mostly HIV subtypes A and B, reviewed in [33]. Several studies have been done investigating MBL and its association with HIV infection in children [60], looking at different aspects and most of these have been done in populations outside Africa, except three studies carried out on Southern African children [39-41]. Some studies investigated association between MBL deficiency and HIV transmission from mother to child, some studied MBL deficiency and HIV disease progression and some have looked at both [33]. Unlike studies that are linking MBL deficiency and HIV infection and disease progression in adults, there is concordance in all the studies among children [33]. Studies on MBL deficiency and HIV transmission showed that MBL deficient genotypes were significantly

increased in HIV infected children compared to either HIV exposed but uninfected children or HIV unexposed controls [33].

For this research project, immunological analysis of plasma samples and genetic analysis of DNA samples for *MBL2* genetic variants among the adult population used the Mupfure Schistosomiasis and HIV (MUSH) cohort [61, 62], while questions regarding the role of MBL deficiency in HIV acquisition and transmission from mother to child used stored blood from and PMTCT program. The blood samples from the PMTCT cross survey provided us with an opportunity to investigate if MBL deficiency is a factor in vertical transmission or acquisition of HIV among infants in Zimbabwe.

1.21 Conceptual Framework

The conceptual framework [63] for our study was based on the graphical disease causality model recommended for use in health sciences research [64, 65], adapted and modified to suite the requirements of this study (Fig.1.1). Diagrams of causal pathways are used to visually summarize hypothetical relations among variables of interest [64]. Nolan *et al* 2004 provided a broad conceptual framework for considering the impact of host genetic diversity on HIV/AIDS, arguing that host genetic variation is an important factor in the response to HIV for every infected individual [66]. The causal graph provides a visual representation of key concepts of the research to be undertaken and the expected outcomes [64, 67,68]. Our study consists of six main concepts namely HIV exposure, *MBL2* genetic polymorphism, adult HIV infection/acquisition, mother to child HIV vertical transmission, HIV/AIDS disease progression and survival. The relationship of these concepts is presented in

the conceptual framework below (Fig. 1). Our research fitted well into this conceptual framework as we aimed to determine role of MBL deficiency on HIV infection as only three such studies have been done in African adults in Tanzania [10] and Gabon [38] and three carried out on African children [39-41]. Our study is the second reported after the Zambian study [41], to investigate the *MBL2* genotypes of HIV positive mothers and their infants in association with HIV vertical transmission.

1.19.1 HIV exposure

Adults are exposed to HIV infection through unprotected sexual contacts, sharing sharp instruments for example drug users and blood transfusion [69, 70]. Unborn babies are exposed to HIV infection in the uterus and/or during delivery and one third of the children born to HIV positive mothers but who test negative for HIV infection can become infected through breastfeeding in the absence of ART [69, 70].

1.19.2 *MBL2* genetic polymorphism

MBL deficiency is an inherited immunological condition that results in appearance of variant forms of the MBL molecule[5-7]. Mutation in the *MBL2* gene and promoter region results in variant *MBL2* alleles and low MBL concentration in plasma[5-7]. MBL deficiency leads to impaired phagocytic ability of the innate immune system therefore increase susceptibility to several infections including HIV[35].

1.19.3 HIV infection/acquisition in adults

Three main routes of HIV infection are known namely sexual contact, through infected body fluids and mother to child transmission [70, 71]. The national HIV prevalence in Zimbabwe is currently 13.6% among adults and the main mode of transmission is heterosexual contacts[71, 72].

1.19.4 Mother to child HIV transmission

An HIV infected mother can transmit infection to her unborn child during pregnancy, during delivery or through breastfeeding [73]. MTCT is the second major route of HIV transmission and the national MTCT rate in Zimbabwe has declined from a high level of 26-29% in 2009 [30] to 6.6% in 2015 [31, 32].

1.19.5 HIV/AIDS disease progression

After infection, in the absence of ART, HIV infection progresses to AIDS disease through four main stages, primary infection, clinically asymptomatic stage, symptomatic HIV infection and progression from HIV to AIDS [69, 70]. As done in several other studies viral load and CD4⁺ T cell counts were used as indicators of disease progression in our adult study population [45, 74]. Our study compared disease progression in the HIV positive adults between those with the wild type *MBL2* alleles and promoter types and those with variant *MBL2* alleles and promoter types.

1.19.6 Survival

Survival after HIV infection greatly varies between individuals, but an infected individual can succumb to AIDS after about 5 years without treatment but with ART survival rate increases [69, 70]. Mortality due to HIV/AIDS was used to compare survival rates among the adults with normal *MBL2* gene and promoter region types and those with variant *MBL2* alleles and promoter types. Survival rates were compared among the HIV positive adults between those with the wild type *MBL2* alleles and promoter region types and those with variant *MBL2* alleles and promoter type

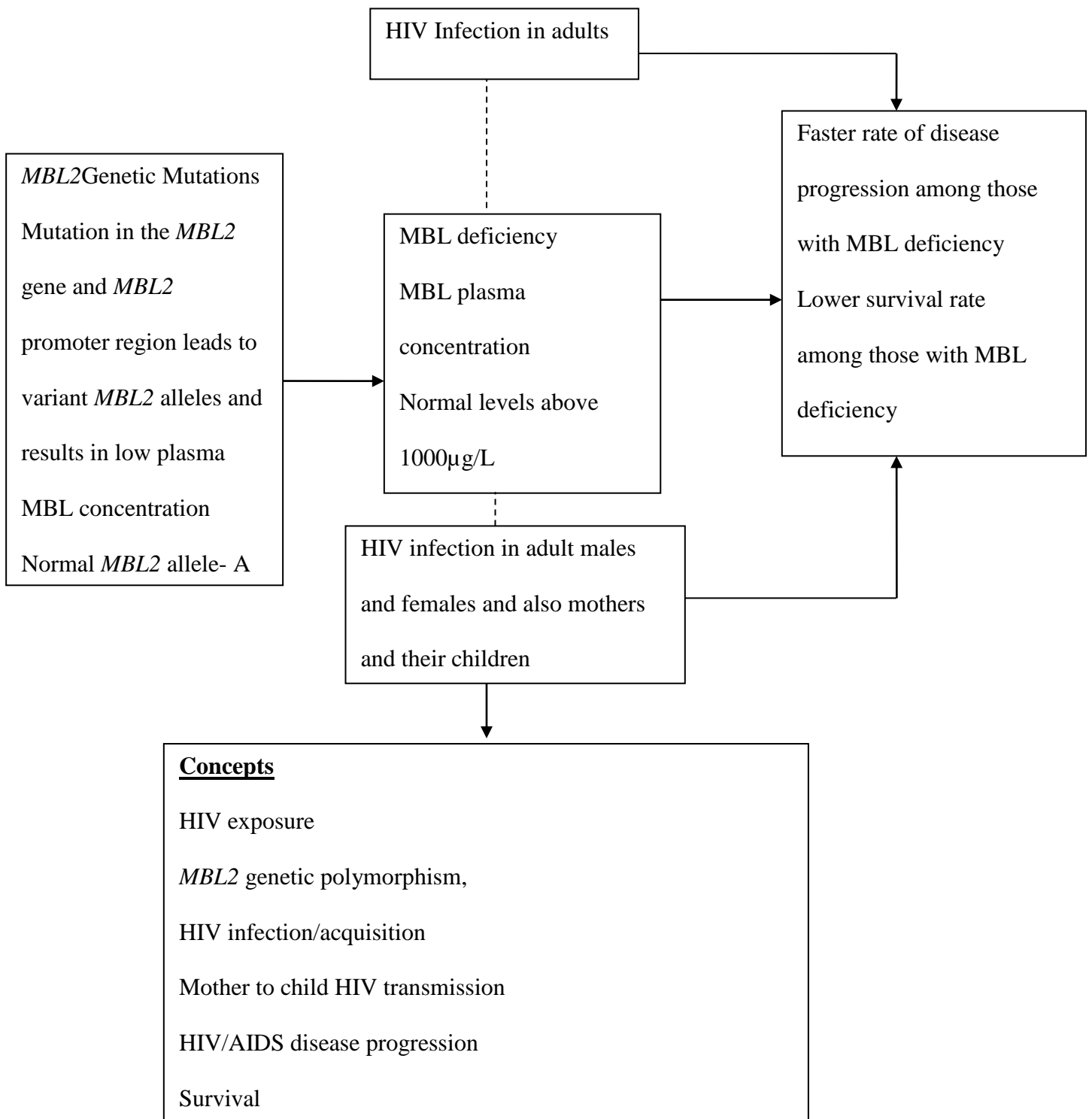


Figure 1.1: The conceptual framework for association between MBL deficiency and HIV infection in adults and children.

CHAPTER 2 CONTEXT: PROFILE OF ZIMBABWE

2.0 Introduction

This chapter provides background information on Zimbabwe and gives a broader picture of the study setting, describing geographical, economic and health context of the country.

2.1 Geographic and Demographic Features

Zimbabwe is located in Sub-Saharan Africa, south of the equator[75]. Zimbabwe is a landlocked country, sharing borders with South Africa to the south, the Republic of Mozambique to the east and the Republic of Zambia to the north (Fig. 2.1).



Figure 2.1.Map of Zimbabwe and Surrounding Countries



Figure 2.2:Map of Zimbabwe showing location of Capital City Harare and other cities

Zimbabwe covers an area of 390 759 square kilometres[75-77]. The country has 10 provinces divided into 58 administrative districts[76]. The districts are divided into wards and the wards are further sub-divided into traditional villages and each village is headed by chiefs[76]. Zimbabwe has 10 major cities and several smaller towns, Harare is the political and commercial capital (Fig.2.2). Other major cities include Bulawayo to the South, Mutare in the East, Marondera, Masvingo, Gweru and Kwekwe (Fig. 2.2).

The population of the country is estimated currently to be 13,7 million, with 52% females[75, 77]. The annual growth of the population is 2.1% as determined in the

2012 national census[77]. The population density is 33 persons per square kilometre[77]. The average number of children per household is 4 with most families in rural areas having 5 children[75, 78]. Zimbabwe has 42% of the population under 18 years of age and the average life expectancy is 54 years for men and 53 years for women years[76]. The high population density puts the country under considerable economic stress, especially when most of the population is rural and the economy is agro-based. Zimbabwe's main languages include Shona, Ndebele, Chichewa, Tonga. English is used as the official language[75].

2.2 Economic Status

Zimbabwe is classified as a developing country[75]. Zimbabwe, a former British colony, obtained its independence from Britain in April 1980 after a long armed guerilla warfare[75]. All sectors of the economy performed well then, until the early 1990s. Zimbabwe is the only country in the Southern Africa Development Community (SADC) region currently experiencing a negative economic growth rate, hyperinflation leading to a huge political and economic crisis since year 2000[75]. Over the last 15 years the Zimbabwean economy has deteriorated from being one of Africa's strongest to being the world's worst with the official inflation rate estimated at more than 1 000% in 2006[75, 79].

More than half of the population (70%) is currently living a life below the poverty datum line (less than one USA dollar/day) in 2016 with about half (50%) living in ultra poverty[75, 79]. The economy is predominantly agriculture but this sector of the economy has been affected by the economic crisis characterised by high costs of

inputs and cash shortages[75, 79]. Much of Zimbabwe's poverty is caused by poor economic governance, unemployment and the HIV/AIDS pandemic[75, 79].

2.3 Health Sector Overview

Zimbabwe is currently experiencing poor health indicators among the general population [76]. The major causes of morbidity and mortality are preventable and curable diseases such as malaria, pneumonia, diarrhoea, tuberculosis, and other infectious diseases which are endemic.

2.4 HIV AIDS Trends in Zimbabwe

HIV and AIDS remain the leading causes of morbidity and mortality among the 15 to 49 years old [31]. The first AIDS case was reported in 1985 in Zimbabwe, from 1985 to the mid-90s the HIV prevalence rose sharply to reach a peak of 27.7% in 1997 and started declining thereafter[4, 31]. The decline in prevalence is attributed to the impact of prevention programs aimed at behavior change (high condom use and reduction in multiple sexual partners), elimination of Mother to Child Transmission services, and successful treatment care and support services[4, 31].The HIV epidemic together with tuberculosis (TB) has become the major cause of morbidity and mortality affecting both adults and children over the last three decades [31]. The main underlying causes of poor health indicators include widespread poverty, unemployment, chronic malnutrition, low educational status mostly in rural areas, poor sanitation, poor access to safe water, and inadequate capacity of the healthcare system to deliver quality and accessible health services[75, 79].

2.5 Organisational Structure of Health in Zimbabwe

The delivery of health services in Zimbabwe relies on locally trained doctors and nurses but we have experienced huge brain drain over the last 20 years as the trained medical professionals have left the country for greener pastures in surrounding countries and overseas[75, 79].. Thus critically paralyzing the health care system with only one physician per 100,000 and 40 nurses per 100,000 populations [31, 75, 79] (Table 2.1). Health service delivery has four main levels of care: rural health centres / rural health posts, rural hospitals servicing three or four health centres, district hospitals and a central hospital in each of the 10 provinces. Coverage of antenatal care is 71% with 60% of babies delivered by skilled attendants (nurses, or doctors)[75, 76], (Table 2.1). Funding for the health services has been through partnerships between the Ministry of Health and Child care and donor agencies including WHO, UNICEF, CDC, UNAIDS, JICA and others[31, 76].

2.6 Health Indicators of Zimbabwe

Health care in Zimbabwe is provided predominantly through the public sector. The major central referral hospitals are Parirenyatwa and Harare hospitals situated in Harare and Mpilo situated in Bulawayo. This study was conducted among adult males and females in Mashonaland Central Province and among mothers and their children enrolled for a national PMTCT evaluation programme which involved all 10 provinces in Zimbabwe.

Table 2.1: Health Indicators for Zimbabwe

Health Indicators for Zimbabwe	
Crude Birth Rate per 1,000 population[31, 75-77]	33
Infant Mortality Rate per 1,000 live births [76, 77]	90
Mortality Rate in <5 year olds per 1,000 live births [76, 77]	72
Life expectancy at Birth (both sexes) [76, 77]	53 years
Maternal Mortality Rate per 100,000 live births [76, 77]	960
Physicians per 100,000 population [75-77]	1
Nurses per 100,000 population [76, 77]	40
GDP per capita[75-77, 79]	953.38US\$
HIV prevalence in 15-49 years in 2014 [4, 75-77]	13.6%

CHAPTER 3 LITERATURE REVIEW

3.0 Introduction

This chapter provides details on literature on collectins, their role in innate immunity, introduces the MBL molecule, its discovery and role in innate immunity.

3.1 Collectins

Mannose Binding lectin (MBL), which is the molecule of interest in this research project, belongs to a group of chimeric molecules comprising globular heads, which contain the carbohydrate recognition domain, and collagen tails and are defined as collectins [80-82]. Collectins are the humoral lectins found in mammals and birds, they are oligomers whose subunits comprise three polypeptide chains each containing a collagenous section and a C-terminal lectin domain[80-82]. Collectins are reported to be related structurally and functionally to the first component of the classical complement pathway, C1q, and serve important roles in innate immunity through opsonization and complement activation[80-82]. Collectins are carbohydrate binding proteins containing a carbohydrate recognition domain at one end and a collagen-like stalk domain at the other end. The lectin domains is capable of binding carbohydrates on microorganisms, while the collagenous regions are ligands for the collectin receptor on phagocytes and also to mediate C1q-independent activation of the classical complement pathway. The mannose-binding lectin (MBL), pulmonary surfactant apoproteins A and D and conglutinin are members of the collectin family, whose function is mainly as pattern recognition molecules involved in the first line of defence in the innate immunesystem [80, 82, 83].

3.2 Mannose Binding Lectin (MBL)

Human MBL is a normal serum protein, synthesized in the liver and released into the bloodstream and belongs to the above mentioned calcium-dependent collectin family [80, 84-86]. The MBL protein is encoded by the MBL2 gene located on chromosome 10 at 10q.11.2q21 [83, 84, 87,88] and MBL1 is a pseudogene [88]. An analysis of the genomic nucleotide sequence and the cDNA sequence of MBL showed that the MBL protein coding region was made up of four exons separated by three introns of 600, 1350 and 800 base pairs [84]. Exon 1 and 2 encode the signal peptide, a cystein and glycine rich domain of repeated amino acids motif, typical for the triple helix formation of collagen structures and exon 3 encodes the neck region and exon 4 encodes the carbohydrate binding domain [84]. The MBL protein is synthesised in the liver as structures consisting of three to six triple helix oligomers[84]. The final MBL protein is made up of oligomers, each with three identical polypeptide 32 kDa chains as evaluated on SDS-PAGE [89]. Many oligomeric forms of MBL with different functional capabilities have been found in human serum/plasma[90-93]. The functions of MBL are strongly influenced by its concentration and its oligomerization both of which are affected by factors like race, age, host immune-competent state, infection status, and also determined by presence of mutations in the gene or promoter region[5-7, 83, 94].

A small proportion of MBL protein is produced from transcripts which originate from exon 0 but the vast majority of liver synthesised MBL protein is from exon 1 initiated transcripts[6].

Low MBL levels in serum/plasma have been associated with various diseases including increased risk of infection with HIV [21], tuberculosis [38], lung diseases [95] and unusual infections in adults [96].

3.3 MBL discovery

In 1968 a patient with a serum-dependent opsonic defect in phagocytosis of yeast particles was described for the first time [97]. The opsonic defect was subsequently linked to the complement system as the C3 complement molecule was deposited in lower amounts on yeast surfaces incubated in sera of individuals with the opsonic defect [98, 99]. Independent of the efforts in humans, a protein was discovered and extracted from rabbit liver using mannan particles from *Saccharomyces cerevisiae* as a probe [100]. This protein was initially given the name mannan binding protein (MBP) that was later renamed to Mannose Binding Lectin [80, 101]. It was later shown that MBL was also present in human serum and that it could activate the complement system when it binds to a mannan surface [85, 102]. Subsequently it was shown that the originally described opsonic/phagocytic defect was due to MBL deficiency and was first described in 1989 [103]. MBL was first documented in 1988 when a cDNA clone that encoded MBL was isolated [89]. In 1989, the following year, independent reports on cloning and sequencing of the human *MBL2* gene were released [84].

3.4 MBL2 gene structure

The *MBL2* gene is made up of 4 exons and 3 introns [80, 101, 104,105], Fig 3.1. The MBL protein is a multi-chain molecule of two to six subunits, each subunit consisting of three identical 32 KDa polypeptide chains that contain a cystein-rich region, a neck

region, a collagenous region and a carbohydrate-binding domain (CBD)[80, 84], (Fig 3.1) The MBL protein can be a monomer, trimer, tetramer, pentamer or hexamer depending on the number of assembled polypeptide chains, the most common assembly is the tetramer with 4 chains and [80, 84]. Disulphide bonds which are found between cysteine residues, situated in the terminal part of the protein, stabilize the collagenous triple helix structure[80, 106]. MBL is able to recognise carbohydrate patterns in the form of terminal mannose groups, found in abundance on the surfaces of a broad range of microorganisms including bacteria, viruses, fungi, parasites[80, 84].

3.5 *MBL1* pseudogene

Pseudogens are defined as functionless versions of genes that have lost their gene expression capacity or ability to code protein in the cells[107]. In man the MBL genetic system consists of one functional gene (*MBL2*) and one pseudogene (*MBLIP1*) which is also expressed, presence of an MBL pseudogene has been reported [88, 108, 109]. The MBL genes, *MBLIP1* and *MBL2* are reported to be most likely products of a gene duplication process [109]. The pseudogene was silenced and selectively turned off through the evolution process by mutations in the glycine residues of the MBL collagen region [108]. *MBLIP1* has been characterised as an expressed pseudogene, consisting of two nonsense mutations in exon 3 and exon 4 and also a splicing defect that leads to the preservation of intron 1 and results in pre-termination of the protein [88] thus explains its non-functional status.

3.6 *MBL2* gene and its variants

The normal *MBL2* allele is known as A and point mutations in the *MBL2* gene result in presence of variant alleles [7], (Fig 3.2). Three single independent nucleotide

substitutions in exon 1 of the *MBL2* gene cause a dominant decrease of functional MBL protein in the circulation namely: at codon 54 due to substitution of glycine with aspartic acid (GGC to GAC, allele *B*) [110], at codon 57 due to substitution of glycine with glutamic acid (GGA to GAA, allele *C*) [111] and at codon 52 due to substitution of arginine with cysteine (CGT to TGT allele *D*) [5]. The variant *MBL2* alleles encode three different structural variants, *B*, *C*, and *D*, (Fig 3.1, Fig 3.2) which result in lower MBL serum/plasma concentrations and the variant alleles have been found to be quite frequent in normal, healthy populations of African, Caucasian, Asian and Eskimo origin and they are present in 20 to 50% of such individuals [7].

It has been suggested, in previous studies, that all three structural variant alleles may disrupt the normal assembly of *MBL2* polypeptide chains into the basic trimer structure or make variant *MBL2* more vulnerable to degradation of the protein during circulation, or both, resulting in a decrease of the serum/plasma MBL concentrations [5-7]. Individuals with *MBL2* structural gene mutations express an unstable protein due to the interruption of the Gly-X-Y repeat motif of the collagenous domain, reducing molecular stability through abnormal disulphide linkages [110, 112, 113]. The *MBL2* variants interfere with the correct assembly of the MBL subunits, disrupts formation of higher *MBL2* oligomers and also disrupts interaction of MBL with MASPs [114]. This instability of the MBL molecule contributes to both low concentration and poor function of MBL observed in individuals with *MBL2* variants. *MBL2* heterozygosity results in 5 to 10 times reduction of functional MBL protein [5-7]. These mutations lead to a reduction of functional MBL to 10% in individuals that are heterozygous for defective alleles compared with the functional MBL found in individuals with two normal functional

26

alleles [5-7]. Reduced serum MBL is not itself pathogenic, but it predisposes individuals to infections and faster disease progression and there are reports of accelerated disease progression in HIV infected individuals with low MBL [21]. Extensive scientific and clinical evidence suggests that administration of MBL to MBL-deficient individuals will restore the MBL function *in vivo* and thereby cure the immuno-deficiency [115, 116].

3.7 *MBL2* promoter region and its variants

Madsen et al., 1994, 1995 were the first to describe the existence of additional polymorphisms in the *MBL2* promoter and 5' Un Translated Region (UTR), upstream of the *MBL2* gene, at positions -550 (*H/L* variants) and -221 (*X/Y* variants (both are G to C nucleotide substitutions))[5, 6], Fig 3.2. The promoter haplotypes *HY*, *LY* and *LX* were the first promoter haplotypes to be documented and they were reported to show associations with high, medium and low levels of MBL in serum or plasma, respectively [7]. Later presence of the *PQ* haplotype, located in the 5' untranslated portion of the gene at position +4, was reported [6], Fig 3.2. These three promoter region polymorphisms are in strong linkage disequilibrium with exon 1 SNPSs, and so far seven haplotypes have been described namely *HYA*, *LYQA*, *LYPA*, *LXPA*, *LYBP*, *LYQC* and *HYPD* [7], (Fig 3.3) [101, 117].

Table 3.1: Classification of *MBL2* haplotypes and plasma MBL concentration

<i>MBL2</i> haplotype	MBL levels classification	MBL levels categories
YA/YA, YAXA	normal plasma MBL levels	above 500µg/L
XA/XA, YA/YO	intermediate or reduced levels	100µg/L- 500µg/L
XA/YO, YO/YO	deficient levels	below 100µg/L

The functional categories of *MBL2* haplotypes

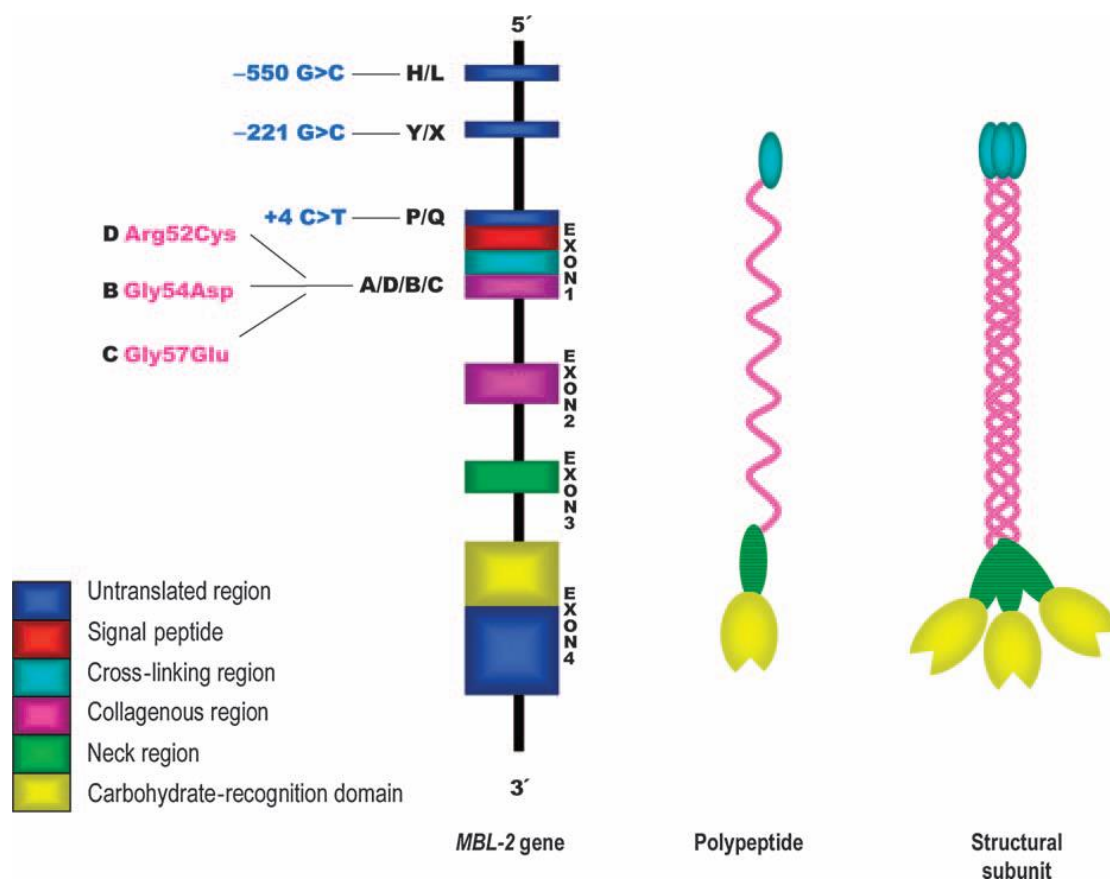


Figure 3.1: Structure of *MBL2* gene, MBL polypeptide and MBL structural subunits

Exon 1 and promoter region of the *mb12* gene

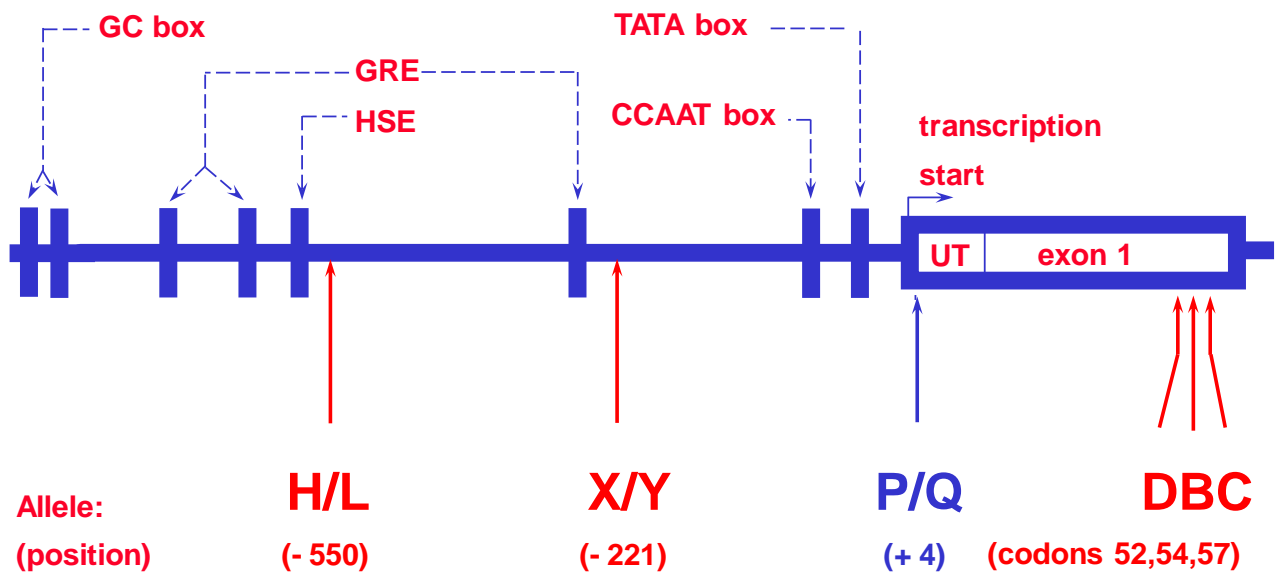


Figure 3.2: Organization of Exon 1 of the *MBL2* gene and *MBL2* promoter region.

The variant alleles are *B*, *C* and *D*. The promoter region alleles are *H/L*, *X/Y* and *PQ*.

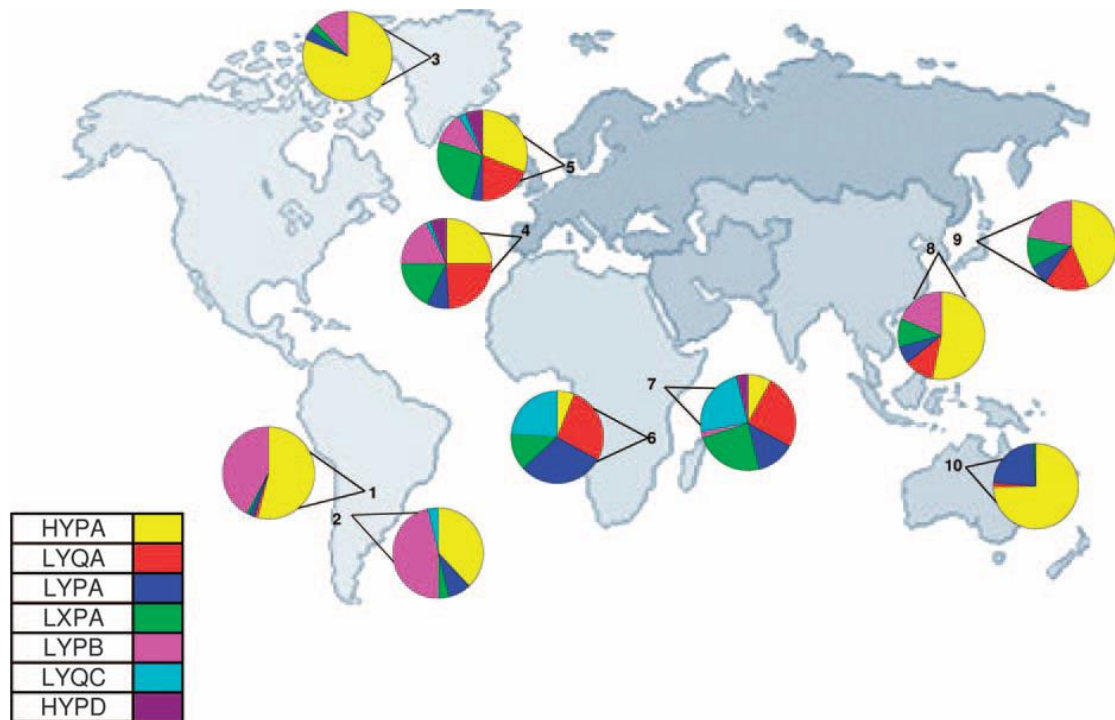


Figure 3.3. Worldwide distribution of *MBL2* genotypes

3.8 *MBL2* genetic selection pressure

There are reports in available literature on the possible advantage of evolution of the *MBL2* variants and the high prevalence of *MBL2* variants on the African continent [38, 118]. *MBL2* variants have been reported to offer protection to infection by intracellular parasites like TB and *Leishmania* such that individuals with variant *MBL2* genotype are less susceptible to these infections as activation of the complement system promotes microbial uptake through opsonophagocytosis, accelerates spread and establishment of the infection [38, 118].

3.9 Biological Role of MBL : MBL and the complement system

The MBL protein plays a key biological role through activating the complement immune system [119], (Fig 5). Complement is part of the innate immune system and

the complement's main functions are defence against infection, bridging innate and adaptive immunity and disposing of immune complexes and the products of inflammatory injury [119]. Complement activation can occur through the classical, the alternative or the lectin pathway and the three pathways are involved in pathogen opsonisation, chemotaxis, activation of phagocytes and direct pathogen lysis through the formation of membrane attack complexes [119], (Fig 3.4, Fig 3.5). MBL is a key factor and an important constituent of the innate immune defence system where it acts as an opsonin [81, 82, 105, 120] and activates the complement system through the lectin pathway, by binding to sugar groups or oligosaccharides on the surface of various bacteria, viruses, fungi and parasites [35, 80, 89, 92, 94, 119-122] including HIV [89]. The ligands for MBL which include high mannose, N-acetylglucosamine oligosaccharides, N-acetylmannoseamine, N-acetylgalactoseamine, maltose, glucose and galactose are present on a variety of micro-organisms [80, 92, 123, 124]. Upon binding to the micro-organism, MBL activates the complement system through the action of MBL-associated serine proteases (MASPs) called MASP1, MASP2 [125], MASP3 [126] and MAP-19 [127] and also interacts with novel receptors on phagocytes [24, 25, 120, 123, 128, 129], reviewed in [105], (Fig 6).

About 5-10% of serum MASP1, MASP2 and MASP3 appear complexed with MBL [130] but MASP-2 is the main initiator of the MBL pathway [131]. Upon MBL ligand binding, the activation of the lectin pathway occurs by the capacity of MASP-2 to cleave C2 and C4 complement components, which causes the transformation of C3 into C3a and C3b, (Fig 6). Very important to note that MASP-2 can only interact with C4 if it is in a complex with MBL and after MBL has been bound to a

carbohydrate ligand, emphasizing the significance of proper MBL structure for effective MBL function [112, 113, 127, 131], (Fig 6).

3.10 Biological role of MBL in HIV infection

MBL is an acute phase protein and a pattern recognition molecule that has been shown to recognize the high mannose glycans present on gp120 molecules of the HIV viruses [89]. MBL has been reported to bind directly to recombinant gp120 molecules, the binding is saturable, mannan inhibitable and can be removed by N-glycanase treatment [89]. In vitro studies have shown that infection by HIV is inhibited by MBL which binds to the gp120 molecules on the surface of target cells thus blocking HIV viral entry to susceptible cells [89]. HV has also been reported to resist complement-mediated attack [32].

3.11 The biological mechanism of MBL binding to HIV

MBL is a pattern recognition biological molecule, it recognises sugar molecules found naturally on the surfaces of bacteria, viruses and fungi [80, 132]. The ligands for MBL which include high mannose, N-acetylglucosamine oligosaccharides, N-acetylmannoseamine, N-acetylgalactoseamine, maltose, glucose and galactose are present on a variety of micro-organisms including HIV [80, 89, 92, 117, 123, 124]. MBL has been shown to recognize and bind the high mannose glycans present on gp120 molecules of the HIV viruses [89], thus activating the complement system as illustrated in (Fig 5, Fig 6), resulting in neutralization and killing of the viruses [34, 130, 133].

3.12 Role of ART on MBL deficiency and HIV infection

Few studies have reported role of MBL deficiency on HIV infection in populations that were on ART [37, 41, 48, 134, 135]. Availability and access to ART in developing countries including in Zimbabwe has greatly improved over the last decade and this has resulted in reduction of HIV prevalence among adults and also considerably reduced HIV mother to child transmission [32, 136]. Heggelund et al reported MBL serum levels significantly increased during HAART therapy and this increase was significantly associated with good response virologically [37].

HIV can be considered as a moving goal post as lots of progress has been made in HIV prevention and treatment, with improved access to ART and declining HIV prevalence [32, 136]. Even though HIV prevalence and MTCT have greatly reduced due to ART it still remains important to carry out immunological investigation of role of host factors because favourable treatment response is not achieved in all patients, drug resistant strains and serious side effects to ART are still being reported [37].

There still remains great need to understand the immune-pathogenesis of HIV infection and role of host genetic factors like MBL deficiency, to help understand the varying responses of patients on ART [37].

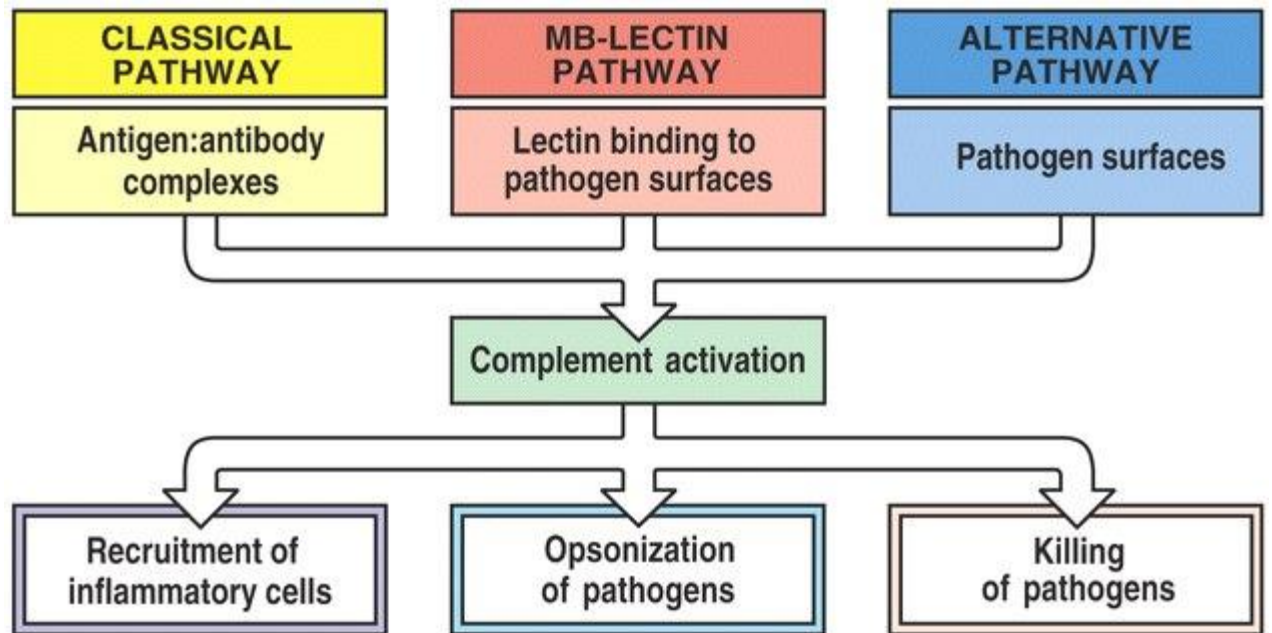


Figure 2-18 Immunobiology, 6/e. (© Garland Science 2005)

Figure 3.4. Role of MBL protein in the complement system. Complement activation can occur through the classical, the alternative or the lectin pathway.

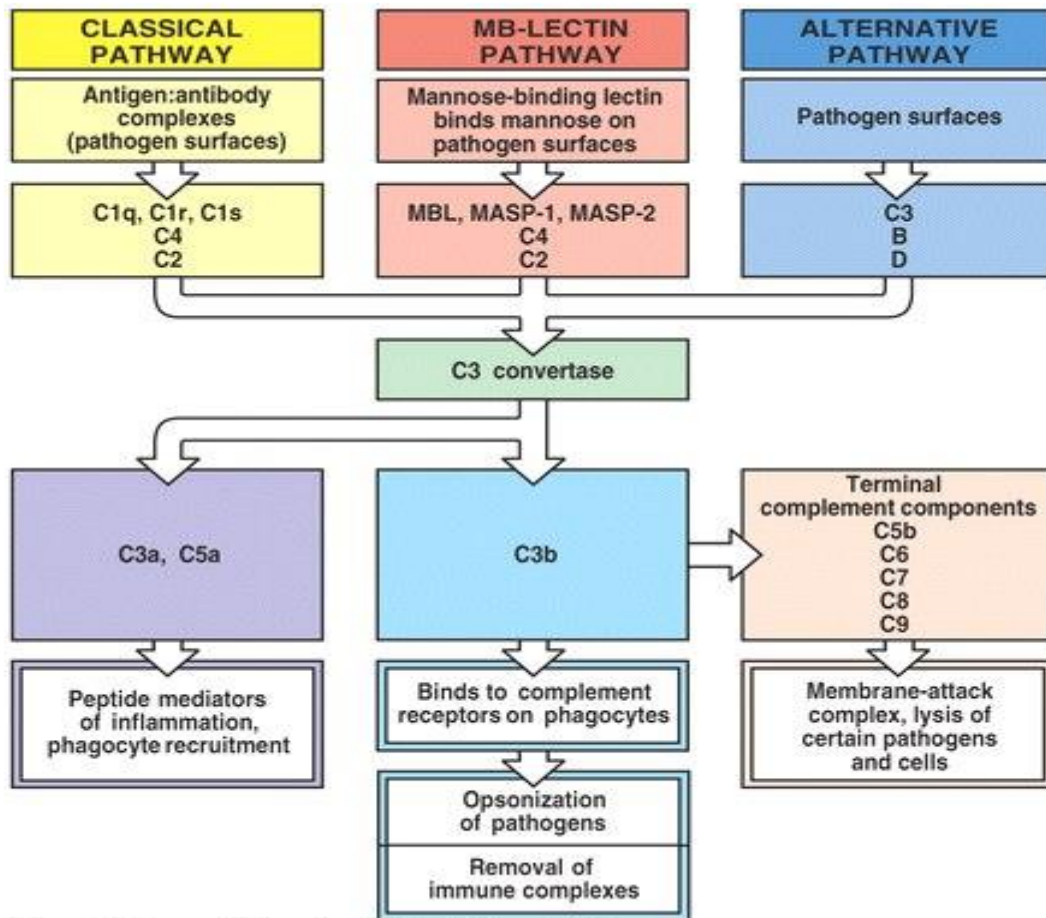


Figure 2-19 Immunobiology, 6/e. (© Garland Science 2005)

Figure 3.5. Role of MBL protein in the complement system. The three complement pathways are involved in pathogen opsonisation, chemotaxis, activation of phagocytes and direct pathogen lysis through the formation of membrane attack complexes.

3.13 Definition of MBL deficiency

There is currently no standard definition of MBL deficiency as several studies have used different cut-offs to define plasma/serum MBL deficiency[105]. Some define MBL deficiency as plasma/serum MBL severe deficiency below 50µg/L[137-140] some below 100µg/L [11] , some below 500µg/L [12, 141, 142] and some below 1000µg/L [115, 143, 144]. For our study we used and defined plasma MBL deficiency to be below 100µg/L [50, 116, 140, 143-145] and this cut off has been used in an MBL Phase 1 clinical trial[116].

3.14 Prevalence of MBL deficiency worldwide

MBL levels in plasma or serum show considerable variation between individuals. Deficiency in MBL exist in about a third of the population in Caucasians and MBL deficiency is particularly common in African populations in which it can exceed 50% [7]. This makes MBL the most common immunodeficiency described to date [34], (Fig 4).

The impact of MBL deficiency appears to be most marked in immune-compromised individuals with co-existing immunodeficiency [144, 146,147]. A high incidence of infectious disease is seen in immune-compromised persons and immunologically immature neonates and infants who are also MBL-deficient. Patients may be immune-compromised due to: immunologic immaturity, acute critical illness, chronic disease, chemotherapy, transplantation, major surgery, and other immune-deficiencies.

3.15 MBL studies on African and Southern Africa populations

According to available literature several studies on role of MBL deficiency in infections have been carried out on African populations, some of these have investigated role in HIV infections. Of these studies, only a few were carried out in Southern Africa [39-41] and only one study has so far been reported from a Zimbabwean population [39].

3.16 Clinical trials on using recombinant MBL as therapy

Two clinical trials have been carried so far to determine the safety of recombinant MBL in human beings, before its recommendation for general use to treat MBL deficiency[116]. A clinical trial reported the effectiveness of MBL replacement therapy and it is recommended for children and adults suffering from recurrent infections and poorly responding to available medications [116].

3.18 MBL deficiency and susceptibility to infections in children

Association of MBL deficiency and infections has been reported in several studies in children[147-151], diarrhoea and failure to thrive [20, 97, 152-154]children with malignancy receiving chemotherapy due to prolonged episodes of febrile neutropenia and bacteremia [144, 155].

3.19 MBL deficiency and susceptibility to infections in adults

Association of MBL deficiency has been investigated in several studies in adults. Low MBL levels and variant *MBL2* genotype associated with increases sepsis, septic shock and fatal outcome in patients with systemic inflammatory response syndrome

(SIRS) [156, 157], fatal outcome patients in intensive care units [158], severe infections after chemotherapy [142], urinary tract infections after pancreas and kidney transplantation [159], increased infections after liver transplant [160, 161], respiratory tract infections [139], pneumonia [162], hepatitis B virus infections [101, 163, 164]

3.20 Mannose Binding Lectin and HIV infection: Biological mechanisms

MBL has been reported to play a role in prevention of infection of target immune cells by the HIV virus [35]. The entry of HIV into target cells is mediated by the glycoproteins gp120 and gp41 which are naturally present on the HIV viral envelope of all HIV strains and are heavily glycosylated consisting of N-linked carbohydrates [35, 130, 133, 165-167]. A number of studies have clearly indicated that MBL binds to the HIV virions [35]. MBL activates the complement system by binding to carbohydrate molecules present on the HIV-1 virion surface thus contributing to HIV-1 clearance [28, 165, 166, 168, 169]. MBL binds and opsonizes the HIV-1 virus hence affecting HIV-1 virus trafficking and antigen presentation during HIV infection [29] leading to destruction or clearance of HIV by complement activation and opsonophagocytosis [29, 35, 89, 130, 167, 170]. Although MBL has been shown not to be able to neutralize the HIV virus however the binding and opsonisation of HIV by MBL may help to alter virus trafficking and viral antigen presentation during HIV infection [29]. MBL has been reported to influence uptake of HIV virions by dendritic cells, which express a cell surface lectin called DC-Specific intracellular adhesion molecule (DC-SIGN) [29]. MBL binds to HIV inhibiting the binding of DC-SIGN and HIV and hence prevents uptake of HIV virions by dendritic cells, thus preventing DC-SIGN mediated transinfection of T cells [29].

MBL has been shown to play a defensive role in HIV infection as MBL and HIV both compete for binding to the CCR5 receptor, the major receptor for HIV-1, thus blocking MBL attachment and entry into target cells [27]. The HIV virions bind to cellular receptor protein CD4 and also to co-receptor CXCR4 and or /CCR5 [133].

In vitro studies suggest a link between MBL levels and HIV pathogenesis, purified MBL has been shown to bind to HIV infected cell lines and can inhibit infection of lymphoblast by HIV, MBL can bind to and activate gp120 complement and MBL binds to both CCR5 and CXCR4-tropic HIV primary strains.

Individuals with MBL deficiency have dysfunctional MBL protein and are more at risk of acquiring HIV infection and HIV disease progression, amongst infected populations [10, 21].

3.21 Mannose Binding Lectin and HIV infection in adults

Several studies have been done on MBL because of its role in innate immunity and the possible association of MBL deficiency with HIV infection in adults [10, 21, 37, 38, 42-44, 46, 47, 49, 50, 59, 134, 171-179], HIV disease progression and has been reviewed in several papers [34, 35, 105, 179]. There is conflicting information on role of MBL deficiency on HIV infection and disease progression among infected adults. Some studies have evaluated association between MBL concentration and HIV infection and found low MBL levels increasing susceptibility to HIV infection [21], some no association [49, 58, 174]. Other studies have determined effect of *MBL2* genetic polymorphism and found an association between MBL genetic mutants and

HIV infection [21, 50, 59, 176], some reported no association [173, 180]. Other studies have looked at both MBL levels and *MBL2* genotypes and reported an association between MBL deficiency and HIV infection [21, 50, 59, 176]. Other studies have looked at *MBL2* promoter region variants and found an association [21, 50, 59, 176], others reported no association [173, 180]. Results from studies in adults on the role of MBL in HIV infection are still conflicting, there are reports of a detrimental effect of the promoter region variants XA [22] and increased susceptibility to HIV transmission [21]. While other studies have reported association of MBL deficiency with increased risk of HIV infection [21], others have reported no association between *MBL2* gene polymorphism and also promoter region polymorphism and HIV infection [36]. Similarly, while some studies have reported increased rate of HIV disease progression to AIDS, others have shown either no effect or delayed rate of disease progression [37]. Most of these studies have been done in European populations, only two studies have been done on African adults, in Tanzania [10] and Gabon [38], which showed increased risk of HIV infection among those with MBL deficiency, but no such study has been reported for any Southern African countries. For this study we evaluated the effect of MBL plasma deficiency, *MBL2* genetic variants and promoter region variants on HIV infection in these adults.

3.22 Role of MBL deficiency in HIV and schistosomiasis co-infections

The adults enrolled in the MUSH cohort comprised of some individuals co-infected with HIV and schistosomiasis, some with schistosomiasis only and controls. We also investigated role of MBL during *S. haematobium* and *S. mansoni* infections. Both HIV-1 and schistosomiasis co-infections are very common in Africa and have been

reported in several studies [181-186]. Schistosoma infection is one of the neglected tropical diseases, which the World Health Organization is targeting for elimination [187-189]. An estimated 85% of the world's estimated 200 million people with schistosomiasis live in Sub-Saharan Africa [190]. *S. haematobium* is associated with urogenital schistosomiasis characterised by severe pathological conditions including hematuria and bladder cancer and *S. mansoni* causes intestinal schistosomiasis characterised by chronic or intermittent abdominal pain bleeding from gastro-oesophageal varices and bloody stool [191]. Schistosomes are complex multi-cellular helminths with several developmental stages well documented in the human host [191].

Schistosomes are reported to carry sugar molecules or glycoconjugates on the surface of all their developmental stages [192, 193] and these glycoconjugates interact with innate immune recognition molecules including MBL [194-196]. In vitro studies have demonstrated complement mediated killing of all stages of the schistosome parasite [195]. Only one study has reported the role of MBL deficiency in schistosomiasis [190] where high MBL levels were associated with protection, but no study could be found in literature that investigated role of MBL deficiency in HIV and schistosomiasis infections. We therefore investigated the role of plasma MBL deficiency and MBL2 polymorphism in HIV-1, *S. mansoni* and *S. haematobium* infections in Zimbabwean adults who were enrolled in the MUSH cohort.

Schistosomiasis immunological pathway and its effect on HIV

Several studies have reported on investigations on schistosoma and HIV co-infections [181, 182, 197-208]. *Schistosoma haematobium* has been reported to cause

urogenital schistosomiasis causing lesions due to granuloma formations which is a risk factor for HIV acquisition [199-206, 209-211].

3.23 Mannose Binding Lectin and HIV infection in children

So many studies have been done investigating MBL and its association with HIV infection in children [22, 33, 39, 40, 45, 212-218], reviewed in [33-35], looking at different aspects and most of these except one [33, 40] has been done in other populations outside Africa. Some studies investigated association between MBL deficiency and HIV transmission from mother to child [40, 213,215]. Unlike studies that are linking MBL deficiency and HIV infection and disease progression in adults, there is concordance in all the studies among children [33]. Studies on MBL deficiency and HIV transmission showed that MBL deficient genotypes were significantly increased in HIV infected children compared to either HIV exposed but uninfected children or HIV unexposed controls [33]. MBL studies have been done on African children [39, 41] and only two reported on Zimbabwean children [3, 39]. For this study we evaluated the effect of MBL plasma deficiency, MBL genetic variants and promoter region variants on HIV infection in a PMTCT survey of mothers and their babies.

3.24 Diversity of the HLA gene

The human leukocyte antigen (HLA) also known as the major histocompatibility complex is found on chromosome 6 [219, 220] . HLA consists of highly polymorphic genes and the protein products play a role in the immune response by presenting foreign antigens to T cells [219, 220]. HLA diversity has been reported to play a significant role in human disease associations [219, 220]. There is an advantage of

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HLA diversity related to the presentation of peptides derived from pathogens to effector T cells [219, 220]. Individuals who are heterozygous for HLA alleles can present more antigens than homozygous individuals, referred to as the HLA heterozygosity advantage [219-222]. Populations that are exposed to a high pathogen burden have been reported to show high HLA diversity, pathogen driven selection [223]. Mechanisms that have resulted in HLA diversity and polymorphism include positive selection, splicing of HLA genes giving rise to diverse isoforms [224]. Other mechanisms of generating HLA diversity include point mutations (substitution, deletion, insertion), gene conversion and gene crossover [225].

3.25 Diversity of the HLA gene in the Zimbabwean population and role in HIV infection

The Diversity of the HLA gene in the Zimbabwean population has been reported in a few studies [226-229] and the HLA gene was reported to be highly polymorphic which is an immunological advantage to the population exposed to high burden of pathogens [226-228]. The HLA loci polymorphic profiles reported in the Zimbabwean populations includes HLA-A, HLA-B, HLA-C [228, 230], DQA1, DQB1, DRB1 and DPB1 [228], HLA-DQA1, LDLR, GYPA, HBBG, D7S8, Gc and D1S80 [227], HLA-E and H:A-G [231], reviewed in [226, 232]. HLA proteins are reported to be important determinants of susceptibility and resistance to HIV infection and disease progression. The highly polymorphic HLA gene has been reported to be strongly predictive of HIV infection outcome in adults [233], with some HLA alleles leading to increased susceptibility and some being protective [234, 235], some HIV infected adults with protective HLA alleles showed capacity to control viral load [234, 235].

The high genetic polymorphism in the HLA loci is the basis of the host's ability to mount an immune response to the different epitopes of HIV.

The study populations for our research comprised of Zimbabwean adult males and females and children who most likely also have high HLA polymorphism which could also play a role in HIV susceptibility.

3.26 Problem Statement

While there have been many studies on MBL and HIV among adults, the results are conflicting. Some studies report increased susceptibility to HIV infection among those with variant *MBL2* genes, others report no association. Unlike MBL studies in the general adult population, results of MBL deficiency and HIV infection among children are concordant and have found increased susceptibility to HIV transmission amongst children with variant *MBL2* genes born to HIV positive mothers, but none of these studies have determined the *MBL2* genotype of the HIV positive mothers.

3.27 Rationale

While there is a lot of advancement in HIV prevention and treatment, there is still need to investigate host genetic molecules in adults and mother-baby pairs that could be playing a role in HIV-1 transmission/acquisition, disease progression and survival.

It was imperative to carry out this study because:

- We needed to quantify the burden of MBL deficiency in this African population comprised of adults and children.
- To contribute to the knowledge gap on the role of MBL deficiency in HIV-1 transmission, disease progression and survival in African populations in adults

and children. The available literature shows that the majority of studies on the association of MBL deficiency and HIV-1 infection in children have been done on populations outside the African continent, in Argentina, USA, Brazil, UK, Italy [33]. Only three studies on children have been done in Sub-Saharan Africa [39-41], this region is still experiencing high prevalence of HIV-1 among adults and vertical transmission from mothers to their babies.

This is the second study after the *Zambian study* [41], to assess *MBL2* genes and promoter typing in mother-infant pairs in HIV vertical transmission/acquisition.

There is dearth of information on the role of MBL in this era when access to ART has greatly improved even in developing countries like Zimbabwe.

3.28 Research Questions

Based on the above, below are the questions that we wanted to answer in this study:

1. What is the prevalence of MBL deficiency among the adult Zimbabwean population?
2. Is MBL deficiency a risk factor for HIV infection among Zimbabwean adults?
3. Does MBL deficiency affect HIV disease progression and survival among the HIV positive adults?
4. What is the prevalence of MBL deficiency among mothers and their infants?
5. Is MBL deficiency a risk factor for HIV transmission from mother to child?

3.29 Broad Objective

The broad objective of this study was to determine the relationship between MBL deficiency and HIV infection in an adult cohort of males and females and among mother-infant pairs in Zimbabwe.

3.30 Study Specific Objectives

1. To determine the prevalence of MBL deficiency among the Zimbabwean adult population.
2. To determine the relationship of MBL deficiency with HIV infection among the Zimbabwean adult population.
3. To determine the effect of MBL deficiency on disease progression and survival among the Zimbabwean adult population over a period of 3 years in the study.
4. To determine prevalence of MBL deficiency among mothers and their infants in a Zimbabwean population.
5. To determine the relationship between MBL deficiency and HIV transmission from mother to child in a Zimbabwean population.

3.31 Hypothesis

Hypothesis 1 : MBL plasma deficiency is a risk of HIV infection, increases disease progression and lowers survival rate in adults.

Hypothesis 11: MBL plasma deficiency is a risk of HIV vertical transmission /acquisition in the children born to HIV positive mothers.

3.32 Scope of this thesis

Genetic analysis of MBL among the adult population will use the available results from the Mupfure Schistosomiasis and HIV (MUSH) study carried out in Zimbabwe between 2001-2007, while questions regarding the role of MBL deficiency in HIV acquisition and transmission from mother to child will use stored dried blood from a PMTCT national survey also carried out in Zimbabwe in 2012. The blood samples from the PMTCT cross sectional survey provides us with an opportunity to investigate if MBL deficiency is a factor in transmission or acquisition of HIV among infants in Zimbabwe.

3.33 Thesis themes

This research and thesis is based on the following broad themes which were based on the five research questions and five research objectives outlined earlier:

- 1. Determination of plasma MBL levels, prevalence of MBL deficiency, prevalence of normal and variant *MBL2* genotypes and promoter region types in Zimbabwean adult males and females.**
- 2. Determination of the role of Mannose Binding Lectin Deficiency in HIV-1 Infection in a Rural Adult Population in Zimbabwe.**
- 3. Determination of the role of Mannose Binding Lectin Deficiency in HIV-1 disease progression and survival in a rural adult population in Zimbabwe.**
- 4. Determination of prevalence of normal and variant *MBL2* genotypes and promoter region in Zimbabwean mothers and their babies.**
- 5. Determination of the role of Mannose Binding Lectin Deficiency in HIV-1 infection and vertical transmission in a PMTCT national survey in Zimbabwe.**

CHAPTER 4 MATERIALS AND METHODS

4.0 Introduction

This chapter describes the methods and materials used for this research project. The methods used in sample collection, laboratory analysis for the adult MUSH study and gives details about the source of samples for the PMTCT MBL study.

4.1 Study description

This research protocol was composed of data from two studies, Mupfure Schistosomiasis and HIV cohort (MUSH) which enrolled adult males and females and a PMTCT cross sectional study comprised of mothers and their infants, both conducted in Zimbabwe (Fig 7). The MUSH cohort was established and blood samples collected and analyzed between 2001 to 2006. Using this cohort we assessed MBL plasma levels, *MBL2* genotypes, *MBL2* promoter types and HIV-1 infection and all MBL assays were carried out at Righospitalet, Copenhagen Denmark. The PMTCT cross sectional study consisted of data on mother-baby pairs from a national PMTCT evaluation survey carried out in Zimbabwe by the CESHAAAR Research Centre in 2012 during which whole blood was collected and dried on filter papers for HIV-1 testing of mother-infant pairs. The dried blood spots from the PMTCT study were used for *MBL2* genotyping at Serum State Institute in Copenhagen, Denmark.

4.2 Study design

This research consisted of two study designs, a longitudinal cohort and a cross sectional design. The cohort design (MUSH sub-study) aimed at assessing the relationship between MBL plasma concentration, *MBL2* genotypes, HIV infection,

HIV disease progression and survival in adults. The cross sectional PMTCT study was laboratory based and used archived whole blood filter samples for *MBL2* genotyping in mothers and their infants from the PMTCT national survey. Samples for the PMTCT study were collected during a one month long evaluation of the PMTCT programme in Zimbabwe carried out in 2012 (Fig. 4.1).

Adult cohort : Mupfure, Mt Darwin
PMTCT survey : National PMTCT Survey



Figure 4.1: Map of Zimbabwe. Showing study area for the adult cohort, Mupfure, Mt Darwin 150km to the North of the capital City Harare. Samples for the PMTCT study were collected from all the 10 provinces of Zimbabwe.



Figure 4.2:Community Sensitization in Mupfure, Mt Darwin

4.3 Study site and Population

Adult cohort : The field work and sample collection was conducted starting from October 2001 to June 2006 in Mupfure and its adjacent areas in Shamva district, Mashonaland Central Province, Shamva District in the north-eastern parts of Zimbabwe[62, 181, 182, 198], (Fig 4.1). The study-population comprised of adults 18 years old and above, residing in the area, who gave their informed consent to be bled and tested for HIV. Blood samples were collected after community sensitization (Fig.4.2), written informed consent and explaining the aims and objectives of the research project. Blood samples were collected at 7 time points over a 3 year period and study participants were not on ART during this period as ART was not yet available in the public health care centres across Zimbabwe.

4.4 PMTCT cross sectional study

Stored samples from mothers and baby pairs were obtained from the CESHAAAR Research Centre in Zimbabwe that had stored whole blood samples for mother –infant pairs from a national PMTCT survey carried out in 2012. All the mothers were HIV positive, 72.3% (450 out of 622 mothers) were on ART, Nevirapine, AZT, Nevirapine and AZT. Of the 622 perinatally exposed children 71.5% (445 out of 622) were on ART during the time of the survey using such drugs as Nevirapine only, Nevirapine and AZT syrup and AZT only.

4.5 Exposures measured

MBL plasma concentration in the adult cohort. The normal MBL concentration in plasma/serum is 1000µg/L and above and MBL deficiency is defined as below 100µg/L [6, 7]. *MBL2* genotype and promoter type in the adult cohort and the PMTCT samples. We were not able to measure MBL plasma concentration for the PMTCT samples as plasma or serum samples were not available, but we used *MBL2* genotype as a proxy for MBL deficiency as the relationship between *MBL2* genotypes and MBL plasma concentration is well documented [6, 7].

4.6 Outcomes measured

The following outcomes were used to define HIV-1 infection in the adult MUSH cohort and HIV transmission/acquisition in the PMTCT study. Schistosomiasis was assessed by determination of presence or absence of eggs in stools and urine and recorded as infected or uninfected. HIV status by standard HIV testing kits. Those defined as HIV positive were the ones with a reactive blood test on standard HIV testing kits. Viral load, measured as HIV RNA, change defined as the change

(increase or decrease) in viral load from the baseline value, measurements were taken at baseline, 6 weeks, 3, 6, 12, 24 and 36 months. CD4 T cell count change defined as the change (increase or decrease) in CD4⁺ T cell count from the baseline value, measurements were taken at baseline, 6 weeks, 3, 6, 12, 24 and 36 months. Survival: dead or alive at end of study. The information on death of each participant was collected from hospital records or first-hand information from family members.

4.7 Potential Confounders

Age, gender, marital status in the MUSH cohort.

Use of ART by the mothers and children in the PMTCT study.

4.8 Sample size calculations

4.8.1 Sample size calculation for the main MUSH study

The sample size for the main MUSH study was based on HIV status as the primary outcome. The Zimbabwe national prevalence of HIV was 25% in 2001 when the MUSH study was proposed and implemented. This prevalence was used to calculate the minimum sample size of adult males and females for inclusion. To calculate the sample size within 5% significance level, the following prevalence formula was used [31]:

$$N = \frac{Z^2 p(1-p)}{d^2}$$

Where:

Z = Z statistic for a level of confidence (95% level of confidence used, therefore Z value is 1.96)

P = expected prevalence of proportion

d = precision

$$N = 1.96 \times 0.25 (1-0.25)$$

$$0.05^2$$

$$N = 288$$

20% more participants were enrolled to compensate for loss to follow up

$$288 + 20\% \text{ of } 288 = 288 + 58 = 346$$

The required minimum sample size for the MUSH study was 346 adult males and females. For the MUSH study, 2281 adult males and females were screened for HIV and schistosomiasis and 379 were recruited into the follow-up cohort. At the end of the enrolment period 379 participants had been enrolled. These had plasma and PBMCs collected and stored for use in the MBL study.

4.8.2 Sample size calculation for the original PMTCT national survey

The target study population for the national survey was biological mothers and their infants 9 to 18 months old. The sample size was based on the need to compare the proportion of living 9 to 18 months old infants born to HIV infected mothers who were HIV infected before versus after implementation of the national PMTCT programme. The focus of the PMTCT survey was primarily on detecting an impact of the national PMTCT programme on HIV prevalence among the 9 to 18 months old infants. Catchment areas for PMTCT clinics were determined in all the provinces in Zimbabwe before randomly selecting catchment areas for inclusion. The study team expected a reduction in HIV prevalence from 25% to 18.75 %, assuming 95% significance level and 80% power, the calculated required minimum sample size was 7800 mothers and their children. For the national PMTCT survey, 8800 dried blood spots were collected from biological mother baby pairs for HIV testing, 880 mothers (10%) were HIV positive (Fig 1), paper III [236, 237]. These are the dried blood spots that were available and in storage at the CESSHAAR Research offices [3].

4.8.3 Sample size calculation for the MBL PMTCT sub-study

The sample size calculation for the MBL sub-study was based on the exposure of interest (MBL deficiency using *MBL2* gene as a proxy) among HIV infected mothers, and the primary outcome was infant HIV status. Based on literature, assuming mother – infant pair are on ART, transmission rates range from 20 to 5% [238]. This study used repository specimens collected as part of the National PMTCT evaluation survey carried out by the CESHAAAR Research Centre in Zimbabwe and the preliminary results showed 10% infant HIV status at 9-12 months of age (personal communication with Dr Cowan). Sample sizes under a range of different scenarios are as summarized (Table 4.1). Assuming an alpha of 0.05 and beta of .90 and 5% significance level and a risk ratio of 2.0 with 10% infant HIV status among the infants born from mothers with normal *MBL2* genotype, we had a sample size, among the exposed population, of 572 mother infant pairs, giving a total 1144 blood filters out of a total of 8800 available filters in storage at CESHAAAR study site in Harare. In order to allow for dried blood spots that would not give adequate DNA for PCR reaction, ten (10%) more samples were selected, to give a final sample size of 632 mother baby pairs calculated using Epi Info 7 programme [31]. DNA was extracted from all 632 mother-baby pairs selected for inclusion into the PMTCT MBL study (Fig. 1). A complete dataset on the HIV status and *MBL2* genotype was available for 622 mother-baby pairs which were used in the final analysis [3].

Table 4.1: Showing different sample size calculations for the PMTCT study carried out using Epi Info Version 7 programme

% Transmission (unexposed)	Risk ratio	Sample Size	
		80% Power	90% power
5	1.5	3100	4096
	2	948	1242
	2.5	496	646
	3	320	414
10	1.5	1452	1914
	2	438	572
	2.5	226	292
	3	144	184
20	1.5	626	824
	2	182	238
	2.5	90	116
	3	56	70

4.8.4 Sampling plan of the PMTCT cross sectional study

MBL2 genotyping on the PMTCT cross sectional study used archived dried blood samples. Simple random probability sampling stratified according to HIV status of the children, was used to select the children whose mothers were HIV positive when these children were born. The list of mothers and their babies was available at the

CESHAAR Research Centre in Harare. Random number tables were used to select the required number of mother-baby pairs from the stratified list. Only those meeting the inclusion criteria will be selected.

4.9 Selection of study sample

4.9.1 Inclusion criteria Adult study

Adult males and females, 18 to 60 years old, were the target population for this study. Only those who had given informed written consent, were willing to submit the necessary blood samples and willing to be bled and tested for HIV, were enrolled into the cohort. The uninfected were included as controls.

4.9.2 Exclusion criteria, Adult study

Those over 60 years old, symptomatic TB and HIV, severe anaemia, bedridden and too sick to come to the clinic were excluded. All women were tested for pregnancy before inclusion, pregnant women were excluded. Other bacterial co-infections were not checked for in this study.

4.9.3 Inclusion criteria PMTCT cross sectional study

Blood samples from HIV positive mothers and their children (HIV positive or negative) below the age of two years were used in this study.

4.9.4 Exclusion criteria, PMTCT cross sectional study

Blood samples from HIV negative mothers and their children.

4.10 Study procedures

4.10.1 Procedures at enrolment

For the adult cohort, adult males and females were enrolled for MBL analysis during a programme evaluating effect of treating schistosomiasis infection on HIV disease progression.

4.10.2 Source of samples for PMTCT study

Stored whole blood samples on filter paper for mother baby pairs were obtained from the CESHAAAR Research Centre in Harare. These samples were collected in 2012 as part of a national PMTCT evaluation programme. For quality control the selected samples used were be checked that they were kept at room temperature, desiccant was in place in the zip lock storage bag and that the blood spots did not have moulds growing on them the due to humid conditions. Plasma samples nor serum samples were not collected on the PMTCT survey, so we did not have samples for determination of MBL concentration in the mothers and their children. Nevertheless, this would not compromise the findings of our research because the association between *MBL2* genotype and MBL deficiency is well documented [6, 7].

4.11 Laboratory methods

4.11.1 Adult study

4.11.1.1 Schistosoma parasitology

Microscopic examination of fixed-volume urine samples filtered on Nytrek filters (VesterGaard Frandsen) was used to identify and quantify eggs of *S. haematobium* by

the syringe urine filtration technique [239], Fig. 4.3. To accommodate the diurnal and day-to-day variation in egg output, the urine samples were collected on 3 consecutive days [240, 241]. We used the modified formol-ether concentration technique was used on 1 stool sample from each participant to detect eggs of *S. mansoni* and other helminth or parasites [242]. Levels of the Circulating Anodic Antigen (CAA) were measured in serum samples using an ELISA assay, to complement the parasitological detection methods mentioned above. The CAA originates from the parasite gut and is used as a bio marker of active schistosome infection [243].



Figure 4.3: Schistosoma parasitology. Processing urine and stool samples at Mupfure Clinic, Mt Darwin, Zimbabwe.

4.11.1.2 HIV-1 serology

HIV testing was performed in respect of the confidentiality principle and pre- and post-test counselling was provided by qualified medical personnel in the participants' native language, Shona. Initially a rapid HIV-1/2 test kit was used on a dry blood spot collected from the participants in the field (Determine, Abbott Laboratories, Tokyo, Japan). This test was then followed by two different rapid HIV tests (namely Oraquick by Orasure and Serodia by Fujirebio) for all who tested initially positive. All those included in the MUSH study had their HIV positive test confirmed by two ELISA tests (Recombigen by Cambridge Biotech and Ortho (Ortho Clinical Diagnostics) on serum. We found no discrepancies in results were found between the initial Determine HIV test and the two subsequent ELISAs. Strict confidentiality was assured to the research participants and was maintained throughout the study. All research participants who requested their HIV results were given after receiving post test counselling from the study nursing staff [1].

4.11.1.3 Quantification of CD4⁺ T cells and viral load

CD4⁺ T cell counts were measured at the Department of Haematology of Parirenyatwa Hospital Harare, Zimbabwe (FacsCalibur, Becton Dickinson) [182, 244,245]. HIV RNA was measured using the Roche Amplicor (F. Hoffmann la-Roche). None of the enrolled participants were receiving antiretrovirals at the time of sample collection as these were not yet available in the public health sector [1, 2].

4.12.1.4 Determination of MBL plasma concentrations

Plasma was stored at -20°C until analysis. MBL concentrations were measured in baseline plasma samples using the double enzyme immuno-assay (EIA) [16]. The lower detection limit in the assay was 20µg/L [16, 246,247]. The micro titre plates (Maxisorp, NUNC, Denmark) were coated with a specific mouse anti-human MBL monoclonal antibody, clone 131-1, IgG1, Kappa (Antibody Shop, Denmark). Plasma samples were diluted in Tris/HCL buffer (Bie and Bernsten) containing EDTA (Bie and Bernsten) and 0,05% Tween 20 (Sigma). A pool of EDTA plasma with a known concentration of MBL was used as the standard. Biotinylated clone 131-1 anti-MBL (Antibody Shop, Denmark) was used as the detection antibody. Horseradish peroxidase labelled streptavidin (Amersham, UK) was added. The substrate uses O-phenylene diamine (OPD) (DakoCytomation) hydrochloride in citrate-phosphate (Bie and Bernsten) buffer pH 5.0. The colour reaction was stopped by the addition of sulphuric acid (Sigma). The optical density (OD) was read on an ELISA reader (MR5000/7000, Dynatech Laboratories, Denmark) at 490 nm with reference filter 630nm and the final output printed (Proprinter 11, IBM Netherlands). The final MBL values are given as µg MBL per µl. In addition, parallel control plates were coated with equivalent amounts of mouse IgG1 and processed as above. The parallel plates are done in order to reveal binding of rheumatoid factors, anti mouse immunoglobulins and non-specific binding of the MBL interfering in the system. ODs were read at 490 nm and reference filter 630 nm [1, 2].

4.11.1.6 Processing and freezing of PBMCs

Twenty millilitres of blood were collected from each participant in sodium heparized tubes to prevent coagulation (Fig.4.4). The white blood cells were separated from the

red cells in 50ml centrifuge tubes with lymphoprep liquid (Greiner Bio-one) with a separation disk. The buffy coat layer was collected into 15ml tubes and the red cells discarded. The cells were further centrifuged in 40ml wash media RPMI (Sigma), 5% AB serum (Blood Transfusion Service, Zimbabwe), 3ml re-suspension media (RPMI + 10% AB serum + 1% penicillin/ streptomycin) the cell suspension was aliquoted into 4 sterile cryotubes, about 750 μ l into each tube and 1ml freeze media (RPMI +25% AB serum + 20% DMSO + 1penicillin/streptomycin) added. Ten micro litres from each cell suspension was used to determine the cell count, viable and nonviable ratios using a haemocytometer. Cells were frozen immediately by gradient freezing method in liquid nitrogen[1, 2].



Figure 4.4. Processing PBMCs at the site laboratory in Mupfure, Mt Darwin.

4.11.1.7 DNA extraction

The salting out procedure [248] was used to isolate DNA from frozen cells of each cohort participant (Fig. 4.5). Frozen cells, in RPMI freezing media, kept in liquid nitrogen until analysis, were first thawed at room temperature. Cells were transferred

to 2ml centrifuge tubes which were filled up with buffer A (1M sucrose, 1M Tris-HCL, 1M MgCl, Triton-X-100 and distilled water) and spun in a centrifuge (Stuers, Sigma) at 3000rpm for 2 minutes. The supernatant was poured off, tubes were filled up with buffer A again, mixed by vortexing to dislodge the pellet, tubes spun in a micro centrifuge at 3000rpm for 2 minutes and the supernatant discarded. Buffer B (3M NaCl, 0,5M EDTA) and 25µl SDS (Bie and Bernstern, Denmark), 10-20µl proteinase K enzyme (Macherey Nagel, Denmark) (depending on the size of the pellet) were added, mixed by vortexing and the tubes incubated at 42°C for 1 hour in a rotation incubator (Hereus Instruments, Holm and Halby, Germany). An additional 10µl proteinase K (Roche, Germany) was added to tubes with visible pellets, vortexed and incubated again. To clean the DNA, 6M NaCl (Fluka chemicals, Denmark) was added. Tubes were topped up with 2-iso-propanol (J.T Baker) and the DNA strands viewed under a work-bench light source. All samples in which DNA was successfully extracted had strands appear when iso-propanol was added[1, 2].

Then 70% ethanol (De Danske Spirifabriker, Denmark) was added to the pellet and the tubes spun down for a further 5 minutes. The DNA pellets were air dried in the tubes for about 15 minutes, 50µl sterile water (H/S Apoteket, Rigshospital, Copenhagen) added to each tube to dissolve the DNA, tubes incubated at 42°C for half an hour. Pellets were checked for complete dissolution in the sterile water[1, 2].



Figure 4.5: DNA extraction at Rigshospitalet in Denmark, Dr Peter Garred laboratory

4.11.1.8 Determination of DNA concentration

The DNA concentration for each sample was determined using a DNA spectrophotometric analyser (Spectrachrom, Shimadzu, Japan). The ideal DNA concentration for MBL genotyping by PCR is 0.3µg/µl, if DNA concentration was more than 0.3µg/µl it was adjusted by adding sterile water. The ideal DNA protein ratio, A260/A280, was between 1,5 and 2. Any measurement below 1,0 indicated that the DNA was contaminated and it was not clean, additional proteinase K was added to further purify the DNA. Purified DNA was stored at -20°C until analysis by PCR[1, 2].

4.11.1.9 Preparation of MBL PCR Master mix

The PCR master-mix was made by mixing the following in 2ml centrifuge tubes: 400µl sterile water (H/S Apoteket, Rigshospital, Copenhagen), 150µl of 1.5mM MgCl₂ (Invitrogen, Denmark), 150µl at 0.07mM final concentration of each deoxynucleotide triphosphate (dNTPs) (Amersham Biosciences, UK Limited), 500 µl PCR Buffer containing 50mM KCL, 10 mM Tris-HCl (pH 8.3), 0.001% (w/v) gelatin p.H.8.3 (Invitrogen, Denmark), 250µl glycerol at 5% final concentration , 50µl cresol red at final concentration 100µg/µl. Cresol red (Invitrogen, Denmark) was added as a colour marker. The PCR Master mix could be kept at 4°C for 2 weeks[1, 2].

4.11.1.10 Preparation of PCR primers

A total of 12 primer solutions (DNA Technology) were prepared for each sample (Table 2.3). The set of 12 primers for MBL variants and promoter region mutations

were prepared by mixing 5 sense and 3 sense primers with water according to manufacturers instructions. Final concentration of each primer was 0.25 μ M for each specific primer. PCR primers were pre-aliquoted in 5 μ l volumes into the PCR plates and the plates could be kept at 4°C for 1 week[1, 2].

4.11.1.11 Preparation of MBL PCR sample reaction mixture

The PCRs were performed in 10 μ l volumes containing 30ng genomic DNA and 0.25 μ mM of the specific primers PCR reaction mixtures for each sample were prepared in PCR microtubes, marked clearly with sample identification numbers. Fifty four μ l of PCR mastermix, 3,5 μ l sample DNA (if the DNA concentration was 0,3 μ g/ μ l), 31 μ l sterile water, and 0.25 Units of Platinum *Taq* DNA polymerase (Invitrogen, Denmark) in each 90 μ l initial reaction mixture volume. Mixture was mixed with a multi-channel pipette three times and 5 μ l of the reaction mixture added to the 12 wells, clearly marked for each sample, which contained 5 μ l of different sets of primers for the genotyping and promoter typing. Only eight samples could be run on each PCR plate. Plates were put in a plate micro centrifuge at 3000rpm for 1 minute to spin down the well contents. Plates were tightly sealed with foil paper and put in a PCR thermocycler for 1 hour 15 minutes[1, 2].

4.11.1.12 Determination of *MBL2* genotype and promoter typing

The genomic DNA was extracted from the collected and frozen Peripheral Blood Mononuclear Cells (PBMCs) using the standard salting out procedure [248]. DNA was amplified by allele-specific oligonucleotide PCR (ASO-PCR) where specific sequences were used for each allele[7, 138]. The *MBL2* genotypes and promoter

region alleles were detected by sequence –specific primer polymerase chain reaction assay where specific sequences were used for each polymorphism according to manufacturer’s instructions. The following detailed steps were carried out:

4.11.1.13 Amplification of *MBL2* genes by allele-specific oligonucleotide PCR (ASO-PCR)

The *MBL2* single nucleotide polymorphisms (SNPs) in the form of structural variants named B (codon 54), C (codon 57) and D (codon 52), as well as the regulatory promoter region variants H/L (-550), X/Y (-221) and PQ (+4), were genotyped by Polymerase Chain Reaction (PCR) using sequence-specific priming, which includes the 12 reactions listed in Table 3.1. The genetic primers for 5 and 3 sense exon 4 of the *MBL2* gene (DNA Technology, Denmark) were included in each PCR reaction as an internal positive control.

The DNA was amplified by allele-specific oligonucleotide PCR (ASO-PCR) in a programmed thermocycler (Gene Amp PCR Systems 9600, Perkin Elmer). All PCRs were initiated by a 2-min denaturation step at 94°C and completed by a 5-min extension step at 72°C. The temperature cycles for the PCR were as follows: 10 cycles of 10 s at 94°C (denaturation) and 60 s at 65°C (annealing and extension), and a further 20 cycles of 10s at 94°C (denaturation), 50s at 61°C (annealing), 30s at 72°C (extension) and 2 minutes at 25°C. Gel electrophoresis was done on the PCR products on the same day or kept at 4°C for a maximum 1 week [1, 2].

4.11.1.14 Visualisation of PCR products by agarose gel electrophoresis

PCR products were separated by a 2% agarose gel (Invitrogen) electrophoresis run and this was carried out in an electrophoresis chamber (Sub-cell Biorad) at 150V,

400mA for 32 minutes. The agarose gel was made by dissolving 6g of agarose powder (Invitrogen) in 300ml TEA buffer (Trizma base, EDTA). The gels were stained with Ethidium bromide (Invitrogen). Molecular weight marker V (Roche Diagnostics, Manheim Germany) at 0.25µg/µl, was included in each electrophoresis run. The gels were then carefully transferred to the UV Transilluminator (Syngene Synoptics Ltd, Denmark) where a photograph of the gel was taken (Figure 4.6). The MBL gene and promoter region typer (Table 3.2) was used to determine the MBL genotype of each sample. All the different MBL gene and promoter region combinations available to date are as listed (Appendix 6)[1, 2].

4.11.1.15 Detection of variant *MBL2* alleles

The genomic DNA was extracted from frozen Peripheral Blood Mononuclear Cells (PBMCs) using the standard salting out procedure [248]. DNA was amplified by allele-specific oligonucleotide PCR (ASO-PCR) and the amplified alleles were detected using allele-specific oligonucleotide PCR (ASO-PCR) procedures [247].

The *MBL2* single nucleotide polymorphisms (SNPs) in the form of structural variants named B (codon 54), C (codon 57) and D (codon 52), as well as the regulatory promoter region variants H/L (-550), X/Y (-221) and PQ (+4), were genotyped by PCR using sequence-specific priming. The *MBL2* genotypes and promoter region alleles were detected by sequence –specific primer polymerase chain reaction assay where specific sequences were used for each allele. Twelve oligonucleotide primer sequence solutions (DNA Technology) were prepared for each sample (Table 4.1).

The 12 primers for *MBL2* variants and promoter region mutations were prepared by mixing 5 sense and 3 sense primers with water according to manufacturer's

instructions. The final concentration of each primer was 0.25uM for each specific primer. Primers for 5 and 3 sense exon 4 of the *MBL2* gene (DNA Technology, Denmark) were included in each PCR reaction as an internal positive control. PCR primers were pre-aliquoted in 5µl volumes into PCR plates and plates could be kept at 4°C for 1 week [1, 2].

4.11.1.16 Amplification of *MBL2* genes by allele-specific oligonucleotide PCR (ASO-PCR)

DNA was amplified by allele-specific oligonucleotide PCR (ASO-PCR) in a programmed thermocycler (Gene Amp PCR Systems 9600, Perkin Elmer). All PCRs were initiated by a 2-min denaturation step at 94°C and completed by a 5-min extension step at 72°C. The temperature cycles for the PCR were as follows: 10 cycles of 10 s at 94°C and 60 s at 65°C, and a further 20 cycles of 10s at 94°C, 50s at 61°C, 30s at 72°C and 2 minutes at 25°C. Gel electrophoresis was done on the PCR products on the same day or kept at 4°C for a maximum 1 week [1, 2].

4.11.1.17 Visualisation of PCR products by agarose gel electrophoresis

PCR products were separated using a 2% agarose gel (Invitrogen) electrophoresis run in an electrophoresis chamber (Sub-cell Biorad) at 150V, 400mA run for 32 minutes. The gels were then carefully transferred to the UV Transilluminator (Syngene Synoptics Ltd, Denmark) where a photograph of the gel was taken. The *MBL2* gene and promoter region typer was then used to determine the *MBL2* genotype of each individual sample (Table 4.2) [1, 2].



Figure 4.6a. Photographs of the MBL gels taken in the UV Transilluminator.

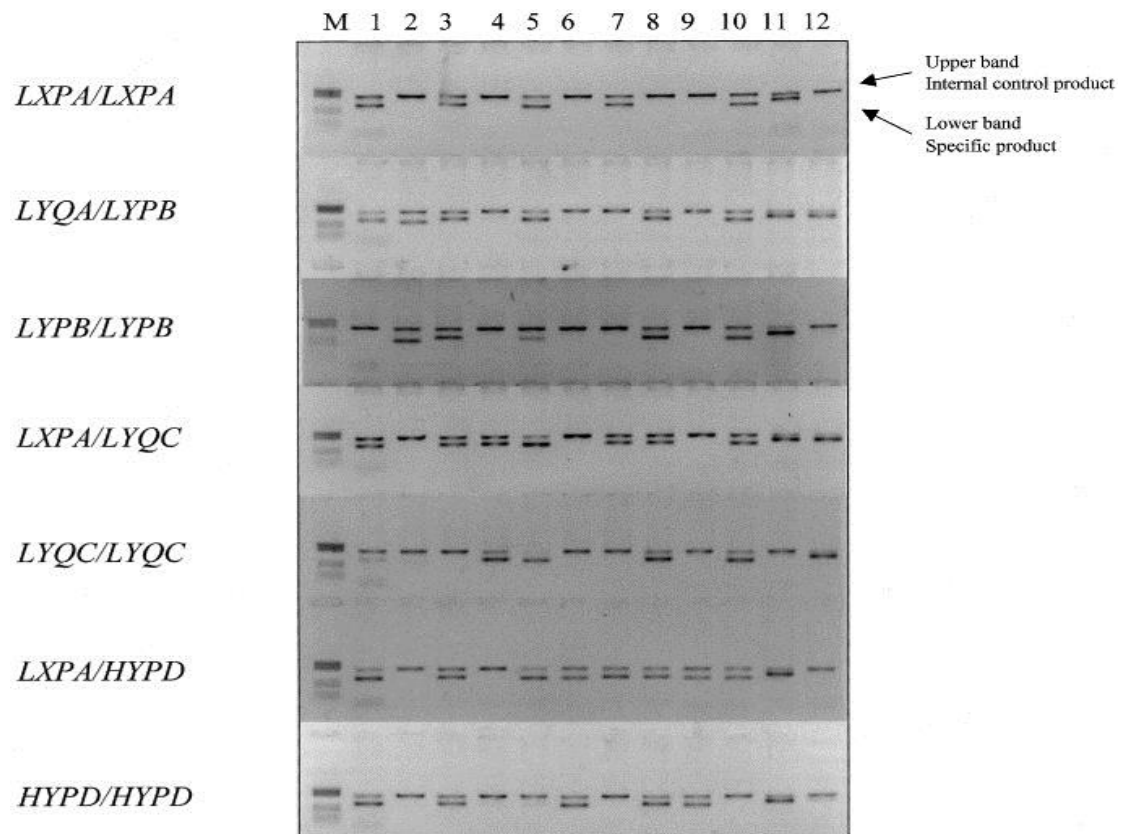


Figure 4.6b: Picture of gel showing the different MBL genotypes obtained in the MUSH cohort

Table 4.2: The 12 oligonucleotide primer sequences and the Exon 1 internal control primer used in this study for the detection of *MBL2* coding, promoter normal and variant alleles.

primer	Specific sequence 5'-3'	Primer localization	Product Size
A54 none B	5'-AGTCGACCCAGATTGTAGGACAGAG-3' 3'-CCTTTTCTCCCTTGGTGC-5'	617-640	278
B	5'-GGGCTGGCAAGACA ACTATTA-3' 3'-GCAAAGATGGGCGTGATGA-5'	617-240	278
A57 None C	5'-AGTCGACCCAGATTGTAGGACAGAG-3' 3'-CCTGGTTCCCCCTTTTCTC-5'	617-240	290
C	5'-AGTCGACCCAGATTGTAGGACAGAG-3' 3'-ACCTGGTTCCCCCTTTTCTT-5'	617-240	290
A52 none D	5'-AGTCGACCCAGATTGTAGGACAGAG-3' 3'-TCCCTTGGTGCCATCACG-5'	617-240	268
D	5'-AGTCGACCCAGATTGTAGGACAGAG-3' 3'-CTCCCTTGGTGCCATCACA-5'	617-240	268
Promoter X	5'-CTCAGGGAAGGTTAATCTCAG-3' 3'-CATTTGTTCTCACTGCCACC-5'	6-25	440
Promoter Y	5'-CTCAGGGAAGGTTAATCTCAG-3' 3'-CATTTGTTCTCACTGCCACG-5'	6-25	443
Promoter H	5'-GGCTTAGACCTATGGGGCTA-3' 3'-GCTTCCCCTTGGTGTTTTAC-5'	76-96	316
Promoter L	5'-GGCTTAGACCTATGGGGCTA-3'	76-96	316

	3'-GCTTCCCCTTGGTGTTTTAG-5'		
P	5'-AGGATCCAGGCAGTTTCCTCTGGAAGG-3' 3'-TAGGACAGAGGGCATGCTC-5'	624-649	335
Q	5'-AGGATCCAGGCAGTTTCCTCTGGAAGG-3' 3'-TAGGACAGAGGGCATGCTT-5'	628-649	331
Exon 1 internal Control	5'-GAGTTTCACCCACTTTTTCACA-3' 3'-GCCTGAGTGATATGACCCTCC-5'	892-913	431

These specific sequences were used for the identification of the *MBL2* and promoter region types, according to the manufacturer's instructions.

Table 4.3: Showing the determination of *MBL2* genotypes and promoter types using the electrophoresis gel bands on the photograph taken by the UV trans-illuminator

MBL genotype	1 A54	2 B	3 A57	4 C	5 A52	6 D	7 X	8 Y	9 H	10 L	11 P	12 Q
AAHYHP	X		X		X			X	X		X	
AAHYLHPQ	X		X		X			X	X	X	X	X
AAHYLP	X		X		X			X		X	X	
AAHYLPQ	X		X		X			X		X	X	X
AAHYLP	X		X		X			X		X	X	
AAHYLYP	X		X		X		X	X		X	X	
AAHYLYHP	X		X		X		X	X	X	X	X	
AAHYLYLPQ	X		X		X		X	X		X	X	X
AAHYLYHP	X		X		X			X	X	X	X	
AAHYLYLP	X		X		X		X			X	X	
AAHYLYHP	X	X	X		X			X	X	X	X	
AAHYLYLPQ	X	X	X		X			X		X	X	X
AAHYLYLP	X	X	X		X			X		X	X	
AAHYLYHP	X	X	X		X		X	X		X	X	
AAHYLYHP	X	X	X		X			X	X		X	
AAHYLYHP	X		X	X	X	X		X	X	X	X	
AAHYLYLQ	X		X	X	X	X		X		X		X
AAHYLYLPQ	X		X	X	X	X		X		X	X	X
AAHYLYLPQ	X		X	X	X	X	X	X		X	X	X
AAHYLYHP	X		X		X	X		X	X		X	
AAHYLYHPQ	X		X		X	X		X	X	X	X	X
AAHYLYHP	X		X		X	X		X	X	X	X	
AAHYLYHP	X		X		X	X	X	X	X	X	X	
AAHYLYLP		X	X		X			X		X	X	
AAHYLYLPQ		X	X	X	X			X		X	X	X
AAHYLYHP	X	X	X			X		X	X	X	X	
AAHYLYLP				X	X			X		X	X	
AAHYLYHPQ	X		X	X	X	X		X	X	X	X	X
AAHYLYHP	X		X			X		X	X		X	

This geno-typer was used to determine the combined MBL genotype and promoter type for each DNA sample.

4.12.0 PMTCT STUDY

4.12.1 Study design and study population

This was a laboratory based sub-study where we used dried blood spot (Fig. 4.7), samples that were collected as part of a national cross-sectional survey [236, 237] on prevention of mother to child transmission (PMTCT) carried out by the CESHAAAR Research Centre, Harare Zimbabwe. The main study was entitled Evaluation of Zimbabwe's Accelerated PMTCT of HIV Program [236, 237, 249]. We carried out *MBL2* genotyping on a subset of the dried blood samples collected during this national PMTCT survey (Fig. 4.7). Details of the PMTCT national survey, including blood sample collection, screening procedures and the study population have been described in detail elsewhere [249]. Briefly, the national PMTCT survey was undertaken in September 2012 and targeted women aged 16 years and above who had children between 9 and 15 months old [236, 237]. The survey examined the uptake of services and behaviours in the national PMTCT programme in Zimbabwe and determined the factors associated with MTCT and maternal antiretroviral therapy (ART) or antiretroviral (ARV) prophylaxis. Dried blood spots were collected from 8800 babies and their biological mothers [236, 237] and of these we used 632 mother-baby pair blood samples [3].

4.12.2 Laboratory procedures

4.12.2.1 Collection of dried blood spots

Venous blood from the heel of the child and a finger of the mother was collected on filter papers (Perkin Elmer 226, Health Sciences, USA), (Fig. 4.7). Blood was collected on five designated spots on the filter papers. The filter papers were air dried overnight and then packed the next day in zip lock bags with desiccant granules to prevent humid formation and

keep the spots dry. The zip lock bags were kept at room temperature until analysis of the spots[3].

4.12.2.3. HIV testing

All the mothers were initially tested for HIV antibodies using EIA HIV test kits (AniLabsystems Ltd, OyToilette 3, FIN-01720, Vantaa, Finland). All the mothers that tested HIV positive on the initial screening tests were confirmed using Enzygnost Anti-HIV ½ Plus ELISA (Dade Behring, Marburg, Germany) and discrepant results were resolved by Western Blot. All the children as they were below two years of age were tested using HIV polymerase chain reaction test kits (Roche Amplicor HIV-1 DNA Test, version 1.5)[3, 236].

4.12.2.4 DNA extraction

1.Sigma Generation kit

Initially DNA was extracted using the Sigma Generation kit. Two 3mm punches [5] were made for each dried blood sample and put in a 200µl 96 well PCR plate (DNA/NDase/RNase/PCR inhibitor free, SARSTEDT, UK). One hundred and forty (140 µl) of assay buffer were added and the plates vortexed for 45 minutes to clean the dried blood spots. Supernatant was discarded and another 140µl assay buffer added, vortexed for 45 minutes and supernatant discarded. Ninety (90µl) Generation DNA Solution 1 added and allowed to stand for 1 minute and supernatant discarded, this step repeated once more. Ninety (90µl) Generation DNA Solution 2 added and allowed to stand for 1 minute and supernatant discarded. Sixty (60µl) of filtered milli-Q water was added and extract vortexed at -20°C and transferred to a PCR machine set for 1 cycle at 99°C for 25 minutes to stabilize

the DNA. The extracted DNA dissolved in the water was stored in a freezer at -20°C until ready for use in PCR.

Initially DNA extraction was done using the above mentioned Sigma Generation kit. We could not amplify by PCR the DNA from this method. Then extraction was done using the second kit SIGMA Extract and Amp kit and DNA extracts from this method could be amplified by PCR. The initial protocol for the lysis step during DNA extraction was 55°C for 15 minutes this was optimised to 75°C for 30 minutes, a modification from the manufacturer's instructions. This change in the conditions for the lysis step gave the best bands in *MBL2* PCR.

Genomic DNA was then extracted from dried blood spots using SIGMA Extract and Amp kit. Briefly, two 3mm punches were made for each dried blood (Fig. 4.8) sample and incubated in 20µl Lysis solution (SIGMA) at 75°C for 30 minutes. Subsequently 180µl of Neutralizing solution (SIGMA) was added and mixed by pipetting in the plate wells and centrifuged for 1 min at 1000rpm. The extracted DNA quantified using the Qubit kit according to manufacturer's instructions and stored at 4°C[3].

Optimization of *MBL2* PCR

The MBL initial PCR method was optimised by initially using the enzyme Taq DNA Polymerase for amplification. We obtained very weak amplification bands using the Taq DNA Polymerase enzyme and the standard *MBL2* genotyping method used at Serum State Institute. We then changed the amplification enzyme to TEMPase Hot Start DNA Polymerase (VWR, Belgium) which gave us better bands for all the *MBL2* SNPS, *HL*, *YX*, *PQ* and *Exon 1*. The amount of template DNA to be used for the PCR reaction was also optimised. We

used 2 μ l, 5 μ l and 10 μ l and obtained the best clear bands by using 2 μ l of the template DNA, 2 μ l was therefore used throughout all the *MBL2* genotyping assays [3].



Figure 4.7 Dried blood spots from mother and baby pairs



Figure 4.8. Preparation of dried blood spots, punching 3mm spots for DNA extraction

Table 4.4: The oligonucleotide primer sequences that were used in this study for detection of *MBL2* coding and promoter normal and variant alleles.

Oligonucleotide name	Primer name	Specific primer sequence 5'-3'	Primer modification
MBL exon 1 codons 52, 54 and 57	Forward	5'AGT ATG GTG GCA GCG TCT TAC TCA G 3'	5' biotin
	Reverse	5'/5Bio/GGC AGT TTC CTC TGG AAG GTA AAG A 3'	
	Sequencing	5' CAG GCA AAG ATG GG 3'	
<i>H/L</i>	Forward	5' /5Bio/GCC AGG GCC AAC GTA GTA A 3'	5'biotin
	Reverse	5' GAG TTT GCT TCC CCT TGG T 3'	
	Sequencing	5' TCC CCT TGG TGT TTT 3'	
<i>Y/X</i>	Forward	5' /5Bio/TCC CCT AAG CTA ACA GCC ATA AG 3'	5' biotin
	Reverse	5' TGA TGA GCA GTG GGG ATC CTA 3'	
	Sequencing	5' AAG ACT ATA AAC ATG CTT TC 3'	

4.13.0 Pyrosequencing Method

4.13.1 Amplification of *MBL2* genes by Initial general PCR

DNA was amplified by PCR using the following reaction mixture (per sample) 41.125 µl Milli-Q water, 0.625µl dNTPs at 10mM each (from Pharmacia Biotech), 0.25µl of 5U/µl HotStar DNA Taq Polymerase Enzyme (QIAGEN), 1µl of 20 pmol of *MBL2* Exon 1, *H/L* and *Y/X* labelled forward and reverse PCR primer

(DNA Technology), 5µl of 10x HotStar PCR buffer and 2µl sample DNA extract. A positive (a Danish DNA sample of known *MBL2* genotype) and a negative control (no DNA added) was included in each PCR run. The oligonucleotide primer sequences used for *MBL2* genotyping are as shown (Supplementary Table 1). The following thermo cycling program was used: an initial denaturation step at 95°C for 15 min, followed by 38 cycles of 95°C 30s, 53°C for 30s and 72°C for 30s, the reaction was completed by a final elongation/extension step at 72°C for 10min. The PCR was expected to produce one specific amplicon of approximately 240 bp, this was evaluated by agarose gel electrophoresis. This PCR product was used for the *MBL2* pyrosequencing reaction [3].

4.13.2 Visualisation of PCR products by agarose gel electrophoresis

The PCR products were separated by a 2% agarose gel (Invitrogen) electrophoresis run in an electrophoresis chamber (Sub-cell Biorad) at 150V, 400mA for 32 minutes including the positive and negative control samples. The gels were then carefully transferred to the UV Transilluminator (Syngene Synoptics Ltd, Denmark) where a photograph of the gel was taken. Samples that showed the required *MBL2* band were used for pyrosequencing [3, 250-254]

4.13.3 Pyrosequencing reaction

In preparation for the pyrosequencing reaction (Fig. 4.9), 25µl of biotinylated PCR product was mixed with 3µl of streptavidin-coated magnetic beads (Streptavidin Sepharose High performance, GE Healthcare Life Sciences) 37µl of binding buffer (Qiagen) and 15 µl Milli-Q water. This mixture was agitated for 10-20 minutes at 1000 rpm on a plate shaker to allow the biotinylated PCR product to bind to the streptavidin sepharose beads. Subsequently, immobilized DNA was transferred, using as PSQ 9 magnetic sample prep tool (Biotage, Pyrosequencing), through a series of washing steps and finally released into a primer plate(Fig. 4.10)(Pyromark

Q96, Qiagen) containing 39µl annealing buffer (Qiagen), 1µl of 16 pmol/µl of each specific pyrosequencing primer i.e Exon1, *HL*, *YX* and *PQ* (DNA Technology) (Supplementary Table S1). This plate was incubated for 2 minutes at 80°C followed by cooling in the pyrosequencing machine (Pyromark Q96, Qiagen) for 10 minutes. Enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase), luciferine and adenosine-5-phosphosulphate (APS) (Pyromark Q96 cartridge reagents kit, Qiagen) were added to the cartridge wells according to instructions from the installed pyro programme (Fig. 4.9). The pyrograms produced by this programme are used in determination of the MBL genotype and promoter type for each sample (Fig. 4.11)[3].

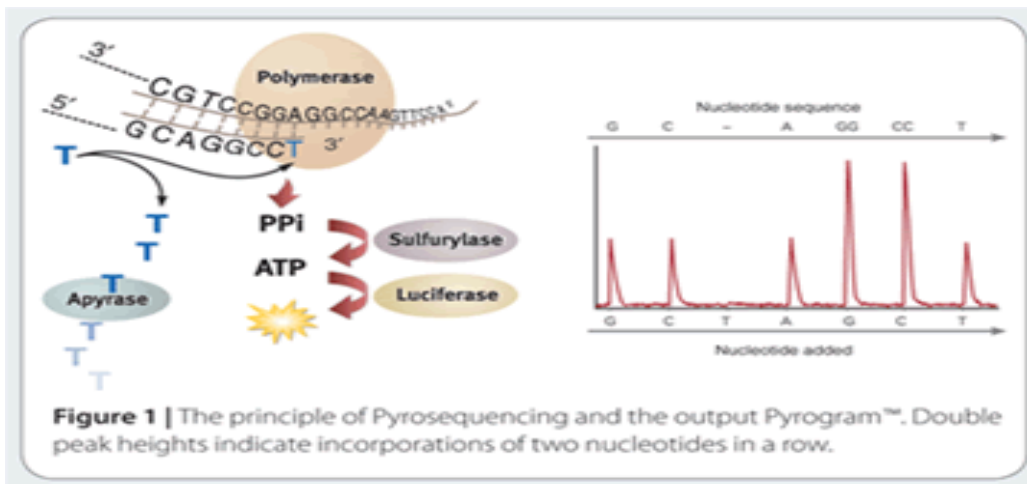


Figure 4.9. Principle of the pyrosequencing reaction.



Figure 4.10. Preparation of pyrosequencing primers



Figure 4.11: Checking quality of the pyrosequencing *MBL2* genotyping pyrograms.

4.13.4 *MBL2* genotypes and haplotype functional groups

Participants were classified into the different *MBL2* Exon 1 genotypes, normal *MBL2* allele designated as *A* and point mutations in the *MBL2* gene and the variants designated as *B* (codon 54, *rs1800450 A>G*), *C* (codon 57, *rs1800451 A>G*), and *D* (codon 52, *rs5030737 T>C*) and the different combinations of variants [6, 7].

Participants were classified into six *MBL2* haplotype functional groups which were combined into three groups for statistical analysis, haplotypes that give normal plasma MBL levels (*YA/YA*, *YA/XA*), intermediate levels (*XA/XA*, *YA/YO*) and deficient levels (*XA/YO*, *YO/YO*) [101, 252]. We did not have plasma nor serum samples to determine plasma MBL concentration for our study population but available literature indicates *MBL2* genotypes can be used as proxy for plasma MBL levels [40, 45, 48, 212-215, 217, 218] and to classify individuals into normal plasma MBL levels, intermediate levels and deficient levels [3, 40, 45, 48, 212-215, 217, 218].

4.14 Data Management and Analysis

All management, data entry, data processing, data cleaning and all statistical analysis for data from the two sub-studies MUSH and PMTCT, were done using Stata 11 statistical package (STATA Corp, Timberlake Consultants)[1, 2]. Normality of plasma MBL levels was checked graphically. Non-parametric tests (Mann-Whitney and Kruskal-Wallis) were used for comparison of median plasma MBL concentrations by age, gender, HIV-1 and schistosoma infection status. Differences in proportions of MBL deficiency between males and females and infection status were done using Chi Square or Fisher's exact tests and confidence intervals were calculated using binomial exact methods. The frequencies of the *MBL2* alleles were obtained by direct gene counting. Conformation to the Hardy-Weinberg Equilibrium was determined using the Chi Square test in the SHEsis online programme [255, 256]. For *MBL2* genotype analysis, normal homozygous *MBL2* was denoted as *A/A*. The variant genotypes *A/B* (*A>G*), *A/C* (*G>A*), *A/D* (*C>T*) were grouped together as *A/O* heterozygous *MBL2* as they all give low plasma MBL levels and all homozygous and compound homozygotes were grouped

together as *O/O*. The differences in frequency of the *MBL2* genotypes, promoter region variants and haplotypes between infection status groups were determined using the Chi Square test or Fisher's exact tests [1, 2].

Model assumptions were checked graphically using residual and Kaplan-Meier plots. Viral load was included in the model as log-transformed units. We used Mann-Whitney and Kruskal-Wallis tests to compare plasma MBL concentrations according to two CD4⁺ T-cell count groups, above or below 350 cells/ μ l [56], the level recommended by WHO then (2007) to initiate ART and viral load above or below the median viral load of this study population of 60 000 copies per ml. Generalised Estimating Equations (GEE) models [257-259] were used to compare rates of change of the CD4⁺ T cell count and viral load measurements over the three-year follow-up period. The role of plasma MBL deficiency and *MBL2* genetic variants on survival over the 3-year period were estimated using the Cox proportional hazard models. Regression analysis was used to test for interaction and confounding between MBL deficiency, *MBL2* genetic variance, age and sex. We used the Wald Chi-square statistic to choose between full and nested models. All tests were conducted at 5% level of statistical significance [1, 2].

For the PMTCT MBL study, to determine the prevalence of MBL deficiency, *MBL2* genotypes were used as proxy for MBL levels, *MBL2* *A/A* genotype was considered as normal and those with *A/O* and *O/O* categorised as deficient MBL. Participants were classified into six *MBL2* haplotype functional groups which were combined into three groups for statistical analysis, haplotypes that give normal plasma MBL levels (*YA/YA*, *YA/XA*), intermediate levels (*XA/XA*, *YA/YO*) and deficient levels (*XA/YO*, *YO/YO*) and promoter region haplotypes [101, 252]. Differences in proportions of MBL deficiency were determined using Chi Square or Fisher's exact tests. The differences in distribution frequency between HIV-1 infected and uninfected children, of the *MBL2* genotypes, promoter region variants and promoter haplotypes, were determined by the Chi Square test or

Fisher's exact tests. The effect of maternal MBL deficiency on HIV status of the children was determined in a logistic regression model after controlling for use of ART. The level of statistical significance used was $p < 0.05$ [3].

Data Analysis for the specific objectives in full detail

1. To determine the prevalence of MBL deficiency among the Zimbabwean adult population.

Exposures to be measured

- MBL plasma concentration in the adult cohort
- MBL deficiency

Confounders to be measured

- Age, gender, education, socio-economic factors

Outcomes to be measured

- HIV status by standard HIV testing kits
- Prevalence of MBL plasma deficiency

Statistical Analysis: Participants with initial mean MBL concentration of $0\mu\text{g/L}$ will be assigned the minimum MBL detection limit value of $20\mu\text{l/L}$. Data analysis will be done initially using MBL plasma concentration as a continuous variable. Normality and variance of MBL concentration will be checked using Skewness and Kurtosis tests, Shapiro Wilk test, box plots and stem and leaf plots. If not normally distributed, Mann-Whitney and Kruskal-Wallis non-parametric tests will be used for comparison of MBL concentrations (medians) by age, gender and HIV infection status. The difference in MBL concentration between infection groups will be given as medians, p values and interquartile ranges. To determine prevalence of MBL deficiency, MBL concentration will be categorised into normal ($1000\mu\text{g/L}$ and above) and deficient (below $1000\mu\text{g/L}$). Differences in proportions of MBL deficiency between HIV positive and HIV negative will be determined using Chi Square or Fishers Exact tests. Factors associated with MBL deficiency will be determined using logistic regression, adjustment for age and gender and results given as odds ratios. Mann-Whitney and Kruskal-Wallis non-parametric tests for unpaired group comparisons, supplemented with logistic regression will be used to compare plasma MBL concentrations between HIV infection status groups, according to CD4^+ T-cell count, above or below $250\text{ cells}/\mu\text{l}$.

2. To determine the relationship of MBL deficiency with HIV infection in the MUSH adult cohort in Zimbabwe.

Exposures to be measured

- MBL plasma concentration in the adult cohort
- MBL deficiency

Confounders to be measured

- Age, gender, education, socio-economic factors

Outcomes to be measured

- HIV status by standard HIV testing kits
- Prevalence of MBL plasma deficiency

Statistical Analysis: Participants with initial mean MBL concentration of 0µg/L will be assigned the minimum MBL detection limit value of 20µl/L. Data analysis will be done initially using MBL plasma concentration as a continuous variable. Normality and variance of MBL concentration will be checked using Skewness and Kurtosis tests, Shapiro Wilk test, box plots and stem and leaf plots. If not normally distributed, Mann-Whitney and Kruskal-Wallis non-parametric tests will be used for comparison of MBL concentrations (medians) by age, gender and HIV infection status. The difference in MBL concentration between infection groups will be given as medians, p values and interquartile ranges. To determine prevalence of MBL deficiency, MBL concentration will be categorised into normal (above 1000µg/L) and deficient (below 1000µg/L). Differences in proportions of MBL deficiency between HIV positive and HIV negative will be determined using Chi Square or Fishers Exact tests. Factors associated with MBL deficiency will be determined using logistic regression, adjustment for age and gender and results given as odds ratios. Mann-Whitney and Kruskal-Wallis non-parametric tests for unpaired group comparisons, supplemented with logistic regression will be used to compare plasma MBL concentrations between HIV infection status groups, according to CD4⁺ T-cell count, above or below 250 cells/µl.

3. To determine the effect of MBL deficiency on disease progression and survival among the Zimbabwean adults.

Exposure to be measured

- MBL plasma concentration in the adult cohort
- MBL genotype and promoter type in the adult cohort

Confounders to be measured

- Age, gender

Outcomes to be measured

- Mortality
- Viral load change
- CD4 T cell count change

Statistical Analysis: Normality and variance of CD4 counts and viral load will be checked using Skewness and

Kurtosis tests, Shapiro Wilk test, box plots and stem and leaf plots. If the CD4 counts and viral load are not normally distributed they will be log transformed to approximate normality. Random effects models will be used to take care of repeated CD4 and viral load measurements and to determine change in CD4 count and viral load at baseline, 3, 6 and 12 months and adjust for factors associated with disease progression. Generalised Estimating Equations (GEE) will also be used to determine association between MBL deficiency and disease progression.

The effect of MBL genetic variants on survival, in HIV-1 positive participants, will be estimated by the Kaplan Meier plots and differences will be tested by the log-rank test. Survival analysis using Kaplan Meier plots and Log rank tests will be done only among the 197 HIV-1 positive participants to determine influence of MBL plasma concentration and MBL polymorphism in the MBL coding and promoter region, on survival. Results will be presented as hazard ratios and CIs after adjusting for gender and age using the Cox regression analysis.

4. To determine prevalence of MBL deficiency among mother and their infants in a Zimbabwean population.

Exposures to be measured

- MBL genotype and promoter type in the PMTCT cohort

Confounders to be measured

- Age, Gender
- Use of ART by the mothers and children in the PMTCT cohort

Outcomes to be measured

- HIV status of the children by standard HIV testing kits

Statistical Analysis: The frequencies of the MBL alleles will be obtained by direct gene counting. For MBL genotype analysis, normal homozygous MBL will be denoted as A/A. The variant genotypes AB, AC, AD

will be grouped together as A/O heterozygous MBL as they all give low MBL plasma levels and all homozygous and compound homozygotes will grouped together as O/O. The fit to the Hardy-Weinburg equilibrium, the differences in distribution frequency of the MBL genotypes, promoter haplotypes between the HIV infected and uninfected will be determined by the Chi Square test. Odds ratios with CIs will be calculated using Logistic regression.

5. To determine the relationship between MBL deficiency and HIV transmission from mother to child.

Exposures to be measured

- MBL genotype and promoter type in the PMTCT cohort

Confounders to be measured

- Age, Gender
- Use of ART by the mothers and children in the PMTCT cohort

Outcomes to be measured

- HIV status of the children by standard HIV testing kits

Statistical Analysis: The frequencies of the MBL alleles will be obtained by direct gene counting. For MBL genotype analysis, normal homozygous MBL will be denoted as A/A. The variant genotypes AB, AC, AD will be grouped together as A/O heterozygous MBL as they all give low MBL plasma levels and all homozygous and compound homozygotes will grouped together as O/O. The differences in distribution frequency of the MBL genotypes, promoter haplotypes and the fit to the Hardy-Weinburg equilibrium will be determined by the Chi Square test. Odds ratios with CIs will be calculated using Logistic regression.

4.15 Additional statistical analysis carried out post publication of the three papers

4.15.1 Fractional polynomials to analyse disease progression

Additional analysis was done treating MBL as a continuous variable [260-263] to determine relationship between change in CD4⁺ T cell count and MBL levels using GEE models.. Fractional polynomials [260-263] were carried out to determine the response of CD4⁺ T cell count to plasma MBL levels using the GEE model,

after controlling for schistosomiasis infection at baseline. MBL was considered as a continuous exposure variable. Fractional polynomials of first and second order were used to test for non-linearity in the response of CD4⁺ T cell count to MBL.

4.15.2 Power analysis for MUSH MBL study

This was done to determine the power of the MUSH study based on HIV as the outcome.

Additionally, for the relationship of MBL to disease progression, this is a cohort study and the analysis is a time to event analysis, with exposure being MBL sufficiency level. So the exposed and unexposed groups were not of equal size. Power analysis was done using `stpower` in Stata to give the power corresponding to different hazard ratios

4.15.3 Power analysis for PMTCT study

This was done to determine the power of the PMTCT study based on HIV as the outcome.

4.16. Ethics Considerations

4.16.1 Ethics clearance for the PhD research project

This PhD research project made use of blood samples from an adult cohort and dried blood samples from a PMTCT national survey of mothers and their babies. The PhD project was granted permission by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/1770) and University of the Witwatersrand Human Research Ethics Committee (clearance number M130348). The details of the clearance processes for the main MUSH study and the PMTCT were as follows:

4.16.2 Ethics clearance and considerations for the main MUSH study

Ethics approval to conduct the MUSH study, on which the MBL study was a sub-study, was granted by the Medical Research Council of Zimbabwe, Ministry of Health and Child Welfare (MRCZ/A/918) local health and political authorities in Shamva District Zimbabwe. In addition, permission was given by the provincial medical director of Mashonaland Central Province, the district medical officer of Shamva District, and the village leaders.

Informed written consent was sought and granted by all research participants. Only those individuals who volunteered to participate after obtaining detailed information about the study including the need for HIV testing was provided, were recruited into the study. All participants were asked to sign a consent form and were informed of their freedom to withdraw at any time even after signing. All participants were given the study information leaflet to take home, read and keep, in a language of their choice (English or Shona). The following ethical issues were also considered and adhered to during the main MUSH study:

4.16.3 Blood sampling

The amount of blood taken for serum/plasma processing, PBMCs and HIV testing was small. However participants had the right to withdraw from the study or refuse to give blood samples at any time.

4.16.4 Testing the participants for HIV

Two tests were done before confirmation of positive diagnosis. During recruitment, the participants were counselled extensively in order to make personal decisions as to receive test results or not. Post-test counselling was also given.

4.16.5 The prevention of HIV and other STIs

All participants were given information on prevention of HIV, those included in the study or not and anyone asking for any information was helped if possible. Treatment for STIs was offered at the research satellite clinic but all complicated cases were referred for further management at Mt Darwin Karanda Hospital.

4.16.6 Treatment for schistosomiasis

Treatment with praziquantel was administered to all infected people after the baseline Survey. Praziquantel was given to pregnant women after delivery as well as at the end of the study.

4.16.7 Ethics considerations for the PMTCT survey and access to dried blood samples

The CESHAAAR PMTCT study was approved by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/1655), University of California and University of London Human Research Ethics Committees. All the mothers who took part in the main PMTCT National Survey gave written

informed consent for specimen collection, storage and future laboratory studies. Permission to access the PMTCT samples was granted by the Directorate at the CESHAAAR Research Centre and their collaborators. Approval to carry out the MBL assays on mother and baby pairs was sought both from the Medical Research Council of Zimbabwe and from the Witwatersrand Human Research Ethics Committee. Permission to ship the dried blood samples to Denmark was also sought from the CESHAAAR Research Centre and from the Research Council of Zimbabwe.

4.16.8 Data protection and absolute confidentiality

Was assured in both the MUSH study and PMTCT survey. Access to participants' personal information was restricted to authorised personnel only. All participants data was anonymized, kept confidential and access to information restricted to authorized personnel.

CHAPTER 5 RESULTS: SUMMARY OF KEY FINDINGS

5.0 Introduction

This chapter gives a summary of the results for this research project. Details of the results are reported in papers 1,2 and 3 attached (Appendices A-C) and the fourth manuscript is under review (appendix D). This chapter also describes in detail all the data analysis carried out after publication of papers 1, 2 and 3. The findings reported below have been divided into three sub sections. The first subsection, by way of introduction and background provides a short demographic description of the two study populations, the adult and PMTCT studies. The second subsection presents the five themes of this thesis based on the five research questions explored during this research and the five specific study objectives. Throughout this chapter references are given of the specific papers in which the detailed reports are given. The third sub-section reports the results from the additional statistical analysis that was carried out after publication of papers 1,2 and 3.

5.1 Socio-demographic characteristics of the two study populations

This sub section provides a brief description of the two study populations involved in this research. Our study comprised of two populations, an adult MUSH population and a PMTCT population of mothers and their 9-18 months old babies. Detailed descriptions of the populations are given in original papers 1,2 and 3. Briefly MUSH study-population comprised of adults 379 adult males and females,18 years old and above, residing in the area, who were willing to be bled and tested for HIV (reported in papers 1 and 2) Median age (IQR) of the MUSH participants was 30 (17 to 41) years old and the majority were females (80%). Baseline demographic characteristics of this study population and prevalence of HIV-1, *S. haematobium* and *S. mansoni* among the 1545 participants screened during the main MUSH study have been described elsewhere [65] and also for this thesis reported in pares 1 and 2.

From the PMTCT study, the median age (IQR) of the mothers was 30 (26 - 34) years and the children mean age (IQR) was 12 (11-15) months old at the time of enrolment. All 622 mothers were HIV-1 infected, 574 babies were HIV negative and 48 were HIV-1 positive babies. For the PMTCT MBL study, stored dried blood samples from mothers and baby pairs which were obtained from the CESHAAAR Research Centre in Zimbabwe that had stored whole blood samples for mother –infant pairs from a national PMTCT survey carried out in 2012 (reported in paper 3).

5.2. Synthesising the two sub-studies

This sub section presents a synthesis of the five thesis themes derived from the five research questions and the five study specific objectives set out to be explored at protocol development stage, explaining the relationship between MBL genetic polymorphism and HIV infection in the adult and PMTCT sub-studies. The five themes have already been introduced in Chapter 3.

5.3. Study themes

We used the MUSH study population to answer research questions 1, 2, and 3 indicated in this section as themes 1, 2 and 3. And we also used the mothers and children PMTCT stored dried blood samples to answer our study research questions 4 and 5 indicated in this section as themes 4 and 5.

5.3.1. Determination of plasma MBL levels, prevalence of MBL deficiency, prevalence of normal and variant *MBL2* genotypes and promoter region types in Zimbabwean adult males and females, MUSH study (THEME 1, PAPER 1).

From the MUSH study a total of 379 plasma and whole blood samples were available for MBL analysis. The median (IQR) plasma MBL concentration was 800µg/L (192-1936µg/L) (paper 1). Prevalence of plasma MBL

deficiency was 18% with high frequency of the *C* (codon 57G>A) mutant allele (20%). The prevalence of normal and variant MBL genotypes are as reported in Table 3 (paper 1).

5.3.2. Determination of the role of Mannose Binding Lectin Deficiency in HIV-1 Infection in a Rural Adult Population in Zimbabwe (THEME 2, PAPER 1).

We found no significant difference in median plasma MBL levels between HIV negative *S. mansoni* (912µg/L) and HIV positive (688µg/L), $p=0.066$ as reported in Table 3 (paper 1). However plasma MBL levels at the assay detection limit of 20µg/L were more frequent among the HIV-1 infected ($p=0.007$) (as reported in paper 1). This adult population had some of the study participants co-infected with *S. haematobium* and *S. mansoni*, this variable was also analysed (Table 1, paper 1). The prevalence of HIV-1, *S. haematobium*, *S. mansoni* and co-infection with both schistosoma species among the 379 participants used for the MBL sub-study was 52%, 58%, 7% and 10%, respectively (Table 1) (Paper 1). Study participants co-infected with *S. haematobium* and had significantly higher MBL levels than the uninfected uninfected (Table 1, paper 1). All *MBL2* variants were not associated with HIV-1 infection (Table 4, paper 1) but promoter variants *LY* and *LL* were significantly associated with *S. haematobium* infection (Table 5, paper 1)

5.3.3. Determination of the role of Mannose Binding Lectin Deficiency in HIV-1 disease progression and survival in a rural adult population in Zimbabwe (THEME 3, PAPER 2).

We assessed 197 HIV positive adults where 83% (164) were women with a median age of 31 years old (paper 2). Prevalence of plasma MBL deficiency (less than 100µg/L) and *MBL2* deficient genetic variants (*A/O* and *O/O* genotypes) was 21% (42 out of 197) and 39% (74 out of 190), respectively (paper 2). We did not observe a significant role to explain individual variation in mortality, change of CD4⁺ T cell count and viral load by

MBL plasma deficiency or *MBL2* genetic variants from baseline to 3 years follow up period in this adult population as reported in Tables 3,4,5, paper 2).

5.3.4. Determination of prevalence of normal and variant *MBL2* genotypes and promoter region in Zimbabwean mothers and their babies (THEME 4, PAPER 3).

From the PMTCT study, the median age (IQR) of the mothers was 30(26 - 34) years and the children mean age (IQR) was 12 (11-15) months old at the time of enrolment. All 622 mothers were HIV-1 infected, 574 babies were HIV negative and 48 were HIV-1 positive babies (Figure 1, paper 3). *MBL2* normal structural allele A and variants B (codon 5A>G), C (codon 57 A>G) and promoter region SNPs -550(H/L) and -221(X/Y) were detected Table 1, paper 3). Prevalence of MBL deficiency was 34% among the mothers and 32% among the children Table 1, paper 3).

5.3.5. Determination of the role of Mannose Binding Lectin Deficiency in HIV-1 infection and vertical transmission in a PMTCT national survey in Zimbabwe (THEME 5, PAPER 3).

We found no association between maternal *MBL2* deficiency and HIV-1 transmission to their children. We found no difference in the distribution of HIV-1 infected and uninfected children between the *MBL2* genotypes of the mothers and those of the children Tables 2 and 3, paper 3). Taken together, the present study in a large sample of mother-infant pairs in Zimbabwe adds to the emerging literature and the hypothesis that *MBL2* variation as predicted by haplotypes do not influence the vertical transmission risk for HIV.

5.4 Additional statistical analysis not included in paper 1

5.4.1 Power analysis for the Adult MBL study

Analysis of power for the MBL adult study based on HIV infection as an outcome, was then done after all laboratory work had been completed. Pocock's formula [109] was used to retrospectively carry out power analysis in sample size (n) required for the adult MBL sub-study : $n = \frac{p_1(1-p_1)+p_2(1-p_2)(Z_\alpha-Z_\beta)^2}{(p_1-p_2)^2}$, where p_1 and p_2 are proportions of MBL deficiency among HIV positive and HIV negative, Z_α and Z_β are values of standard normal distribution at α = Significance level and β = power. Given that the final $n = 378$ (HIV+ = 197, HIV – 181), $p_1 = 0.43$ and $p_2 = 0.33$, the sample size had power of 61% to detect a difference in distribution of MBL deficiency between the two HIV status groups. The adult MBL study therefore had 61% power to detect a difference in prevalence of MBL deficiency between the HIV infected and the HIV negative.

5.4.2 Power analysis of the adult MBL study corresponding to different hazard ratios

Analysis of power of the adult MBL study was done corresponding to different hazard ratios. This was done using the 3 MBL plasma categories, initially hazard ratio of MBL 100-500ug/ml against MBL plasma normal levels. The adult MBL study therefore had 9% power to detect a difference in prevalence of MBL deficiency between the HIV infected and the HIV negative after taking consideration of the different hazard ratios.

Then using hazard ratio of MBL <100µg/ml against MBL plasma normal levels. The adult MBL study therefore also had 9% power to detect a difference in prevalence of MBL deficiency between the HIV infected and the HIV negative after taking consideration of the different hazard ratios.

5.5 Additional statistical analysis not included in paper 2

This section provides details about the additional analysis that were carried out after publication of papers 1, 2 and 3, now incorporated in this integrating narrative.

5.5.1 Role of MBL deficiency in disease progression after controlling for schistosomiasis

Additional GEE analysis was carried out to determine role of MBL deficiency in change in CD4⁺ T cell count and viral load, after controlling for schistosomiasis. Our results showed that MBL deficiency had no role in change in CD4 T cell count (Table 5.1) and viral load (Table 5.2), after controlling for schistosomiasis.

5.5.2 Multiple GEE regression analysis

This analysis was carried out including all variables in the analysis. Our results showed the MBL deficiency had no role in change in CD4 T cell count (Table 5.3) and viral load (Table 5.4) after controlling for schistosomiasis.

5.5.3 Analysis of disease progression, treating MBL as a continuous variable using fractional polynomials

Additional analysis was done treating MBL as a continuous variable [260-263] to determine relationship between change in CD4⁺ T cell counts and MBL levels using GEE models. In the models used there was no evidence of non-linearity so MBL was fitted as a continuous explanatory variable without transformation. We found no relationship between change in CD4⁺ T cell count, viral load and MBL levels in our GEE models, after controlling for schistosomiasis infection.

TABLE 5.1. The factors associated with change in CD4⁺ T cell counts from baseline to 3 years follow up period, after controlling for schistosomiasis.

Factor	N	Parameter Estimate (cell counts)	95% CI	P value
Plasma MBL level				
Normal plasma MBL level >100µg/L (reference)	113	353	310-396	<0.001
MBL intermediate level 100-500µg/L	43	-38	-115-38	0.325
MBL deficient level <100µg/L	41	-14	-98-69	0.731
MBL exon 1 genotype				
MBL normal genotypes A/A	116	351	307-395	<0.001
MBL deficient genotypes A/O and O/O combined	74	-16	-87-53	0.644
MBL haplotypes				
MBL normal haplotypes (YAYA/YAXA)	110	347	303-391	<0.001
1.MBL deficient genotype (XA/XA, YA/YO) (intermediate levels	72	-3	-66-58	0.902
2.MBL deficient genotype XA/YO, YO/YO (deficient levels)	8	-30	-203-143	0.732
MBL promoter region genotypes				
MBL normal promoter region H/Y	14	315	193-437	<0.001
MBL promoter variants LY/LX	176	32	-95-159	0.621

Age at t0				
Below 25 years old	21	423	321-525	<0.001
Above 25 years old	177	-90	-198-16	0.098
Sex				
Female	16	361	324-397	<0.001
Male	34	-108	-196--21	0.015

Change in CD4⁺ T cell count (cells/ μ l) over the 3 year follow up period, after controlling for schistosomiasis. Reference plasma MBL levels is normal levels above 500 μ g/L. Reference *MBL2* genotype is normal wild type genotype *A/A* and *YAYA/YAXA*. Reference *MBL2* promoter region genotype is *H/Y*. Reference age was below 25 years old. Reference sex was females.

TABLE 5.2 The factors associated with change in viral load from baseline to 3 years follow up period, after controlling for schistosomiasis.

Factor	n	Parameter Estimate viral load	95% CI	P value
Plasma MBL level				
Normal plasma MBL level >100µg/L (reference)	113	4.58	4.41-4.75	0.000
MBL intermediate level 100-500µg/L	43	0.19	-0.11-4.90	0.220
MBL deficient level <100µg/L	41	0.02	-0.31-0.35	0.890
<i>MBL2</i> exon 1 genotype				
MBL normal genotypes <i>A/A</i>	116	4.63	4.45-4.79	0.00
MBL deficient genotypes <i>A/O</i> and <i>O/O</i> combined	74	-0.025	-0.30-0.25	0.860
<i>MBL2</i> haplotypes				
MBL normal haplotypes (<i>YAYA/YAXA</i>)	110	4.62	4.44-4.79	0.000
MBL deficient genotype (<i>XA/XA</i> , <i>YA/YO</i>) (intermediate levels)	72	-0.01	-0.28-0.25	0.925
MBL deficient genotype <i>XA/YO</i> , <i>YO/YO</i> (deficient levels)	8	0.03	-0.66-0.73	0.932
<i>MBL2</i> promoter region genotypes				
MBL normal promoter region <i>H/Y</i>	14	4.67	4.21-5.14	0.000
MBL promoter variants <i>LY/LX</i>	176	-0.068	-0.55-0.41	0.780

Age at t0				
Below 25 years old	21	4.49	4.10-4.88	0.000
Above 25 years old	177	0.15	-0.26-0.57	0.464
Sex				
Female	16	4.54	4.40-4.69	0.000
Male	34	0.471	0.13-0.81	0.006

Change in viral (load log₁₀ scale) over the 3 year follow up period, after controlling for schistosomiasis. Reference plasma MBL levels is normal levels above 500µg/L. Reference *MBL2* genotype is normal wild type genotype *A/A* and *YAYA/YAXA*. Reference *MBL2* promoter region genotype is *H/Y*. Reference age was below 25 years old. Reference sex was females.

Table 5.3. GEE Multiple Regression analysis of the factors associated with change in CD4⁺ T cell counts from baseline to 3 years follow up, after controlling for schistosomiasis infection.

Factor	n	Parameter Estimate (cell counts)	95% CI	P value
Plasma MBL level				
Normal plasma MBL level >100µg/L (reference)	113	441	278-604	<0.001
MBL intermediate level 100-500µg/L	43	-37	-128-53	0.421
MBL deficient level <100µg/L	41	-7	-141-126	0.915
MBL exon 1 genotype				
MBL normal genotypes A/A	116	356	312-400	<0.000
MBL deficient genotypes A/O and O/O combined	74	-24	-168-120	0.741
MBL haplotypes				
MBL normal haplotypes (YAYA/YAXA)	110	353	309-396	<0.001
1.MBL deficient genotype (XA/XA, YA/YO) (intermediate levels	72	10	-94-114	0.848
2.MBL deficient genotype XA/YO, YO/YO (deficient levels)	8	4	-204-214	0.965
MBL promoter region genotypes				
MBL normal promoter region H/Y	14	318	196-440	<0.001
MBL promoter variants LY/LX	176	19	-104-144	0.757

Age at t0				
Below 25 years old	21	429	327-531	<0.001
Above 25 years old	177	-88	-198-20	0.112
Sex				
Female	16	367	331-403	<0.001
Male	34	-111	-199--23	0.013

Change in CD4⁺ T cell count (cells/ μ l) over the 3 year follow up period, after controlling for schistosomiasis infection. Reference plasma MBL levels is normal levels above 500 μ g/L. Reference *MBL2* genotype is normal wild type genotype A/A and YAYA/YAXA. Reference *MBL2* promoter region genotype is H/Y. Reference age was below 25 years old. Reference sex was females.

Table 5.4. GEE Multiple regression analysis of the factors associated with change in viral load from baseline to 3 years follow up, after controlling for schistosomiasis

Factor	n	Parameter Estimate viral load	95% CI	P value
Plasma MBL level				
Normal plasma MBL level >100µg/L (reference)	113	4.37	3.75-5.00	<0.001
MBL intermediate level 100-500µg/L	43	0.30	-0.06-0.67	0.108
MBL deficient level <100µg/L	41	0.22	-0.31-0.75	0.426
<i>MBL2</i> exon 1 genotype				
MBL normal genotypes <i>A/A</i>	116	4.63	4.45-4.79	<0.001
MBL deficient genotypes <i>A/O</i> and <i>O/O</i> combined	74	-0.26	-0.92-0.40	0.443
<i>MBL2</i> haplotypes				
MBL normal haplotypes (<i>YAYA/YAXA</i>)	110	4.62	4.44-4.79	<0.001
MBL deficient genotype (<i>XA/XA</i> , <i>YA/YO</i>) (intermediate levels)	72	0.08	-0.47-0.63	0.772
MBL deficient genotype <i>XA/YO</i> , <i>YO/YO</i> (deficient levels)	8	0.06	-0.85-0.96	0.901
<i>MBL2</i> promoter region genotypes				
MBL normal promoter region <i>H/Y</i>	14	4.67	4.21-5.14	<0.001
MBL promoter variants <i>LY/LX</i>	176	-0.04	-0.52-0.44	0.872

Age at t0				
Below 25 years old	21	4.49	4.10-4.88	<0.001
Above 25 years old	177	0.15	-0.27-0.57	0.476
Sex				
Female	16	4.54	4.40-4.69	<0.001
Male	34	0.50	0.15-0.84	0.004

Change in viral (load log₁₀ scale) over the 3 year follow up period. Reference plasma MBL levels is normal levels above 500µg/L. Reference *MBL2* genotype is normal wild type genotype A/A and YAYA/YAXA. Reference *MBL2* promoter region genotype is H/Y. Reference age was below 25 years old. Reference sex was females.

5.6 Additional statistical analysis not included in paper 3

Pocock's formula [109] was used to retrospectively carry out power analysis in sample size (n) required for the

PMTCT MBL sub-study: $n = \frac{p_1(1-p_1)+p_2(1-p_2)(Z_\alpha-Z_\beta)^2}{(p_1-p_2)^2}$, where p_1 and p_2 are proportions of normal MBL and

MBL deficiency among mothers, Z_α and Z_β are values of standard normal distribution at α = Significance level and β = power. Given that the final $n = 622$ (normal MBL = 411, deficient MBL = 211), $p_1 = 0.09$ and $p_2 = 0.07$, the sample size had power of 17% to detect a difference in distribution of MBL deficiency between the two HIV status groups of the children. The PMTCT MBL study therefore had 17% power to detect a difference in prevalence of MBL deficiency between the HIV infected and the HIV negative children.

CHAPTER 6: DISCUSSION, CONCLUSIONS, PUBLIC HEALTH IMPLICATIONS OF THE STUDY AND RECOMMENDATIONS FOR FURTHER RESEARCH.

6.1 Introduction

This chapter provides in brief, a discussion on the key results obtained from this research project. A detailed comparison of our results on both the adult and PMTCT MBL studies to results available in literature in relation to the specific objectives and study themes set out for this research, are provided in papers 1, 2 and 3 (Appendix A-D). This chapter also describes the conclusions we drew from the adult MBL study and the PMTCT MBL study on the role of MBL deficiency on HIV infection, disease progression and survival in adults and HIV vertical transmission. In addition this chapter also discusses the public health implications of these findings for policy and recommendations for further research.

The results from the adult MUSH study showed a high prevalence of plasma MBL deficiency, high frequency of *MBL2* genetic variant *C* (G>A). These findings are consistent with other observations that the *C* (G>A) variant allele is the predominant variant allele in Sub-Saharan Africa. We found no evidence of an association between MBL deficiency and HIV-1 infection, however plasma MBL levels at assay detection limit were associated with HIV-1 infection indicating a possible role of MBL deficiency in HIV infection. Lower plasma MBL levels were protective against both *S. haematobium* and *S. mansoni* infections and higher plasma MBL levels associated with increased susceptibility to schistosoma infections. All the other MBL gene and promoter region variants detected played no role in both HIV and schistosoma infections but presence of promoter region variants *LY* and *LL* increased susceptibility to both *S. haematobium* and *S. mansoni* infections. The available evidence on the association of polymorphism in the *MBL2* promoter region with HIV-1 infection is still conflicting. In light of all this conflicting evidence it would be difficult to recommend the use of MBL plasma levels, *MBL2* structural variants and promoter region mutations as a biomarker of HIV infection and for the

monitoring of ARV therapy together with viral load and CD4⁺ T cell lymphocyte counts in the population represented by our study participants. Our results on role of plasma MBL levels in schistosoma infections are in conflict with the only available report that found lower plasma MBL levels to increase susceptibility to schistosoma infections. There is therefore, need to carry out a much bigger study to verify these results. In addition, future immunological studies looking at the association between MBL deficiency and other diseases are highly recommended for the Zimbabwean population where persistent recurrent bacterial, viral, fungal and other parasitic infections remain prevalent affecting both adults and children (discussed in more detail in paper 1)[1].

The results from an analysis of the role of MBL deficiency in HIV disease progression and survival cannot support recommendations to use *MBL2* polymorphism as a prognostic tool for AIDS progression using CD4⁺ T cell counts and viral load as has been recommended by others[49] (discussed in more detail in paper 2)[2].

Finally, our results showed a high prevalence of *MBL2* genetic variant *AC* among the mothers and their children, in accordance with other studies of *MBL2* genetic variation in African populations [6, 39]. This *MBL2* variant form is associated with MBL deficiency; however the high prevalence of MBL deficiency among the mothers and their children in our study was not associated with HIV-1 vertical transmission. It is important to explore the genetic make-up of the Zimbabwean population in view of the high prevalence of several viral, parasitic and bacterial infections considering the conflicting evidence associating MBL deficiency and protection against some infections and increased susceptibility in others (discussed in more detail in paper 3) [3].

6.2 Study Limitations

1. The main limitation of our study was inclusion of few men in the main MUSH study [1, 181, 182, 198]. There is a gold panning area in the neighbouring district, most men work away from their rural homes and were not available during our study recruitment phase. This has the potential to bias our findings but every effort was done to enrol all eligible men into the main MUSH study.
2. It was not possible to determine the effect of MBL deficiency on HIV treatment outcome because the ARVs were not readily available in Zimbabwe during that time.
3. Another limitation in this study was that serum/plasma samples were not available from the PMTCT study to determine MBL concentration for the PMTCT mothers and their babies. However, *MBL2* exon 1 region and promoter region polymorphisms can be used as proxy to classify participants into normal plasma/serum MBL levels, intermediate and deficient [5-7, 138, 264-267].
4. The low statistical power of 60% for the adult MBL study and 9% for the PMTCT MBL study is another limitation of our study. We therefore cannot generalise our findings to the general Zimbabwean population. Our major recommendation is therefore the need in future to carry out a study with a larger adult sample size and a larger PMTCT sample size.

6.3 Public Health Implications

In our study we did not find a role of MBL deficiency in HIV infection in the adult population. In our study we also did not find a role of MBL deficiency in survival and disease progression among the adults infected with HIV. We did also did not find a role of MBL deficiency in HIV vertical transmission. In conclusion we did not

find a role for MBL genetic variants in HIV infection nor in HIV vertical transmission from mothers to their children in our study population.

However this still remains a critical and interesting area of research around HIV prevention strategies as other studies have reported a protective role of normal MBL levels and normal *MBL2* genotypes in HIV infection [21].

6.4 Recommendations for Future Studies

This section gives an outline of recommendations and suggestions for future studies arising out of the findings of this study:

1. A bigger study with power above 80% to be carried out involving more adults to determine the role of MBL deficiency on HIV infection. Our adult MUSH MBL study had 61% power to determine a difference in HIV susceptibility.
2. A bigger study with power above 80% to be carried out involving more mother baby pairs to determine the role of MBL deficiency on HIV vertical transmission. The PMTCT MBL study had 9% power to determine a difference in HIV susceptibility between the HIV infected children and the HIV negative children.
3. Future work to include genotyping of other related loci which encode proteins along the same complement pathway for example the MASP loci. As dysfunctional MASP genotypes would also cause low levels of MBL.
4. Future work to also look at MBL genotypes and MBL levels in TB infections since Zimbabwe has a high TB prevalence.

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Appendices

- A. Published paper 1 and Supplementary materials.
- B. Published paper 2 and Supplementary materials.
- C. Published paper 3 and Supplementary materials.
- D. Manuscript under review.
- E. University of Witwatersrand Ethics clearance
- F. University of Witwatersrand Faculty approval
- G. Medical Research Council of Zimbabwe approval letter 2013
- H. MRCZ protocol amendment
- I. MRCZ Continuing approval letter 2014
- J. MRCZ Continuing approval letter 2015
- K. MRCZ Continuing approval letter 2016
- L. Letten Foundation Supporting letter
- M. RCZ permit to ship dried blood samples
- N. University of California access to blood samples
- O. Invitation to Serum State Institute (SSI) Denmark
- P. Information leaflet and Consent Form MUSH study (Shona version)
- Q. Other Papers on the MUSH study co-authored related to my PhD work, but not included in this thesis
- R. TURNITIN report

Appendix A:Published paper 1 and published supplementary materials

RESEARCH ARTICLE

Role of Mannose-Binding Lectin Deficiency in HIV-1 and Schistosoma Infections in a Rural Adult Population in Zimbabwe

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Abstract

Background Polymorphism in the MBL2 gene lead to MBL deficiency, which has been shown to increase susceptibility to various bacterial, viral and parasitic infections. We assessed the role of MBL deficiency in HIV-1 and schistosoma infections in Zimbabwean adults enrolled in the Mupfema Schistosomiasis and HIV Cohort (MUSH Cohort).

Methods

HIV-1, *S. haematobium* and *S. mansoni* infections were determined at baseline. Plasma MBL concentration was measured by ELISA and MBL2 genotypes determined by PCR. We calculated and compared the proportion of plasma MBL deficiency, MBL2 structural variant alleles B (codon 54A>G), C (codon 57A>G), and D (codon 52T>C) as well as MBL2 promoter variants -550(H/L), -221(X/Y) and +4(P/Q) between HIV-1 and schistosoma co-infection and control groups using Chi Square test.

Results

We assessed 379 adults, 80% females, median age (IQR) 30 (17–41) years. HIV-1, *S. haematobium* and *S. mansoni* prevalence were 26%, 43% and 18% respectively in the MUSH

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baseline survey. Median (IQR) plasma MBL concentration was 800 µg/L (192–1936 µg/L). Prevalence of plasma MBL deficiency was 18% with high frequency of the C (codon 57G>A) mutant allele (20%). There was no significant difference in median plasma MBL levels between HIV negative (912 µg/L) and HIV positive (688 µg/L), $p=0.066$. However, plasma MBL levels at the assay detection limit of 20 µg/L were more frequent among the HIV-1 infected ($p=0.007$). *S. haematobium* and *S. mansoni* infected participants had significantly higher MBL levels than uninfected. All MBL2 variants were not associated with HIV-1 infection but promoter variants LY and LL were significantly associated with *S. haematobium* infection.

Conclusion

Our data indicate high prevalence of MBL deficiency, no evidence of association between MBL deficiency and HIV-1 infection. However, lower plasma MBL levels were protective against both *S. haematobium* and *S. mansoni* infections and MBL2 promoter and variants LY and LL increased susceptibility to *S. haematobium* infection.

Introduction

HIV-1 and schistosomiasis co-infections are very common in Africa and have been reported in several studies [1–6]. Sub-Saharan Africa is the region hardest hit by the HIV/AIDS pandemic where 63% of the 33 million infected people live [7]. HIV infection has remained a major public health challenge since its discovery in 1983 [8]. The HIV pandemic is still ravaging most parts of Southern African countries with current prevalence in these countries between 10–20% [7]. Several reports indicate that individuals worldwide differ in their susceptibility to HIV infection and it is widely agreed that genetic polymorphisms in the host genes important in immune regulation can increase or reduce the risk of HIV infections [9, 10]. An understanding of the immunological factors fuelling the HIV-1 epidemic in Africa is very important in an effort to curb the HIV-1 scourge.

Schistosomiasis is one of the neglected tropical diseases, which the World Health Organization is targeting for elimination [11–13]. An estimated 85% of the world's estimated 200 million people with schistosomiasis live in Sub-Saharan Africa [11, 13]. *S. haematobium* is associated with urogenital schistosomiasis characterised by severe pathological conditions including haematuria and bladder cancer and *S. mansoni* causes intestinal schistosomiasis characterised by chronic or intermittent abdominal pain, bleeding from gastro-oesophageal varices and bloody stool [14]. Schistosomes are complex multicellular helminths with several developmental stages well documented in the human host [14].

Mannose-Binding Lectin (MBL) is a key component of the innate immune system and polymorphism in the MBL2 gene and promoter region lead to MBL deficiency [15–17]. MBL deficiency is associated with impaired function of the innate immune system and leads to increased susceptibility to several infections [18–20]. MBL acts as an opsonin by binding to sugar groups naturally found on the surface of various infectious bacteria, viruses and parasites and activates the complement system [21, 22] through association with MBL-associated serine proteases MASP-1, MASP-2 and MASP-3 [23–25]. Sub-Saharan Africa has a high burden of viral [26–28], bacterial [27–29] and parasitic infections [12, 13, 27]. Assessment of polymorphisms in the MBL2 gene and promoter region to determine functional MBL deficiency has

been carried out in different populations with few studies in Sub-Saharan Africa [17,30,31]. Schistosomes carry sugar molecules or glycoconjugates on the surface of all their developmental stages [32,33] and these glycoconjugates interact with innate immune recognition molecules including MBL [34–36]. In vitro studies have demonstrated complement mediated killing of all stages of the schistosome parasite [35].

Several studies have been conducted worldwide [37–43] including several African populations [17,30,44–48] looking at MBL2 genetic variants and plasma/serum MBL concentration and their association to increased infection susceptibility. The role of MBL deficiency in infection has been shown to be different across different infections [44]. For example MBL deficiency has been shown to increase risk of recurrent respiratory infections [49] and malaria [50] whilst being protective in TB [44], leprosy [51] and leishmaniasis [52,53]. Results on the role of MBL deficiency in HIV infections are conflicting [54,55]. In addition, studies conducted in Sub-Saharan Africa, in Mozambique [17,56,57], South Africa [48,58] and Zimbabwe [31] have been done in children and none among adults. One study reports investigation on MBL deficiency and schistosome infections in Nigeria [59].

In this paper we describe the role of plasma MBL deficiency and MBL2 polymorphism in HIV-1, *S. mansoni* and *S. haematobium* infections in Zimbabwean adults enrolled in the MUSH cohort. The infection rates in Zimbabwe are currently 15% for HIV-1 [60] and 40% and 20% for *Schistosoma haematobium* and *Schistosoma mansoni* respectively [61]. *S. haematobium* is the most common species followed by *S. mansoni* in Zimbabwe [61–64]. In the context of these infections the role of MBL deficiency in these infections in our adult population.

Materials and Methods Study design and study population

This was a sub-study of the Mupfure Schistosomiasis and HIV Cohort (MUSH Cohort) established in 2001 aimed at studying the immunological interactions between HIV-1 and schistosomiasis infections [35,36,38,44,45]. We used plasma and whole blood samples collected at baseline in 2001–2002 to determine prevalence of MBL deficiency in this population. Details of the MUSH Cohort and procedures are described elsewhere [1,2,65]. Briefly, recruitment into the MUSH study, sample collection and laboratory assays took place between October 2001 and November 2007. A total of 1574 adult males and females were screened for HIV and schistosomiasis infections at baseline (Fig. 1) and 379 met the MUSH eligibility criteria: adult males/females above 18 years old, HIV positive or negative, positive for *S. haematobium* or *S. mansoni* or both or uninfected, no active TB infection, non-pregnant women and not currently on schistosomiasis treatment. After screening for HIV-1 and schistosomiasis status, participants were categorised into four groups: HIV-1 only, schistosomiasis only, co-infected with both diseases and controls with none of these two infections.

This sub-study was approved by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/1770) and the University of Witwatersrand Human Research Ethics Committee (M130348). Permission was given by the Provincial Medical Director of Mashonaland Central Province and the District Medical Officer of Shamva District, the village health laboratory procedures. All individuals in the main MUSH study gave written informed consent or specimen collection, storage and future laboratory studies.

HIV testing. Participants were screened for *S. haematobium*, *S. mansoni* and HIV-1 as described earlier [1,65]. Briefly, HIV pre- and post-test counselling was done in the participants'

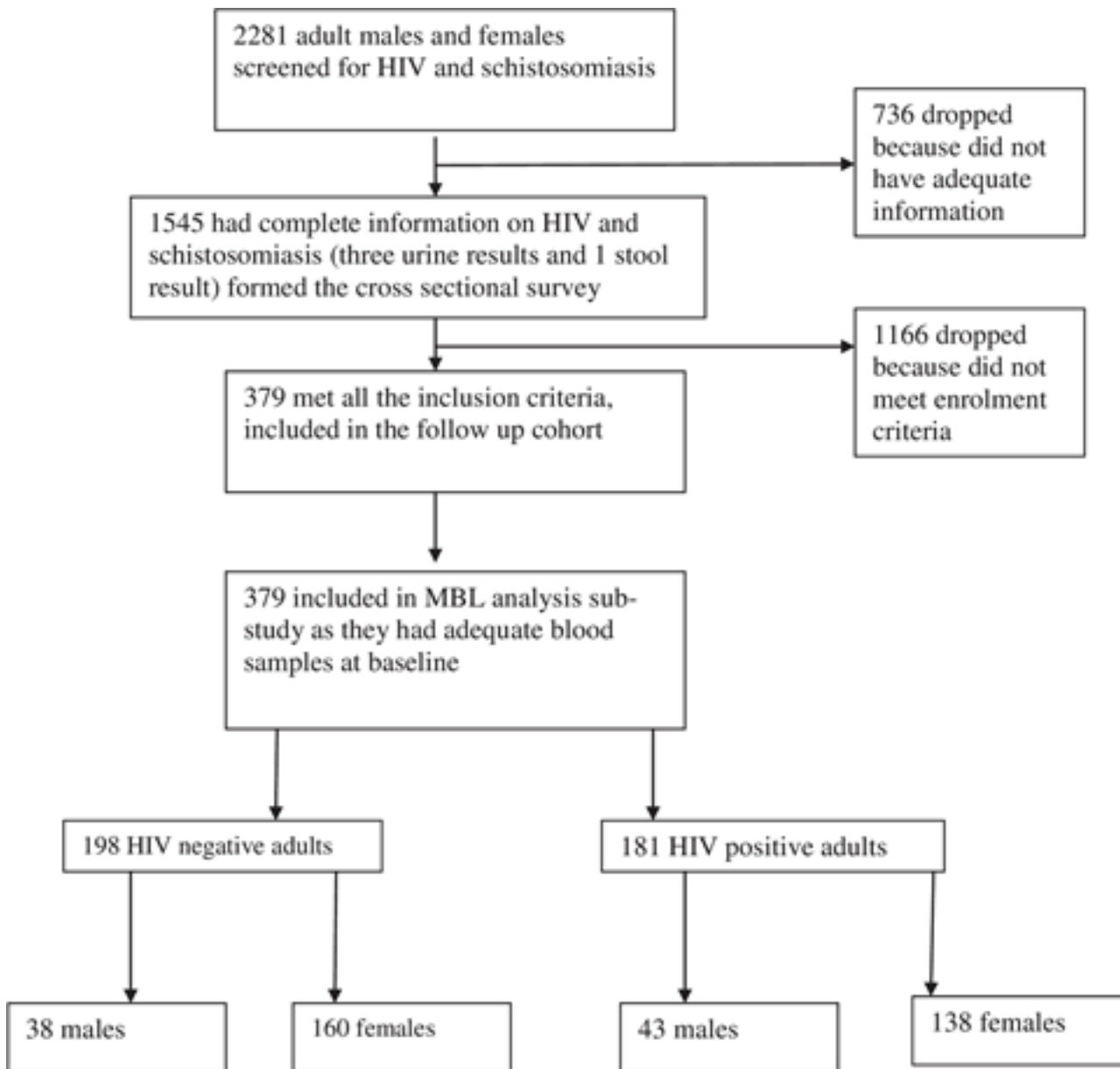


Fig 1. Participant Flow diagram. Flow chart showing the recruitment procedures for the adult cohort. A total of 2281 community dwelling rural adult males and females were screened for HIV and schistosomiasis, 379 males and females who met the inclusion criteria were enrolled and their baseline blood samples used for MBL plasma and MBL2 genotype analysis.

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native language (Shona) by qualified medical personnel. A rapid HIV-1/2 test kit was used on a dry blood spot in the field (Determine, Abbott Laboratories, Tokyo, Japan), followed by two different rapid HIV tests Oraquick (by Orasure) and Serodia (by Fujirebio) for all who tested initially positive. No discrepancies in results were found between the initial Determine HIV test and the subsequent ELISAs. Strict confidentiality was maintained throughout the study. All those who requested their HIV results were given after post-test counselling from the nursing staff.

Schistosoma parasitology. Microscopic examination of fixed-volume urine samples filtered on Nytrel filters (Vester Gaard Frandsen) was used to identify and quantify eggs of *S.*

haematobium by the syringe urine filtration technique [66]. Due to the diurnal and day-to-day variation in egg output, the urine samples were collected on 3 consecutive days [67,68]. The modified formalin-ether concentration technique was used on 1 stool sample from each participant to detect eggs of *S. mansoni* and other helminth parasites [69]. Levels of Circulating Anodic Antigen (CAA) were measured in serum samples using an ELISA assay, to complement parasitological detection methods. CAA originates from the parasite gut and is used as a marker of an active schistosome infection [70].

Determination of plasma MBL concentration. Baseline plasma samples stored at 20°C were used for measurement of plasma MBL concentrations using the double enzyme immunoassay (EIA) (Antibody Shop, Denmark) as described previously [30,71]. Microtitre plates (Maxisorp, NUNC, Denmark) were coated with a mouse anti-human MBL monoclonal antibody, clone 131-1, IgG1, Kappa (Antibody Shop, Denmark). Plasma samples were diluted 1/25 and 1/400 in Tris/HCL buffer (Bie and Bernsten) containing EDTA (Bie and Bernsten) and 0.05% Tween 20 (Sigma) [71]. A pool of EDTA plasma with a known concentration of MBL was used as the standard. Biotinylated clone 131-1 anti-MBL (Antibody Shop, Denmark) was used as the detection antibody. Horseradish peroxidase labelled streptavidin (Amersham, UK) was added. The substrate uses O-phenylenediamine (OPD) (Dako Cytomation) hydrochloride in citrate-phosphate (Bie and Bernsten) buffer pH 5.0, the colour reaction was stopped by addition of sulphuric acid (Sigma). Parallel control plates were coated with the equivalent amount of mouse IgG1 and processed as above. Parallel plates are done in order to reveal binding of rheumatoid factors, anti-mouse immunoglobulins and non-specific binding of MBL interfering in the system. Optical density (OD) was read on an ELISA reader (MR5000/7000, Dynatech Laboratories, Denmark) at 490 nm with reference filter 630 nm. Final MBL values are given as µg MBL per litre. To determine prevalence of MBL deficiency, plasma MBL concentration was categorised into normal (above 500 µg/L), intermediate (100 µg/L–500 µg/L) and deficient (below 100 µg/L) [30,72–74].

MBL genotyping. Genomic deoxyribonucleic acid (DNA) was used for MBL2 genotyping. The DNA was extracted from frozen baseline Peripheral Blood Mononuclear Cells (PBMCs) using the standard salting out procedure [75]. The MBL2 genotypes and promoter regional lesions were detected by allele-specific oligonucleotide PCR (ASO-PCR) where specific sequences were used for each allele [42]. Briefly, the PCR master mix was made by mixing the following: 400 µl sterile water (H/S Apoteket, Rigshospital, Copenhagen), 150 µl of 1.5 mM MgCl₂ (Invitrogen, Denmark), 150 µl of 0.07 mM final concentration of each deoxynucleotide triphosphate (dNTPs) (Amersham Biosciences, UK Limited), 500 µl PCR Buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% (w/v) gelatin p.H. 8.3 (Invitrogen, Denmark), 250 µl glycerol at 5% final concentration, 50 µl cresol red at final concentration 100 µg/µl. Cresol red (Invitrogen, Denmark) was added as a colour marker. The PCR Master mix could be kept at 4°C for 2 weeks.

Then a total of 12 primer solutions (DNA Technology) were prepared for each sample (S1 Table). This set of 12 primers for MBL2 variants and promoter region mutations were prepared by mixing 5' sense and 3' sense primers with water according to the manufacturer's instructions. Final concentration of each primer was 0.25 µM for each specific primer. Primers for 5 and 3 sense exon 4 of the MBL2 gene (DNA Technology, Denmark) were included in each PCR reaction as an internal positive control. PCR primers were pre-aliquoted in 5 µl volumes into the PCR plates and the plates could be kept at 4°C for 1 week.

MBL PCR and sample reaction mixtures were then prepared as 10 µl volumes containing 30 ng genomic DNA and 0.25 µM of the specific primer, 54 µl of PCR master mix, 3.5 µl sample DNA (if the DNA concentration was 0.3 µg/µl), 31 µl sterile water, and 0.25 Unit of Platinum Taq DNA polymerase (Invitrogen, Denmark) in each 90 µl initial reaction mixture volume.

PCR reaction mixture was mixed and added to the 12 wells, clearly marked for each sample, which contained 5 µl of different sets of primers for the genotyping and promoter typing. Only eight samples could be run on each PCR plate. Plates were put in a plate microcentrifuge at 3000 rpm for 1 minute to spin down the well contents. Plates were tightly sealed with foil paper.

DNA was then amplified by PCR in a programmed thermocycler (GeneAmp PCR Systems 9600, PerkinElmer). All PCRs were initiated by a 2-minute denaturation step at 94°C and completed by a 5-minute extension step at 72°C. The temperature cycles for the PCR were as follows: 10 cycles of 10s at 94°C and 60s at 65°C, and a further 20 cycles of 10s at 94°C, 50s at 61°C, 30s at 72°C and 2 minutes at 25°C. Gel electrophoresis was done on the PCR products on the same day or kept at 4°C for a maximum 1 week. The PCR products were separated by a 2% agarose gel (Invitrogen) electrophoresis run in an electrophoresis chamber (Sub-cell Biorad) at 150V, 400mA for 32 minutes. The gels were then carefully transferred to the UV Transilluminator (Syngene Synoptics Ltd, Denmark) where a photograph of the gel was taken and used to determine the MBL2 genotype of each sample. MBL2 genotypes A/O, O/O and XAYO/YOYO were classified as MBL deficient producers and the normal MBL2 denoted A/A. MBL2 single nucleotide polymorphisms (SNPs) in the form of structural variants named B (codon 54, rs1800450 A>G), C (codon 57, rs1800451 A>G) and D (codon 52, rs5030737 T>C), as well as the regulatory promoter region variants H/L (-550, rs11003125 G>C), X/Y (-221, rs7096206 C>G) and PQ (+4, rs7095891 T>C) [76,77] were determined [42]. All the single nucleotide polymorphisms (SNPs) and their reference sequence numbers are found on the online database <http://www.ncbi.nlm.nih.gov/snp/>.

Statistical analysis

All statistical analysis was done using Stata 11 statistical package (STATA Corp, Timberlake Consultants). Normality of plasma MBL levels was checked graphically. Non-parametric tests (Mann-Whitney and Kruskal-Wallis) were used for comparison of median plasma MBL concentrations by age, gender, HIV-1 and schistosoma infection status. Differences in proportions of MBL deficiency between males and females and infection status were done using Chi Square or Fisher's exact tests and confidence intervals were recalculated using binomial exact methods. The frequencies of the MBL2 alleles were obtained by direct gene counting. Conformation to the Hardy-Weinberg Equilibrium was determined using the Chi Square test in the SHEsis online programme [78,79]. For MBL2 genotype analysis, normal homozygous MBL2 was denoted as A/A. The variant genotypes A/B (A>G), A/C (G>A), A/D (C>T) were grouped together as A/O heterozygous MBL2 as they all give low plasma MBL levels and all homozygous and compound homozygotes were grouped together as O/O. The differences in frequency of the MBL2 genotypes, promoter region variants and haplotypes between infection status groups were determined using the Chi Square or Fisher's exact tests.

Results

A total of 379 plasma and whole blood samples were available for MBL analysis. Median age (IQR) of the participants was 30 (17 to 41) years old and the majority were females (80%). Baseline demographic characteristics of this study population and prevalence of HIV-1, *S. haematobium* and *S. mansoni* among the 1545 participants screened during the main MUSH study have been described elsewhere [65].

Table 1. Comparison of median plasma MBL concentrations between groups.

Variable	n	Median MBL levels $\mu\text{g/L}$	IQR $\mu\text{g/L}$	p-value
Sex				
Males	824		159–	
Females	76	800	196–200–	0.561
emales	302		1936	
Age				
5 years old	920		209–	
15 years old	82	776	260–166–	0.202
>25 years old	297		1864	
HIV status				
Positive	688		147–	
Negative	197	912	190–272–	0.065
	181		1968	
Schistosoma infection status				
No infection	6569		139–	
S. haematobium only	89	1210	115–2213–	
S. mansoni only	205	8	219–2297–	0.036
S. haematobium+/ HIV+	24	824	151–	
Coinfected with both species	302	944	216–20251–	
Schistosoma and HIV status				
S. haematobium+/ HIV+	103	707	1176–163–	0.037
S. haematobium-/ HIV+			1152	
S. haematobium positive	44	912	213–	
S. haematobium-/ HIV-	265	656	232–20139–	0.006
S. haematobium negative only	89		1152	
Circulating Anion Gap (CAA)				
Below 40 pg/ml (CAA positive)	293	848	161–2232	
Below 40 pg/ml (CAA negative)	63	784	346–1152	0.308
Eggs per 10 ml urine, S. Haematobium infection				
0 (no infection)	108		145–	
705 < 10 (light infection)	123		126–4230–	
896 10–			211–2182–	
<50 (medium infection)	55	1408	2528	
>50 (heavy infection)	9	295	87–2816	0.109

The concentration of MBL in plasma was measured in $\mu\text{g/L}$. Differences in median plasma MBL concentration in the different infection status categories were analysed by using non-parametric tests Mann-Whitney for two categories and Kruskal-Wallis for three or more categories.

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Prevalence of HIV-1, S. haematobium and S. mansoni in the MBL sub-study

The prevalence of HIV-1, S. haematobium, S. mansoni and co-infection with both schistosoma species among the 379 participants used for the MBL sub-study was 52%, 58%, 7% and 10%, respectively (Table 1).

Plasma MBL levels

Results for plasma MBL concentration are available for 378 individuals; one sample repeatedly gave inconsistent plasma level results and was dropped from this analysis. The median (IQR) plasma MBL concentration in all the investigated individuals was 800 $\mu\text{g/L}$ (192–1936 $\mu\text{g/L}$). There was no difference in the median plasma MBL concentration between males and females ($p=0.553$) and by to age ($p=0.204$) (Table 1).

Association between HIV-1, schistosoma infections and plasma MBL concentration

There was no difference in the median plasma MBL concentration between HIV infected and uninfected individuals ($p=0.065$). However, there were 37 participants with plasma MBL levels at the assay detection limit of $20\mu\text{g/L}$. This level of plasma MBL was found more frequently among HIV-1 infected 14% (27 of 196, 95% CI 8.4–18.2), compared with HIV-1 uninfected 6% (10 of 181, 95% CI 2.7–9.9) ($\chi^2=7.24, p=0.007$), indicating a possible role of MBL deficiency in HIV-1 infection.

There was a significant difference ($p=0.037$) in median plasma MBL levels between the four schistosoma infection status groups, namely, no infection, *S. haematobium* only, *S. mansoni* only and those co-infected with both species. Those with *S. mansoni* infection only had the highest median plasma MBL level of $1016\mu\text{g/L}$ followed by those with *S. haematobium* only median MBL level $912\mu\text{g/L}$, $p=0.037$ (Table 1). There were also significant differences in median plasma MBL levels between the four HIV-1 and *S. haematobium* co-infection groups, with those positive for *S. haematobium* and negative for HIV having the highest median plasma MBL level ($944\mu\text{g/L}$, $p=0.037$, Table 1). Further comparison was done between the *S. haematobium* infected and uninfected individuals after excluding those with *S. mansoni* or co-infected with both species. There was a significant difference in plasma MBL levels, those *S. haematobium* infected having higher median MBL level than the uninfected, $p=0.006$ (Table 1) indicating protective role of low plasma MBL levels. There was no difference in plasma MBL levels when comparison was done between CAA levels groups above or below 40pg/ml ($p=0.381$) and four egg count groups among those with *S. haematobium* infection only ($p=0.114$, Table 1).

Association between HIV-1, schistosoma infections and plasma MBL deficiency

Plasma MBL concentration was categorised into normal (above $500\mu\text{g/L}$), intermediate ($100\mu\text{g/L}$ – $500\mu\text{g/L}$) and deficient (below $100\mu\text{g/L}$) (Table 2) (72, 73). The prevalence of plasma MBL deficiency in all participants analysed was 18% (67 of 378, 95% CI: 14–22%), 20% (77 of 378, 95% CI: 16–24%) had intermediate plasma MBL levels and 62% (234 of 378, 95% CI: 56–66%) had normal plasma MBL levels above $500\mu\text{g/L}$. There was no difference in proportion of males with MBL deficiency 20% (15 of 76, 95% CI: 11–30%) and females with MBL deficiency 17% (52 of 302, 95% CI: 13–22%).

We found no difference in distribution frequency of participants according to HIV ($p=0.070$) and among *S. haematobium* infected and uninfected participants after excluding *S. mansoni* and co-infections ($p=0.446$) between the three plasma MBL levels categories, normal, reduced and deficient (Table 2). There was also no difference when a similar analysis was done among the schistosoma infection status groups ($p=0.351$) and among HIV and *S. haematobium* co-infection groups ($p=0.546$).

MBL2 polymorphism

MBL2 genotyping was successfully done on 366 samples out of 379 and these were used in this analysis, the other samples could not be amplified. All the known MBL2 coding alleles, wild-type A/A, B (codon 54, rs1800450 A>G), C (codon 57, rs1800451 A>G) and D (codon 52, rs5030737 T>C), were found in this study population. In the MBL2 coding region, 233/366 (64%) were classified as A/A genotype, 3 (0.8%) were A/B, 117 (32%) were A/C, 1 (0.3%) A/D and 12 (3%) C/C (Table 3). There were neither B/B, C/D, D/D nor B/D variants. The frequency

Table 2. Distribution of participants between three plasma MBL levels, participants stratified according HIV, S. haematobium and S. mansoni infection and co-infection status.

HIV status	n	Normal MBL levels	Reduced MBL levels	Deficient MBL levels	P value
HIV positive	113	41	43 (22%)		
197 HIV negative	181	(57%) 121	(21%) 36	24 (13%)	0.070
S. haematobium infection		(67%)	(20%)		
S. haematobium positive	131	38 (19%)	36 (17%)		
S. haematobium negative	205	(64%) 51 (5	17 (19%)	21 (24%)	0.446
S. mansoni infection	89	7%)			
S. mansoni positive	51 (57%)	1	17	21 (24%)	
S. mansoni negative	89	31 (64%)	1	36 (18%)	
S. haematobium only	205	5 (63%)	19 (6%)	3 (13%)	
S. mansoni only	205	23 (62%)	5 (12%)	3 (8%)	0.351
S. haematobium and S. mansoni co-infection	24	11 (30%)	11 (30%)		
Co-infected with both species	37	17 (17%)	21 (21%)		
haematobium+HIV+/S.	64 (63%)	8 (18%)	13 (30%)		
haematobium-HIV-/S.	44	1 (20%)	15 (15%)		
haematobium+	23 (52%)	9 (20%)	8 (18%)	0.546	
HIV-/S. haematobium-	103	67 (65%)			
Prevalence of MBL deficiency, plasma MBL concentration was categorised into normal (above 500 µg/L), intermediate (100 µg/L - 500 µg/L) and deficient (below 100 µg/L), analysed by the Chi Square or Fisher's exact tests, n = 378.	45	28 (62%)			

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of MBL2 genetic variants was 36% and 64% had the normal homozygous A/A genotype. The normal homozygous MBL2 A/A genotype was found at frequencies of 64% (233 of 366, 95% CI: 58.5–68.5%), heterozygous MBL2 A/O variants at 33% (121 of 366, 95% CI: 28.3–38.1) and homozygous O/O variants at 3.3% (12 of 366, 95% CI: 1.7–5.7%) (Table 3). All the MBL2 SNPs detected among the HIV negative in this study were in Hardy-Weinberg Equilibrium (structure-allele sp = 0.400, -550H/Lp = 0.820, -221Y/Xp = 0.550 and +4P/Qp = 0.170 (S2 Table).

Frequency of MBL2 genotypes and haplotypes

MBL2 wild-type genotype A/A had the highest frequency of 64% (233 of 366, 95% CI: 77.1–83%) and genotype A/D the lowest frequency of 0.1% (1 of 366, 95% CI: 0.003–0.76, Table 3). All the currently known MBL2 promoter region alleles, -550H, -550L, -221Y, -221X, +4P and +4Q, were detected. Promoter region allele L had the highest frequency 95% (694 of 732, 95% CI: 92.9–96.3%) compared to 5% (38 of 732, 95% CI: 3.69–7.1%) for the H allele (Table 3). The LL MBL2 promoter region genotype (89%) had the highest frequency and HH (0.3%) the lowest frequency. Eleven different haplotypes were detected in these individuals including three rare haplotypes namely LXQA, LXQC and LXPC. Haplotype LYPA had the highest frequency (35%), followed by LYQA and LYPB, LYPC and HYPD the lowest (0.1%) (Table 3). MBL2 genotypes A/A showed the highest levels of plasma MBL concentrations and O/O genotypes had the lowest.

Association between HIV-

1, schistosoma infections and MBL2 genotypes and promoter region genotypes

Analysis was done to determine if MBL2 genetic and promoter region variants were associated with HIV-1 infection. The distribution of the three genotypes (A/A, A/O and O/O) did not differ between the HIV-1 infected and uninfected (p = 0.429, Table 4). The MBL2 haplotypes were further combined and subdivided into three groups namely genotype that given normal

Table 3. Gene, promoter alleles and haplotype frequencies obtained for MBL2 polymorphism amongst the enrolled Zimbabwean participants.

	N	Frequency%
Exon1 allele		
A normal		80
	587	0.4
B(A>G)		20
	3C(0.1
G>A)		
	141	59
D(C>T)		51
	1Pr	88
omoter alleles		
-550H	38-	75
550L		3
	694	
-221X		64
	134	0.8
-221Y		32
	598	0.2
+4P		3
	343	
+4Q		0.31
	389	0.18
MBL2 Exon 1 genotype		
AA wild-type		6.13
	233	0.03
AB(A>G)		.220
	3A	.226
C(G>A)		.553
	117	.3
AD(C>T)		
	1C	35.5
C(C>T)		25.0
	12P	14.6
romoter region genotypes		
-550HH	1-	.15.
550HL	37	64.2
-550LL		0.30
	328	.30.
-221YY		1
	242	0.1
-221XY		
	112	
MBL2 gene and allele frequencies obtained by direct gene counting, frequencies expressed as percentages.		
	12+	
	40P	
	10.1371/journal.pone.0122659.t003	
	74+	
4QQ		
	97+	
4PQ		
	195	
MBL2 Haplotypes		
1.MBL2*LYPA		
	260	
2.MBL2*LYQA		
	183	

Table 4. Distribution of participants between MBL2 genotypes and promoter region haplotypes, participants stratified according to HIV-1 infection status.

MBL2 genotypes/haplotypes	n	HIV negative	HIV positive	P value
A/A	233		116(50%)	
A/O		117(50%)	66(55%)	
O/O	121	55	8 (67%)	
YAYA/YAXAXA/YA	(45%)12	4		0.429
YOXAYO/YOYOProm	(33%)223		110(49%)	
Other genotypes-		113(51%)	82(53%)	0.347
550HH	131			
-	1	59(45%)1	0(0%)15	
550HL-	2	4(30%)3	(41%)	
550LL-	7		175(53%)	
550HY-		22(59%)3		0.141
550LY-	28		12(39%)	
221LX-		153(47%)	67(53%)	
221YY-	31			0.229
221XY-		19(61%)3	122(50%)	
221XX-	26		6(34%)	
550HY		154(47%)		0.521
-	9		15(39%)	
221LY*LX+		3(33%)24	175(53%)	
4C/C(PP)+	2			0.105
4TT(QQ)		120(50%)	97(59%)	0.545
+4C/TPQ	112		42(43%)	

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This analysis was done using the Chi Square and Fisher's exact tests, n = 366

plasma MBL levels (YA/YA, YA/XA), intermediate levels (XA/XA, YA/YO) and deficient levels (XA/YO, YO/YO) (20, 77). There was no difference in distribution frequency when comparison was done among the HIV-1 stratified and the three MBL2 genotype groups YAYA/YAXA, XAXA/YAYO and XAYO/YOYO (p=0.347, Table 4). None of these MBL2 genotypes and promoter variants were associated with HIV-1 infection (Table 4).

We found no association between MBL2 genotypes and P/Q promoter genotypes and schistosoma infections (Table 5). However, there was a significant association between *S. haematobium* and *S. mansoni* infections and LL (p=0.051) and LY-LX (p=0.026) promoter genotypes. Therefore promoter region genotypes LL, LY and LX which encode for low plasma MBL levels were significantly associated with *S. haematobium* and *S. mansoni* infections.

Analysis among *S. haematobium* infected and uninfected after excluding those with *S. mansoni* and co-infection with both species showed a significant difference in distribution frequency of the LY promoter region variant, participants with LY promoter genotype being more susceptible to *S. haematobium* infection (p=0.048, Table 6).

Analysis among HIV-1 and *S. haematobium* co-infections, after excluding *S. mansoni* infections and schistosoma co-infections showed no difference in distribution frequency of MBL genotypes and promoter genotypes between the four co-infection status groups (Table 7). MBL2 genotypes and promoter region genotypes were not associated with HIV-1 and *S. haematobium* co-infections.

Table 5. Distribution of participants between MBL2 genotypes and promoter region haplotypes according to schistosoma infection status.

MBL2 Genotype/Haplotype	N	No infection (controls)	S. haematobium only	S. mansoni only	Coinfected with both species	P value
A/A	216	51 (24%)	128 (59%)	15 (7%)	22 (10%)	0.893
A/O	117	33 (28%)	62 (53%)	9 (8%)	13 (11%)	
O/O	11	4 (36%)	5 (45%)	0 (0%)	2 (18%)	
O/O	11	5 (28%)	6 (55%)	0 (0%)	22 (11%)	0.903
AYA/YAXA	208	0 (0%)	1 (100%)	0 (0%)	0 (0%)	
XAXA/YAYO	125	44 (35%)	8 (53%)	0 (0%)	1 (3%)	
XAYO/YOYO	11	50 (45%)	7 (57%)	0 (0%)	35 (11%)	0.051
Promoter region genotypes	11	24 (22%)	58 (53%)	7 (6%)	1 (4%)	
-550HH	1	9 (9%)	4 (4%)	6 (6%)	9 (9%)	
550HL	34	0 (0%)	6 (60%)	1 (10%)	27 (12%)	0.072
-550LL	309	15 (4%)	19 (54%)	0 (0%)	8 (8%)	
-550HY	28	16 (57%)	3 (62%)	3 (4%)	1 (3%)	
221LY	307	17 (23%)	103 (55%)	8 (9%)	35 (11%)	0.026
-221LX	9	55 (29%)	13 (7%)	7 (9%)	13 (15%)	
-221YY	228					

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106
-221XX 10-
550HY 35-
221LY*LX 309
+4PP 69+
4QQ 88
+4PQ 187

Discussion

This study showed that plasma MBL deficiency, all MBL genetic and promoter region variants detected were not associated with HIV-1 infection in this population, however participants with plasma MBL levels at the assay detection limit were significantly more frequent among the HIV-1 infected. Higher plasma MBL levels, LY and LL promoter genotypes were associated with increased susceptibility to both S. haematobium and S. mansoni infections. To our knowledge, this is the first study that has investigated the role of MBL deficiency in HIV-1 and schistosoma infections and second study that assessed the role of MBL deficiency in schistosoma infections [59]. Our results also confirm previously reported association of MBL polymorphism with MBL levels [15–17].

In view of available literature, we hypothesized that plasma MBL deficiency due to single locus substitutions in MBL2 resulting in varying levels of circulating MBL would have an effect on susceptibility to HIV-1 and schistosoma infections in this study population. The results of our study showed no difference in plasma MBL concentration between the HIV positive and HIV negative individuals, consistent with other reports [80–82]. In contrast, some have reported protective effect of normal plasma MBL levels and increased susceptibility due to low MBL levels [30, 37, 83, 84], but some reported a deleterious effect of normal plasma MBL levels where high MBL levels were associated with acquiring HIV infection [39].

In addition, we investigated the role of plasma MBL deficiency in adult singly infected or co-infected with HIV-1, S. haematobium and S. mansoni and uninfected controls. Our results showed a significant difference in plasma MBL levels with those S. mansoni and S. haematobium positive having significantly higher MBL levels than the uninfected participants. When

Table 6. Distribution of participants between MBL2 genotypes and promoter region haplotypes according to schistosoma infection status.

MBL2 genotype/haplotype	n	S. haematobium negative	S. haematobium positive	P value
A/A	179	51(28%)	128(72%)	0.466
A/O		33(35%)	62(65%)	
A/O	95	4(40%)	6(60%)	
O/O		9(28%)	3	
O/O	10Y	5(34%)	4	
AYAYAXA		(40%)	67(68%)	
AYAYAXA	172	0(0%)	15	
XAXA/YAYO		(45%)	73	
XAXA/YAYO	102	(29%)	14	
XAYO/YOYO		(52%)	72	
XAYO/YOYO	10P	(29%)	2	
Promoter genotypes		29%	65	
-550HH	1-	35%	21	0.048
550HL	33	24%	2	
-550LL	250	5%	15	
-550LL		4%	73	
-550HY	27-	9%	16	0.161
221LY	7%	17		
-221LX	250	5%		0.078
-221LX	7	55(35%)	40(65%)	
-221YY			50(75%)	0.289

This analysis was done using the Chi Square or Fisher's exact tests, n = 284

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221XX 89-

221XX 8

-550HY 34-

221LY*LX 250-

+4PP 59-

4QQ 67

+4PQ 158

analysis was done among the S. haematobium infected and uninfected, after excluding those infected with S. mansoni and those co-infected with both schistosoma species, the S. haematobium infected participants had significantly higher plasma MBL levels. The clinical relevance of our findings is that higher plasma MBL levels led to increased susceptibility to schistosome infections and lower levels were protective. Our results are in contrast to the only similar report available [59] which found lower MBL levels in the infected participants and higher MBL levels were protective against S. haematobium infection in a Nigerian population [59]. The Nigerian study had a sample size of 346 almost similar to our study with 379 participants. The reasons for these contrasting findings on role of plasma levels in schistosomiasis are not clear but may be due to population differences. There have been reports on the possible advantage of evolution of the MBL2 variants and the high prevalence of MBL2 variants resulting in reduced plasma MBL concentrations on the African continent [44,85]. We can only postulate that selection pressure favouring MBL2 variants and reduced plasma levels that occurred in African populations offers protection against the numerous intracellular infections present in this population at large. Thus in our study population we found a high prevalence of plasma MBL deficiency at 18% and the reduced plasma MBL levels were found to be protective against schistosome infections.

Our findings of high plasma MBL deficiency are consistent with findings from other African populations [16,17,30]. Population surveys have shown that the concentration of MBL in plasma/serum ranges from <20 µg/L to 10000 µg/L [42] and plasma/serum MBL concentration less than 100 µg/L is considered deficient [72,73]. The plasma MBL level median of 800 µg/L and the wider range of 20–7600 µg/L for our study population are consistent with studies in African

Table 7. Distribution of participants between MBL2 promoter region haplotypes according to HIV and S. haematobium co-infection status.

MMBL2 genotype/haplotype	N	HIV-/S. haematobium-	HIV+/S. haematobium+	HIV-/S. haematobium+	HIV+/S. haematobium-	Phae value
A/A	179		64(36%)	64(36%)	24(13%)	
A/O	27(15%)	95	30(32%)	32(34%)	17(18%)	
O/O		16(17%)	3(30%)	3(30%)	3(30%)	
YAYA/YAXAXAXA/YA	10		1(36%)	2(36%)	3(36%)	0.802
YOXAYO/YOYO.Pro		1(10%)	3(32%)	4(33%)	23(13%)	
moter genotypes-	72		(30%)	(30%)	3(30%)	0.830
550HH	26(15%)	102				
-	17(17%)	100	0(0%)	1(100%)	0(0%)	
550HL-		8(20%)	21(90%)	11(33%)	(21%)	
550LL-	50		36(51%)	87(35%)	37(15%)	
550HY-	36(14%)	27	9(89%)	8(30%)		0.384
221LY-		8(30%)	6(43%)	9(36%)	6(22%)	
221LX-	50	35(14%)	7(59%)	32(29%)	64(37%)	
221YY-		1(14%)	1(34%)	3(38%)		0.295
221XY-	87		4(50%)	2(37%)	32(17%)	
221XX-	32(17%)	89	7(21%)	25(25%)	12(11%)	
550HY		11(12%)	9(90%)	35(35%)	87(37%)	0.729
-	8		2(20%)	35(35%)	23(21%)	
221LY*LX+		1(13%)	3(26%)	39(39%)	24(37%)	
4PP+4QQ	4			36%		0.227
+4PQ		8(24%)	2(51%)	52(33%)	2(56%)	0.685
This analysis was done using the Chi-Square or Fisher's exact tests, n = 284	50				10(15%)	
	36(14%)	59				

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7(12%)

7(10%)

158(19%)

populations [17,30]. Plasma MBL concentration did not differ between males and females nor by age as reported by others [30].

When analysis was done comparing the proportions of those with plasma MBL concentration at the assay detection limit, a strong association with being HIV positive was detected, supporting our hypothesis of MBL consumption and reduction during HIV infection, consistent with the findings [82]. The possible explanation for the association between this severe MBL deficiency and being HIV positive in our study could be that the low MBL levels may indicate that there is consumption of the MBL protein molecule when it engages in opsonic clearance of the HIV virus, leading to MBL consumption [82] and not accumulation as proposed by others [39,86]. Available evidence shows that MBL assist in HIV clearance through activation of the complement system and MBL bound to HIV can be cleared from the circulation by the C1q receptor, a molecule that has been shown to have high affinity for MBL [87,88] and MBL binds to and neutralises HIV in vitro [89]. However, other recent studies have conflictingly shown that MBL levels remain relatively stable during the course of HIV infection and does not support the theory of MBL consumption during HIV infection [82]. We also noted differences in MBL2 levels between CD4 HIV categories, CD4+ T cell count and viral load in our study, similar to findings by others [39], in contrast to other findings [39].

Plasma MBL concentration was categorised into normal (above 500 µg/L), intermediate (100 µg/L-500 µg/L) and deficient (below 100 µg/L) [72,73]. Our results showed no association between this categorisation of plasma MBL level, HIV-1 and schistosoma infections. Our results are in contrast to a report that showed increased susceptibility to HIV infection in people

with MBL deficiency [30]. We could not find any similar studies comparing these MBL categories with schistosoma infections.

Assessment of MBL2 polymorphisms showed a high prevalence MBL2 deficient genotypes A/O and O/O at 18%, due to high frequency of the C (G>A) variant allele (20%) and high frequencies of variant promoter alleles X and L. All the currently known MBL2 alleles, wild-type A, B (A>G), C (A>G), and D (C>T) and promoter region alleles -550H, -221Y, -550L, -221X, +4P and +4Q, were found in this population. Presence of a high frequency of the variant C MBL2 allele in our population is consistent with findings from other studies on Africans with frequencies as high as 24% in Mozambicans [17], 27% in Gambian adults [45] and 15–38% in several East and West African countries [44,45]. The MBL genotypes A/A, A/O and O/O in our study correlated with high, intermediate and deficient plasma MBL levels confirming results on other African populations [17,30]. Promoter region variants have been reported to significantly affect MBL plasma/serum concentration [16], also confirmed by our results. The H, Y, L, Y and LX promoters are associated with high, medium and low MBL expression respectively [17]. In our study, the L allele (94%) had the highest frequency and H allele (5%) had the lowest frequency, consistent with other reports on African populations [16].

We found no association between presence of MBL2 structural variants and promoter variants with HIV infection, in accordance with other findings [38,90], in contrast to reports of significant associations [37,83,84,91]. Similar results to ours of no association between MBL2 polymorphism and HIV infection, reported in a Colombian population, were explained as being due to existence of redundant constitutive acquired immune defence systems that can complement or take up the innate defence functions provided by MBL, which are likely favoured under conditions of high pathogen exposure [38]. It is indeed true that polyparasitism is common in most rural populations in Zimbabwe [61].

There were no differences in distribution of MBL2 promoter region alleles and variants between the HIV positive and HIV infected participants and no role of MBL promoter region variants in increasing susceptibility to HIV infection, in accord with other reports [92,93]. In contrast promoter region variants LX/LX have been reported to have higher frequency among HIV-infected adults than controls [94]. The H, Y, L, Y and LX promoters are associated with high, medium and low MBL2 expression respectively [16]. All the currently known MBL2 promoter region variants H, L, Y, X, P and Q were detected in our study, the L allele (47%) had the highest frequency and H allele (3%) had the lowest frequency, in accord with a report on African populations [16]. The H allele is found predominantly in white populations who also have very low L and X alleles [16]. Our results of no association between presence of MBL2 promoter region variants and HIV infection are in accord with other reports in adults [93] but in contrast and children with promoter type L were protected from HIV rapid progression [95].

The reason for the differences in plasma MBL levels, MBL2 genotypes and HIV association outcomes is not clear [54,55,96] but this may be because studies have been carried out in different populations, homosexual groups [37] and some in heterosexuals [30,38,91] and also the methods employed in these studies are different with some measuring plasma MBL level only [30,39,81,82,97,98], others assessing structural alleles only [38,91,99], some looked at both [37,80,83,84,90] others in addition looked at MBL2 promoter region alleles [92,100]. Our study investigated all three MBL parameters, plasma MBL levels, polymorphism in the MBL2 exon 1 gene and promoter region variants. Therefore the conflicting reports on association of HIV infection with MBL levels and genetic polymorphism may be due to differences in HIV transmission route, sample size, ethnicity, environmental conditions and study design as postulated by others [38].

MBL2 genetic variants A/O and O/O and the other promoter variants were not associated with schistosoma infections in our study similar to findings in a Nigerian population [59].

However our findings showed that the promoter genotypes L/Y and LL which code for low plasma levels, both showed significant association with *S. haematobium* infection, similar to findings in the Nigerian. In contrast to our findings of no association the promoter genotypes H/L and P/P were reported to be protective and P/Q carriers showed increased susceptibility [59].

Participants were further stratified into six MBL2 haplotype groups which were combined into three groups for analysis namely, haplotypes that give normal plasma MBL levels (YA/YA, YA/XA), intermediate levels (XA/XA, YA/YO) and deficient levels (XA/YO, YO/YO) [20,77]. None of the three MBL2 haplotype groups were associated with HIV-1 nor schistosoma infections in contrast to a report of increased susceptibility where the XA/XA promoter variant had a detrimental effect in HIV vertical transmission [100]. Our results showed no association between HIV-1 and schistosoma infections and these seven MBL2 haplotypes detected in this study, in contrast to findings of significant association between MBL2-HYPA and *S. haematobium* infection, those with MBL2-HYPA haplotype were reported to be at a lower risk for *S. haematobium* infection, presence of MBL2-HYPA was protective [59].

MBL deficiency has been reported in several studies to be strongly associated with increased susceptibility to several other infections like respiratory infections and recurrent infections in adults and children, infections that are highly prevalent in Zimbabwe. Available literature therefore shows evidence of low MBL levels to be therefore protective in some diseases but increases susceptibility in some infections. This varying evidence shows that the clinical significance of the MBL2 variants may possibly depend on the population investigated and the type of disease. We found plasma MBL deficiency and MBL2 gene and promoter region variants to play no role in HIV-1 infection, but high plasma MBL levels and the heterozygous promoter genotype LY and homozygous LL increased susceptibility to schistosoma infections.

The main limitation of our study was inclusion of few women in the main MUSH study. There is a gold panning area in the neighbouring district, most men work away from their rural homes and were not available during our study recruitment phase. This has the potential to bias our findings but every effort was done to enrol all eligible men into the main MUSH study and MBL sub-study.

In conclusion, this study showed a high prevalence of plasma MBL deficiency, high frequency of MBL2 genetic variant C (G>A). These findings are consistent with the observation that the C (G>A) variant allele is the predominant variant allele in Sub-Saharan Africa. We found no evidence of an association between MBL deficiency and HIV-1 infection, however plasma MBL levels at assay detection limit were associated with HIV-1 infection indicating a possible role of MBL deficiency in HIV infection. Lower plasma MBL levels were protective against both *S. haematobium* and *S. mansoni* infections and higher plasma MBL levels associated with increased susceptibility to schistosoma infections. All the other MBL gene and promoter region variants detected played no role in both HIV and schistosoma infections but presence of promoter region variants LY and LL increased susceptibility to both *S. haematobium* and *S. mansoni* infections. The available evidence on the association of polymorphism in the MBL2 promoter region with HIV-1 infection is still conflicting. In light of all this conflicting evidence it would be difficult to recommend the use of MBL plasma levels, MBL2 structural variants and promoter region mutations as a biomarker of HIV infection and for the monitoring of ARV therapy together with viral load and CD4⁺ T cell lymphocyte counts in the population represented by our study participants. Our results on role of plasma MBL levels in schistosoma infections are in conflict with the only available report that found lower plasma MBL levels to increase susceptibility to schistosoma infections. There is therefore, need to carry out a much bigger study to verify the results. In addition, future immunological studies looking at the association between MBL deficiency and other diseases are highly recommended for the

Zimbabwean population where persistent recurrent bacterial, viral, fungal and other parasitic infections remain prevalent affecting both adults and children.

Supporting Information

S1 Dataset. MSEXcel format.xls. Dataset showing study variables HIV-1, schistosoma, plasma MBL and MBL2 genotyping results.
(XLSX)

S1 Fig. Box Plot showing differences in median plasma MBL levels according to MBL2 genotype. MBL2 genotypes were categorized into three functional groups A/A, A/O and O/O. There was a statistically significant difference in median plasma MBL concentration between the 3 genotype groups, with the A/A genotype having the highest median plasma MBL concentration and O/O the lowest (AA: 1552 µg/L, A/O: 139 µg/L, O/O: 20 µg/L, $p < 0.0001$). Differences in median MBL concentration by MBL2 genotype were analysed by Kruskal-Wallis non-parametric tests ($n = 366$). IQR = Interquartile range.
(TIFF)

S2 Fig. Box Plot showing comparison of median plasma MBL levels according to these seven main MBL2 haplotypes found in this study. HYP A haplotypes showed the highest median plasma MBL concentration (median MBL 2464 µg/L, IQR 1336–3368 µg/L) and LYQC haplotype had the lowest levels, at assay detection limit of 20 µg/L. We found varying median MBL concentrations due to the effect of HY, LY and LX promoters.
(TIFF)

S3 Fig. Box Plot showing MBL2 genotypes and plasma MBL concentration. The MBL2 genotypes were further combined and subdivided into three groups namely genotype that given normal plasma MBL levels (YA/YA, YA/XA), intermediate levels (XA/XA, YA/YO) and deficient levels (XA/YO, YO/YO) (20, 77). There was a statistically significant difference in median plasma MBL concentration between the 3 haplotype groups, with the (YA/YA, YA/XA) haplotypes showing the highest median plasma MBL concentration 1568 µg/L, followed by (XA/XA, YA/YO) (151 µg/L) and (XA/YO, YO/YO) (20 µg/L) showing deficient levels ($p < 0.0001$). (TIFF)

S1 Table. The 12 oligonucleotide primer sequences and the Exon 1 internal control primers, that were used in this study for detection of MBL2 coding and promoter normal and variant alleles. These primer sequences and specifications were for identification of the MBL2 and promoter region types, according to manufacturer's instructions (DNA Technology, Denmark).
(DOCX)

S2 Table. MBL2 genotypes and promoter SNPs and HWE. The HWE for the above MBL2 SNPs was determined among the HIV negative participants.
(DOCX)

S3 Table. Detailed summary of the MBL2 genotypes, haplotypes and corresponding plasma MBL concentrations ($n = 366$). Gene and allele frequencies were obtained by direct gene counting. The three main MBL2 genotype groups AA, AO and OO were further subdivided according to the MBL2 gene and haplotype combinations detected. Twenty-four different complete MBL2 genotypes were detected as shown above. As expected, the HYP A/HYP A genotype which codes for the homozygous normal A/AMBL2 genotype, had the highest median plasma MBL concentration (median MBL 2464 µg/L, IQR 1336–3368 µg/L) and LYQC/LYQC had the

lowest levels, (MBL 20 µg/L). HYP A haplotypes showed the highest median plasma MBL concentration (median MBL 2464 µg/L, IQR 1336–3368 µg/L) and LYQ C haplotype had the lowest levels (MBL 20 µg/L). We found varying median MBL concentrations due to the effect of HY, LY and LX promoters. (DOCX)

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Author Contributions

Conceived and designed the experiments: RBLZGHOMPKEGHUSMENKPGCETM. Performed the experiments: RBLZGPGHOM. Analyzed the data: RBLZGPGHOMCCSRTM. Contributed reagents/materials/analysis tools: RBLZGHOMPKEGHUSMSRENKPGCEBSPTM. Wrote the paper: RBLZGPGCCSRMCTM.

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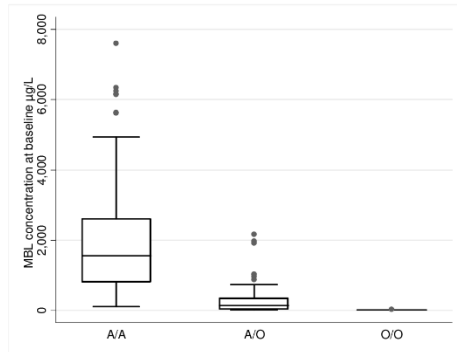
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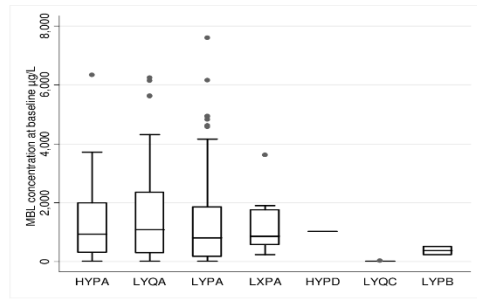
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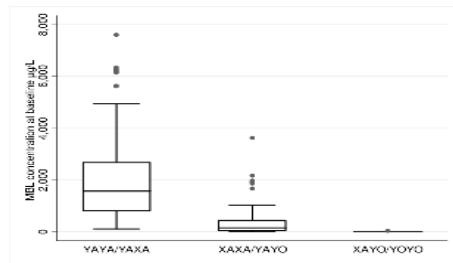


S1 Fig. Box Plot showing differences in median plasma MBL levels according to *MBL2* genotype.

MBL2 genotypes were categorized into three functional groups A/A, A/O and O/O. There was a statistically significant difference in median plasma MBL concentration between the 3 genotype groups, with the A/A genotype having the highest median plasma MBL concentration and O/O the lowest (AA: 1552µg/L, A/O: 139µg/L, O/O: 20µg/L, $p < 0.0001$). Differences in median MBL concentration by *MBL2* genotype were analysed by Kruskal-Wallis non-parametric tests ($n=366$). IQR = Interquartile range.



S2 Fig. Box Plot showing comparison of median plasma MBL levels according the seven main *MBL2* haplotypes found in this study. HYP A haplotype showed the highest median plasma MBL concentration (median MBL 2464µg/L, IQR 1336-3368µg/L) and LYQ C haplotype had the lowest levels, at assay detection limit of 20µg/L. We found varying median MBL concentrations due to the effect of HY, LY and LX promoters.



S3 Fig. Box Plot showing *MBL2* genotypes and plasma MBL concentration.

The *MBL2* genotypes were further combined and subdivided into three groups namely genotypes that give normal plasma MBL levels (YA/YA, YA/XA), intermediate levels (XA/XA, YA/YO) and deficient levels (XA/YO, YO/YO) (20, 77). There was a statistically significant difference in median plasma MBL concentration between the 3 haplotype groups, with the (YA/YA, YA/XA) haplotypes showing the highest median plasma MBL concentration 1568µg/L, followed by (XA/XA, YA/YO) (151µg/L) and (XA/YO, YO/YO) (20µg/L) showing deficient levels (p<0.0001).

Table S1. The 12 oligonucleotide primer sequences and the Exon 1 internal control primers, that were used in this study for detection of *MBL2* coding and promoter normal and variant alleles.

	primer	Specific sequence	Product Size (bp)
1	A54	5'-AGTCGACCCAGATTGTAGGACAGAG-3'	278
	none B	3'-CCTTTTCTCCCTTGGTGC-5'	
2	B	5'-GGGCTGGCAAGACA ACTATTA-3' 3'-GCAAAGATGGGCGTGATGA-5'	278
3	A57	5'-AGTCGACCCAGATTGTAGGACAGAG-3'	290
	None C	3'-CCTGGTTCCCCCTTTTCTC-5'	
4	C	5'-AGTCGACCCAGATTGTAGGACAGAG-3' 3'-ACCTGGTTCCCCCTTTTCTT-5'	290
5	A52	5'-AGTCGACCCAGATTGTAGGACAGAG-3'	268
	none D	3'-TCCCTTGGTGCCATCACG-5'	
6	D	5'-AGTCGACCCAGATTGTAGGACAGAG-3' 3'-CTCCCTTGGTGCCATCACA-5'	268
7	Promoter	5'-CTCAGGGAAGGTTAATCTCAG-3'	440
	X	3'-CATTGTCTCACTGCCACC-5'	
8	Promoter	5'-CTCAGGGAAGGTTAATCTCAG-3'	443
	Y	3'-CATTGTCTCACTGCCACG-5'	

9	Promoter	5'-GGCTTAGACCTATGGGGCTA-3'	
	H	3'-GCTTCCCCTTGGTGTTTTAC-5'	316
10	Promoter	5'-GGCTTAGACCTATGGGGCTA-3'	
	L	3'-GCTTCCCCTTGGTGTTTTAG-5'	316
11	P	5'-AGGATCCAGGCAGTTTCCTCTGGAAGG-3'	
		3'-TAGGACAGAGGGCATGCTC-5'	335
12	Q	5'-AGGATCCAGGCAGTTTCCTCTGGAAGG-3'	
		3'-TAGGACAGAGGGCATGCTT-5'	331
<hr/>			
	Exon 1	5'-GAGTTTCACCCACTTTTTCACA-3'	
	internal control	3'-GCCTGAGTGATATGACCCTCC-5'	431

These primer sequences and specifications were for identification of the *MBL2* and promoter region types, according to manufacturer's instructions (DNA Technology, Denmark).

Table S3.Detailed summary of the *MBL2* genotypes, haplotypes and corresponding plasma MBL concentrations (n=366)

Structural genotype	Promoter	Complete genotype	(n)	MBLconcentration median $\mu\text{g/L}$	MBL concentration IQR ($\mu\text{g/L}$)
AA	HY/HY	HYP A/HYP A	4	2464	1336-3368
		LYP A/LYP A	16	1149	672-2280
	LY/LY	LYP A/LYP A	16	1149	672-2280
		LYP A/LYP B	1	-	225
		LYP A/LYP C	1	-	512
	LYQA/LYQA	LYP A/LYP A	16	1149	672-2280
		LYP A/LYP B	1	-	225
		LYP A/LYP C	1	-	512
	LYPA/LYQA	LYP A/LYP A	16	1149	672-2280
		LYP A/LYP B	1	-	225
		LYP A/LYP C	1	-	512
	LX/LX	LXP A/LXP A	12	856	568-1760
		LXP A/LXP B	1	-	286
LXP A/LXP C		1	-	1024	
HY/LY	HYP A/LYP A	3	656	136-2080	
	HYP A/LYP B	1	-	225	
	HYP A/LYP C	1	-	512	
HYPA/LYQA	HYP A/LYP A	3	656	136-2080	
	HYP A/LYP B	1	-	225	
	HYP A/LYP C	1	-	512	
HYPA/LXPA	HYP A/LXP A	5	1680	720-2400	
	HYP A/LXP B	1	-	286	
	HYP A/LXP C	1	-	1024	
LY/LX	LYP A/LXP A	27	1040	688-2000	
	LYP A/LXP B	1	-	286	
	LYP A/LXP C	1	-	1024	
LYPA/LXQA	LYP A/LXP A	27	1040	688-2000	
	LYP A/LXP B	1	-	286	
	LYP A/LXP C	1	-	1024	
LYQA/LXQA	LYP A/LXP A	27	1040	688-2000	
	LYP A/LXP B	1	-	286	
	LYP A/LXP C	1	-	1024	
A/O	HY	HYP D/LYP A	1	-	1024
		HYP A/LYP C	10	244	20-352
		HYP A/LXP C	1	-	192
	LY	LYP A/LYP B	1	-	512
		LYP A/LYP C	1	-	225
		LYP A/LYP C	48	150	24-416
		LYP A/LYP C	1	-	202

		LYQA/LYQC	26	188	105-528
	LX	LYP A/LXPC	2	162	62-263
		LYP A/LXQC	27	27	20-83
		LYQA/LXQC	3	128	20-1920
O/O		LYQC/LYQC	12	20	20-20
Haplotypes					
HYP A	HY		36	1024	310-1992
HYPD	HY		1	-	1024
LYQA	LY		85	1056	346-2320
LYP A	LY		217	816	163-1936
LXP A	LX		12	856	568-1760
LYPB	LY		2	368	225-512

Gene and allele frequencies were obtained by direct gene counting. The three main *MBL2* genotype groups AA, AO and OO were further subdivided according to the *MBL2* gene and haplotype combinations detected. Twenty-four different complete *MBL2* genotypes were detected as shown above. As expected, the HYP A/HYP A genotype which codes for the homozygous normal A/A *MBL2* genotype, had the highest median plasma MBL concentration (median MBL 2464 μ g/L, IQR 1336-3368 μ g/L) and LYQC/LYQC had the lowest levels, (MB220 μ g/L). HYP A haplotype showed the highest median plasma MBL concentration (median MBL 2464 μ g/L, IQR 1336-3368 μ g/L) and LYQC haplotype had the lowest levels (MBL 20 μ g/L). We found varying median MBL concentrations due to the effect of HY, LY and LX promoters.

Table S2.*MBL2* genotypes and promoter SNPs and HWE

<i>MBL2</i> variant	Reference	Alleles	Alternative	Location	HWE
	SNP ID		nomenclature		controls P
	number				
	(rs#)				
-550	11003125	G/C	H/L	promoter	0.830
-221	7096206	C/G	X/Y	promoter	0.550
+4	7095891	A/G	P/Q	Exon 1	0.170
Exon 1					
B(codon 54)	1800450	A/G	A/B	Exon 1	0.400
C(codon 57)	1800451	G/A	A/C		
D(codon 52)	5030737	C/T	A/D		

The HWE for the above *MBL2* SNPs was determined among the HIV negative participants.

Table S3.Detailed summary of the *MBL2* genotypes, haplotypes and corresponding plasma MBL concentrations (n=366)

Structural genotype	Promoter	Complete genotype	(n)	MBLconcentration median µg/L	MBL concentration IQR (µg/L)
AA	HY/HY	HYP A/HYP A	4	2464	1336-3368
		LYP A/LYP A	16	1149	672-2280
	LY/LY	LYP A/LYP A	47	2048	1344-2960
		LYP A/LYP A	59	1856	992-3024
		LXP A/LXP A	12	856	568-1760
		HYP A/LYP A	3	656	136-2080
		HYP A/LYP A	13	1520	1088-1888
		HYP A/LXP A	5	1680	720-2400
	LY/LX	LYP A/LXP A	27	1040	688-2000
		LYP A/LXP A	42	1016	752-1904
LYP A/LXP A		1	-	286	
LYP A/LXP A		1	-	1024	
A/O	HY	HYP D/LYP A	1	-	1024
		HYP A/LYP C	10	244	20-352
		HYP A/LXP C	1	-	192
	LY	LYP A/LYP B	1	-	512
		LYP A/LYP B	1	-	225
		LYP A/LYP C	48	150	24-416
		LYP A/LYP C	1	-	202
	LYQA	LYP A/LYP C	26	188	105-528
		LXP A/LXP C	2	162	62-263
		LXP A/LXP C	27	27	20-83
LYQA	LXP A/LXP C	3	128	20-1920	
	LXP A/LXP C	12	20	20-20	
O/O		LYP C/LYP C	12	20	20-20
Haplotypes					
HYP A	HY		36	1024	310-1992
HYP D	HY		1	-	1024
LYP A	LY		85	1056	346-2320
LXP A	LX		217	816	163-1936
LXP A	LX		12	856	568-1760

Gene and allele frequencies were obtained by direct gene counting. The three main *MBL2* genotype groups AA, AO and OO were further subdivided according to the *MBL2* gene and haplotype combinations detected. Twenty-four different complete *MBL2* genotypes were detected as shown above. As expected, the HYPA/HYPA genotype which codes for the homozygous normal A/A *MBL2* genotype, had the highest median plasma MBL concentration (median MBL 2464 μ g/L, IQR 1336-3368 μ g/L) and LYQC/LYQC had the lowest levels, (MB220 μ g/L). HYPA haplotype showed the highest median plasma MBL concentration (median MBL 2464 μ g/L, IQR 1336-3368 μ g/L) and LYQC haplotype had the lowest levels (MBL 20 μ g/L). We found varying median MBL concentrations due to the effect of HY, LY and LX promoters.

Appendix B: Published paper 2 and Supplementary materials

HIV-1 Disease Progression and Survival in an Adult Population in Zimbabwe: Is There an Effect of the Mannose Binding Lectin Deficiency?

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Michael Christiansen,⁶ Bernard Ngara,³ Exnevia Gomo,⁷ Henrik Ullum,⁸ Christian Erikstrup,⁹
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Abstract

HIV infection remains a major global health burden since its discovery in 1983. Sub-Saharan Africa is the region hardest hit by the HIV/AIDS pandemic where 63% of the 33 million infected people live. While there is marked person-to-person variability in susceptibility, progression, and survival with HIV infection, there is a paucity of predictive diagnostics associated with these clinical endpoints. In this regard, the deficiency in plasma Mannose Binding Lectin (MBL) is a common opsonic defect reported to increase susceptibility infections, including HIV. To the best of our knowledge, we report here the first study on the putative role of MBL deficiency on HIV progression and survival in an African adult population. We hypothesized that MBL deficiency has a role to play in HIV infection by increasing HIV disease progression and decreasing survival. We assessed the role of MBL deficiency on HIV disease progression and survival in a Zimbabwean adult population enrolled in the Mupfure Schistosomiasis and HIV (MUSH) cohort. We analyzed blood samples for MBL levels, *MBL2* genotypes, HIV-1 status, viral load, and CD4⁺ T cell counts. Participants were followed for 3 years wherein the endpoints were measured at baseline, 6 weeks, and 3, 6, 12, 24, and 36 months. Disease progression was measured as the rate of decline in CD4⁺ T cell counts and the rate of increase in HIV viral load. We assessed 197 HIV positive adults where 83% (164) were women with a median age of 31 years. Prevalence of plasma MBL deficiency (less than 100 µg/L) and *MBL2* deficient genetic variants (*A/O* and *O/O* genotypes) was 21% (42 out of 197) and 39% (74 out of 190), respectively. We did not observe a significant role to explain individual variation in mortality, change of CD4⁺ T cell count, and viral load by MBL plasma deficiency or *MBL2* genetic variants from baseline to 3 years follow up period in this adult population. We suggest the need for global OMICS research and that the present findings attest to the large between-population variability in a host of factors that can predispose individuals susceptible to HIV progression and mortality. We therefore cannot recommend at this time the use of plasma MBL levels or *MBL2* genetic variants as a prognostic marker in HIV infection, disease progression, and survival in this adult population in Africa.

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Introduction

SUB-SAHARAN AFRICA IS THE REGION hardest hit by the HIV/AIDS pandemic where 63% of the 33 million infected people live (UNAIDS, 2012). HIV infection has remained a major public health challenge since its discovery in 1983 (Gallo et al., 1983). Several reports indicate that individuals worldwide differ in their susceptibility to HIV infection and it is widely agreed that genetic polymorphisms in the host genes that are important in immune regulation, can influence the risk and progression of HIV infections (Cao Qin et al., 1995; Dzwonek et al., 2006; Israels et al., 2012; Tang and Kaslow, 2003). There has been growing interest in genomics research in recent years in Africa due to the realization that diseases are influenced by both genetic and social factors (Dandara et al., 2014). Knowledge translation and multi-omics research are sorely needed within the real-life context of the African continent (Ahmed et al., 2014; Bosch et al., 2014; Dandara et al., 2014; ElRakaiby et al., 2014).

Mannose Binding Lectin (MBL), an important constituent of the innate immune system, has been widely investigated in different populations to determine association between MBL deficiency and susceptibility to several other infections (Antony et al., 2013; Garred et al., 1997; 2003; Hu et al., 2010; Zinyama-Gutsire et al., 2015) and the role of MBL deficiency in HIV disease progression (Boniotto et al., 2000; Catano et al., 2008; Garred et al., 1997; Hundt et al., 2000; Maas et al., 1998; Mangano et al., 2008; Prohaszka et al., 1997; Senaldi et al., 1995; Tan et al., 2009; Vallinoto et al., 2008) and survival (Garred et al., 1997; Maas et al., 1998).

HIV disease progression is characterized by decline in CD4⁺ T cell count and increase in HIV viral load (Lyles et al., 2000; Mellors et al., 1995; 1996; Mellors et al., 1997; Nakagawa, 2014; Phillips et al., 1991; Sabin et al., 2000) eventually leading to death in the absence of treatment (Lyles et al., 2000; Mellors et al., 1995; 1996; 1997; Nakagawa, 2014; Phillips et al., 1991).

Available literature on the association between MBL deficiency and disease progression is conflicting, some report faster HIV disease progression due to MBL deficiency (Garred et al., 1997; Hundt et al., 2000; Tan et al., 2009; Vallinoto et al., 2008) but others found no association (Catano et al., 2008; Nielsen et al., 1995; Pastinen et al., 1998; Senaldi et al., 1995). Others report association between MBL deficiency and decreased survival in HIV infected people (Garred et al., 1997) but conflictingly others reported increased survival in those with variant *MBL2* genotypes and MBL deficient individuals (Maas et al., 1998).

We hypothesized that MBL deficiency had a role to play in HIV infection by increasing HIV disease progression and decreasing survival. We therefore assessed the role of plasma MBL deficiency and *MBL2* genetic variants, on HIV-1 disease progression and survival in a Zimbabwean adult population enrolled in the Mupfure Schistosomiasis and HIV cohort (MUSH) cohort.

Materials and Methods

Study design

This was a sub-study of the Mupfure Schistosomiasis and HIV (MUSH) cohort, established between 2001 and 2007. The main aim of the MUSH cohort was to investigate im-

munological interactions between HIV-1 and schistosomiasis (Kallestrup et al., 2005a; 2005b; Zinyama-Gutsire et al., 2015). Details including screening procedures, the setting, and the study population have been described in detail elsewhere (Kallestrup et al., 2005a; 2005b).

Plasma and whole blood samples collected at baseline and the six follow-up time points were used for measurement of viral load and CD4⁺ T cell count. The MUSH study was approved by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/918) and this sub-study was also approved separately (MRCZ/A/1770), in addition to having been approved by the University of Witwatersrand Human Research Ethics Committee (M130348). All individuals gave written informed consent for specimen storage and future laboratory studies. The participants were all naïve to treatment for HIV-1 infection as there were no anti-HIV drugs available in public hospitals in Zimbabwe at the time of sample collection.

Laboratory procedures

Participants were screened for HIV-1 as described earlier (Kallestrup et al., 2005a; 2005b). Briefly, HIV testing was performed confidentially and pre- and post-test counselling was provided in the participants' native language (Shona) by qualified medical personnel. Initially, a rapid HIV-1/2 test kit was used on a dry blood spot in the field (Determine, Abbott Laboratories, Tokyo, Japan). This test was followed by two different rapid HIV tests Oraquick (by Orasure) and Serodia (by Fujirebio) for all who tested positive initially. CD4⁺ T cell counts and HIV RNA at the six follow-up points were used for this analysis. CD4⁺ T cell counts were measured in whole blood (FacsCalibur, Becton Dickinson) and HIV RNA was measured in plasma using Roche Amplicor (F. Hoffmann la-Roche) at the Department of Haematology of Parirenyatwa Hospital Harare, Zimbabwe.

Viral load and CD4⁺ T cell counts were available at baseline, 6 weeks, and 3, 6, 12, 24, and 36 months follow-up for the 198 HIV positive participants. HIV disease progression was defined as the magnitude of decrease in CD4⁺ T cell counts and increase in viral load (Mellors et al., 1995; 1996; 1997). Participants were classified into three CDC categories, namely A, B, and C according to CDC classification (CDC 1993; 1999). Category A were HIV positive but asymptomatic, category B showed HIV symptomatic conditions, and category C showed AIDS-indicator conditions (CDC 1993; 1999).

Plasma MBL concentration was measured in baseline plasma samples using the double enzyme immuno-assay (EIA) (Garred et al., 1999; Zinyama-Gutsire et al., 2015) using plasma stored at -20°C until analysis. The lower detection limit in the assay was 20 µg/L (Garred et al., 1997; 1999; Madsen et al., 1998; Zinyama-Gutsire et al., 2015). Plasma MBL concentration was categorized into normal (above 500 µg/L), intermediate (100 µg/L–500 µg/L) and deficient (below 100 µg/L) (Eisen et al., 2008; Egli et al., 2013; Garred et al., 1997; Kruse et al., 2002; Zinyama-Gutsire et al., 2015).

Genomic DNA was extracted from frozen peripheral blood mononuclear cells (PBMCs) collected at baseline using the standard salting out procedure (Miller et al., 1988). The *MBL2* genotypes and promoter region alleles were detected by allele-specific oligonucleotide PCR (ASO-PCR) where specific sequences were used for each allele (Madsen et al.,

1998; Steffensen et al., 2000; full details provided earlier (Zinyama-Gutsire et al., 2015).

Statistical analysis

All statistical analysis was done using Stata 11 statistical package (STATA Corp, Timberlake Consultants, Texas, USA). Model assumptions were checked graphically using residual and Kaplan-Meier plots. Viral load was included in the model as log-transformed units. We used Mann-Whitney and Kruskal-Wallis tests to compare plasma MBL concentrations according to two CD4⁺ T-cell count groups, above or below 350 cells/ μ L (Sabin et al., 2000), the level recommended by WHO then (2007) to initiate ART and viral load above or below the median viral load of this study population of 60,000 copies per mL.

Frequencies of the *MBL2* genotypes, alleles, and promoter region genotypes and haplotypes were obtained by direct gene counting. For *MBL2* genotype analysis, normal homozygous *MBL2* was denoted as *A/A* and the variant *MBL2* genotypes *A/B*, *A/C*, *A/D* were grouped together as *A/O* homozygous and compound homozygotes were grouped together as *O/O* (Supplementary Table S1; supplementary material is available online at www.liebertpub.com/omi). Conformation to the Hardy-Weinberg equilibrium was determined using the chi square test in the SHEsis online programme (Li et al., 2009; Shi and He, 2005).

Generalized Estimating Equations (GEE) models (COHERE, 2014; Hansen and Holmskov 1998; Ma et al., 2012; Martin et al., 2014) were used to compare rates of change of the CD4⁺ T cell count and viral load measurements over the 3-year follow-up period. The role of plasma MBL deficiency and *MBL2* genetic variants on survival over the 3-year period were estimated using the Cox proportional hazard models.

Regression analysis was used to test for interaction and confounding between MBL deficiency, *MBL2* genetic variance, age, and sex. We use the Wald Chi-square statistic to choose between full and nested models. All tests were conducted at 5% level of statistical significance.

Results

Baseline demographic characteristics of the main MUSH cohort are reported elsewhere (Kallestrup et al., 2005; 2006). The majority of the HIV-infected participants were females (83%), median age was 31 (27–29) years. The plasma MBL concentration was available for 197 HIV positive individuals and *MBL2* genotyping results were available for 190 individuals. The median (interquartile range, IQR) plasma MBL concentration was 688 μ g/L (147–1904 μ g/L) among these HIV-1 positive individuals. Prevalence of plasma MBL deficiency (less than 100 μ g/L) and *MBL2* deficient variants (*A/O* and *O/O* genotypes) was 21% (42 out of 197) and 39% (74 out of 190) respectively. Plasma MBL concentration among these 197 HIV-1 infected individuals did not differ between participants in CDC categories A versus categories B/C combined, CD4 count groups below or above 350 cells/ μ L (Table 1) and viral load did not differ between the three plasma MBL groups ($p=0.867$) (Table 2).

Role of MBL deficiency in CD4⁺ T cell count change

GEE models were used for disease progression analysis. In bivariate analysis, there was a significant change from

TABLE 1. COMPARISON OF MEDIAN PLASMA MBL CONCENTRATIONS AMONGST THE HIV-1 INFECTED INDIVIDUALS ACCORDING TO CDC HIV/AIDS DISEASE CATEGORIES AND CD4⁺ T CELL COUNT

Variable	n (%)	Median (IQR) MBL levels μ g/L	p-value
<i>CDC category</i>			
A	132 (67)	688 (20–5792)	0.970
B and C combined	65 (33)	656 (20–3120)	
<i>CD4⁺ T cell count</i>			
Above 350 cells/ μ L	92 (47)	728 (20–5792)	0.468
Below 350 cells/ μ L	105 (53)	576 (20–3840)	

The concentration of MBL in plasma was measured in μ g/L. Differences in median plasma MBL concentration in the different infection status categories were analyzed by using nonparametric Mann-Whitney test.

baseline in CD4 to 359 cell/count ($p<0.001$) among those with normal MBL levels. The change in CD4 among those with intermediate and deficiency MBL levels were 37 ($p=0.338$) and 15 ($p=0.717$) cell/count less than those with normal MBL levels, however the difference was not significant at 5% level (Table 3). Similarly, plasma MBL deficiency levels category 100–500 μ g/L ($p=0.338$), *MBL2* structural variants *AO/OO* ($p=0.633$), *MBL2* deficient genotype *XA/XA*, *YA/YO* ($p=0.851$), MBL deficient genotype *XA/YO*, *YO/YO* ($p=0.705$), and promoter region variants *LY* LX* combined ($p=0.602$) had no role in change in CD4⁺ T cell count change over the 3-year follow-up period (Table 3).

We found a significant difference in the rate of change in CD4⁺ T cell count by sex, males had mean (95%, CI) CD4⁺ T cell decrease of -110 cells/ μ L (-197 – 22 , $p=0.014$) less than that of females. Multivariate analysis also showed MBL deficiency had no role in change in CD4⁺ T cell count and viral load (Table 3). There was insufficient evidence at 5% level to explain change of CD4⁺ T cell count by MBL plasma deficiency and *MBL2* genetic variants from baseline to 3-year follow-up period.

Role of MBL deficiency in change in viral load

In bivariate analysis, there was a significant change from baseline in viral load to 4.58 ($p<0.001$) among those with normal MBL levels. The changes in viral load among those

TABLE 2. COMPARISON OF VIRAL LOAD ACCORDING TO PLASMA MBL CONCENTRATIONS AMONGST THE HIV-1 INFECTED INDIVIDUALS

Variable	n	Median (IQR) viral load copies per mL	p-value
Normal plasma MBL levels	113	43250 (12700–192750)	0.867
Intermediate plasma MBL levels	41	49000 (12800–193750)	
Deficient plasma MBL levels	43	65000 (14350–157500)	
Total	197		

Viral load was measured in copies per mL. Differences in median viral load in the three plasma MBL categories shown above were analyzed using nonparametric Kruskal-Wallis test.

TABLE 3. FACTORS ASSOCIATED WITH CHANGE IN CD4⁺ T CELL COUNTS FROM BASELINE TO 3 YEAR FOLLOW-UP PERIOD

Factor	n	Parameter Estimate (cell counts)	95% CI	P value
<i>Plasma MBL level</i>				
Normal plasma MBL level > 100 µg/L (reference)	113	359	317–403	<0.001
MBL intermediate level 100–500 µg/L	43	–37	–114–39	0.338
MBL deficient level < 100 µg/L	41	–15	–99–68	0.717
<i>MBL exon 1 genotype</i>				
MBL normal genotypes A/A	116	356	312–400	0.000
MBL deficient genotypes A/O and O/O combined	74	–17	–87–53	0.633
<i>MBL haplotypes</i>				
MBL normal haplotypes (YAYA/YAXA)	110	353	309–396	0.000
1.MBL deficient genotype (XA/XA, YA/YO) (intermediate levels)	72	–6	–206–139	0.851
2.MBL deficient genotype XA/YO, YO/YO (deficient levels)	8	–33	–206–139	0.705
<i>MBL promoter region genotypes</i>				
MBL normal promoter region H/Y	14	318	196–440	0.000
MBL promoter variants LY/LX	176	33	–93–160	0.602
<i>Age at t0</i>				
Below 25 years old	21	429	327–531	0.000
Above 25 years old	177	–90	–198–16	0.097
<i>Sex</i>				
Female	16	367	331–403	0.000
Male	34	–110	–197–22	0.014

Change in CD4⁺ T cell count (cells/µL) over the 3 year follow-up period. Reference plasma MBL levels is normal levels above 500 µg/L. Reference MBL2 genotype is normal wild type genotype A/A and YAYA/YAXA. Reference MBL promoter region genotype is H/Y. Reference age was below 25 years old. Reference sex was females.

with intermediate and deficiency MBL levels were 0.19 ($p=0.221$) and 0.02 ($p=0.892$) log₁₀ copies/mL more than those with normal MBL levels; however the difference was not significant at 5% level (Table 4). The mean (95%, CI) increase in viral load among those with plasma MBL defi-

ciency less than 100 µg/L was 0.02 log₁₀ copies/mL (–0.31–0.35, $p=0.892$) compared to those with normal plasma MBL levels (Table 4).

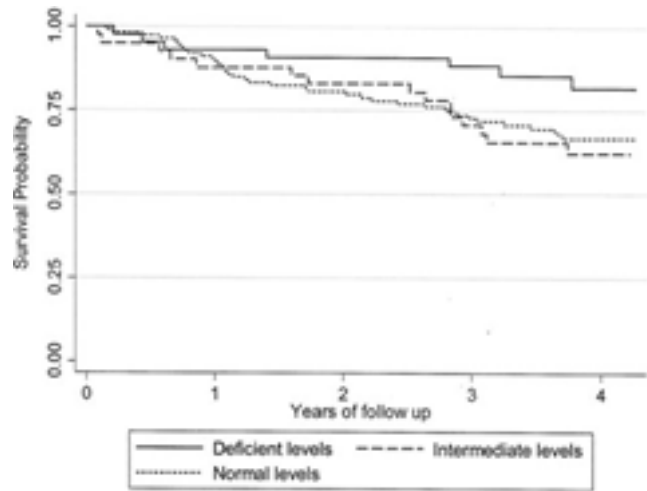
Similarly, plasma MBL deficiency levels 100–500 µg/L ($p=0.221$), MBL2 structural variants AO/OO ($p=0.847$),

TABLE 4. FACTORS ASSOCIATED WITH CHANGE IN VIRAL LOAD FROM BASELINE TO 3 YEARS FOLLOW-UP PERIOD

Factor	n	Parameter Estimate viral load	95% CI	P value
<i>Plasma MBL level</i>				
Normal plasma MBL level > 100 µg/L (reference)	113	4.58	4.41–4.75	0.000
MBL intermediate level 100–500 µg/L	43	0.19	–0.11–4.90	0.221
MBL deficient level < 100 µg/L	41	0.02	–0.31–0.35	0.892
<i>MBL2 exon 1 genotype</i>				
MBL normal genotypes A/A	116	4.63	4.45–4.79	0.00
MBL deficient genotypes A/O and O/O combined	74	–0.027	–0.30–0.24	0.847
<i>MBL2 haplotypes</i>				
MBL normal haplotypes (YAYA/YAXA)	110	4.62	4.44–4.79	0.000
MBL deficient genotype (XA/XA, YA/YO) (intermediate levels)	72	–0.01	–0.28–0.25	0.911
MBL deficient genotype XA/YO, YO/YO (deficient levels)	8	0.027	–0.66–0.72	0.937
<i>MBL2 promoter region genotypes</i>				
MBL normal promoter region H/Y	14	4.67	4.21–5.14	0.000
MBL promoter variants LY/LX	176	–0.067	–0.55–0.41	0.785
<i>Age at t0</i>				
Below 25 years old	21	4.49	4.10–4.88	0.000
Above 25 years old	177	0.15	–0.26–0.57	0.463
<i>Sex</i>				
Female	16	4.54	4.40–4.69	0.000
Male	34	0.471	0.13–0.81	0.006

Change in viral (load log₁₀ scale) over the 3 year follow-up period. Reference plasma MBL levels is normal levels above 500 µg/L. Reference MBL2 genotype is normal wild type genotype A/A and YAYA/YAXA. Reference MBL2 promoter region genotype is H/Y. Reference age was below 25 years old. Reference sex was females.

FIG. 1. Kaplan Meier plot of the 197 HIV-1 infected participants by plasma MBL concentration. Survival time was calculated from baseline data to death or upon censoring. Participants were subdivided into three groups according to plasma MBL concentration, normal levels (above 500 $\mu\text{g/L}$), reduced/intermediate (100–500 $\mu\text{g/L}$), and deficient (below 100 $\mu\text{g/L}$). One hundred and thirteen (113) participants had normal plasma MBL concentrations, 41 had reduced/intermediate levels, and 43 had deficient levels. Log Rank test 0.1129.



MBL deficient genotype *XA/XA*, *YA/YO* ($p=0.911$), MBL deficient genotype *XA/YO*, *YO/YO* ($p=0.937$), and promoter region variants *LY*LX* combined ($p=0.785$) had no role in change in viral load over the 3-year follow-up period (Table 4). However, we found a significant difference in increase in viral load between males and females, mean log (95% CI) decrease in viral in males was 0.471 \log_{10} copies/ml (0.131–0.81, $p=0.014$) ($p=0.006$) greater than in females. Multivariate analysis also showed MBL deficiency had no role in change in both CD4^+ T cell count and viral load (results not shown).

Analysis of mortality in this study population

Participants in the main MUSH cohort were followed up for a total of 631 person-years with a median follow-up time of 3.6 years with 58 deaths, 32 due to respiratory illness, 12 unknown causes, and one suicide, as reported in more detail earlier (Erikstrup et al., 2007). The rate of death was 29% (58 out of 197) among the HIV infected compared to 1% (2 out of 181) among HIV-negative participants.

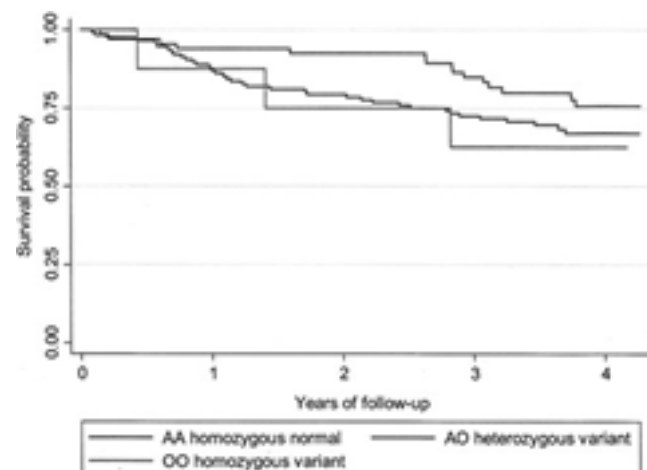
Survival analysis in this study population

Analysis was done only among the 197 HIV-1 positive participants to determine the effect of plasma MBL concentration and *MBL2* polymorphism in the *MBL2* coding and

promoter region, on survival. There was no difference in rate of death amongst the HIV-positive participants according to plasma MBL concentration when participants were divided into three plasma MBL concentration groups normal ($n=113$), intermediate ($n=41$) and deficient levels ($n=43$) (Fig. 1, Kaplan Meier plot, Log Rank test 0.1129) and according to MBL levels quartiles (Supplementary Fig. S1, Kaplan Meier plot, Log Rank test 0.2916). There was also no difference in rate of death, measured as time to death, between those with normal *MBL2* genotype compared to those with the combined *MBL2* variants (Fig. 2, Kaplan Meier plot, Log Rank test 0.263).

The survival of those with the homozygous *XA/XA* promoter type, which is a low expression promoter, was compared to those with the *A/A*, *A/O*, and *O/O* genotypes. There was no difference in rate of death when *XA/XA* was compared to individuals with normal and variant *MBL2* genotypes, (Supplementary Fig. S2, Kaplan Meier plot Log Rank test 0.4175). It is known that individuals can have one or two or none *MBL2* X promoter. Analysis was done in the homozygous wild-type *A/A* genotype group to compare the dose-dependent effect of the number of X promoters on survival among the HIV-positive participants. There was no difference in rate of death between those with 1, 2, or none X promoter (Fig. 3, Kaplan Meier plot, Log Rank test 0.6125).

FIG. 2. Kaplan Meier plot of HIV-1 infected participants comparison by *MBL2* genotype. Survival time was calculated from baseline data to death or upon censoring. One hundred and sixteen participants (116) had normal *A/A* *MBL2* genotype, 66 had *A/O* variant *MBL2* genotype, and 8 had *O/O* genotype. Log rank test 0.263.



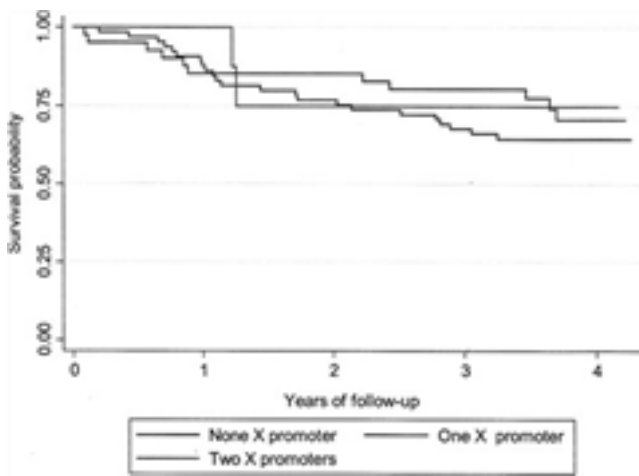


FIG. 3. Kaplan Meier plot showing the comparative dose-dependent effect of number of XA haplotypes on survival in the normal A/A *MBL2* genotype group only, within the HIV positive stratum. Log rank test 0.6125. Zero X $n=65$, One X $n=41$, Two X $n=8$.

The *MBL2* haplotypes were further combined and subdivided into three groups, namely haplotypes that give normal plasma *MBL2* levels (YA/YA, YA/XA), intermediate levels (XA/XA, YA/YO), and deficient levels (XA/YO, YO/YO), showed no differences in rate of death (Fig. 4, Kaplan Meier plot, Log Rank test 0.3176).

Cox regression models, adjusted for gender and age, were fitted to determine if MBL deficiency predicted mortality amongst the HIV positive. For this analysis, participants were subdivided into two groups for each variable (Table 5). MBL concentration ($p=0.72$), *MBL2* genotype ($p=0.69$), and *MBL2* promoter haplotypes ($p=0.58$) were all not predictors of mortality in this study amongst those who were HIV positive (Table 5). The other predictors of mortality for these participants were reported earlier (Erikstrup et al., 2007; 2008) such as low CD4⁺ T-cell count, viral load level, hemoglobin, and CDC category.

In addition, we found no correlation between plasma MBL concentration and the CD4⁺ T-cell counts (Spearman's rho=0.0275, $p=0.702$). Similarly, there was no correlation between plasma MBL concentration and plasma HIV-1 RNA (Spearman's rho=0.01, $p=0.822$). Also CD4⁺ T cell count (Table 6, χ^2 test $p=0.962$, Fig. 5) and plasma viral load (χ^2 test $p=0.675$, Fig. 5) did not differ according to *MBL2* genotype.

Discussion

To our knowledge, this is the first study in adults to investigate role of MBL deficiency on disease progression and survival in an adult African population. Our results showed that plasma MBL deficiency, low producer *MBL2* variant genotypes, and *MBL2* promoter region variants played no role in change in CD4⁺T cell, change in viral load, disease progression, and survival in this ART-naïve adult population. Plasma MBL concentration was not a predictor of HIV status, CD4⁺ T cell count, and viral load.

Plasma MBL deficiency and *MBL2* genetic variants had no role in HIV disease progression in our study population defined as decrease in CD4⁺ T cell count and increase in viral load, in accord with other findings (Catano et al., 2008; Nielsen et al., 1995; Senaldi et al., 1995) but in contrast to other reports of a significant association (Garred et al., 1997; Hundt et al., 2000; Tan et al., 2009; Vallinoto et al., 2008). The studies that showed similar results to ours were done in the USA among those with European origins and African Americans (Catano et al., 2008), UK (Senaldi et al., 1995), and Denmark (Nielsen et al., 1995). Studies that showed contrasting results to ours were done in Denmark (Garred et al., 1997), China (Tan et al., 2009), Germany (Hundt et al., 2000), and Brazil (Vallinoto et al., 2008).

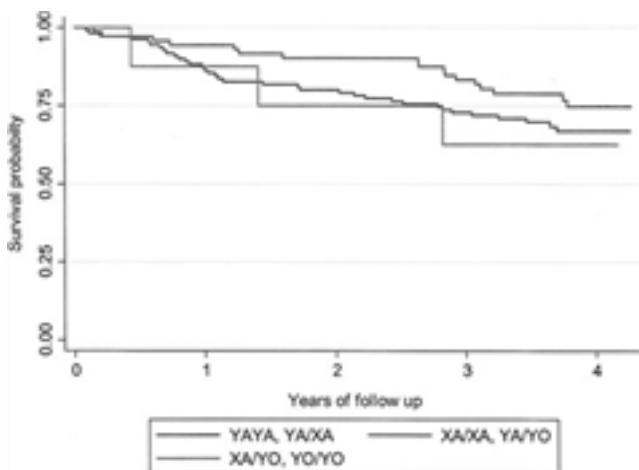


FIG. 4. Kaplan Meier plot of HIV-1 infected participants comparison by *MBL2* genotype. The *MBL2* haplotypes were further combined and subdivided into three groups, namely haplotypes that give normal plasma MBL levels (YA/YA, YA/XA), intermediate levels (XA/XA, YA/YO), and deficient levels (XA/YO, YO/YO). Survival time was calculated from baseline data to death or upon censoring. One hundred and ten participants (110) had (YA/YA, YA/XA) *MBL2* genotype, 72 had variant (XA/XA, YA/YO) *MBL2* genotype, and 8 had (XA/YO, YO/YO) genotype). Log rank test 0.3176.

TABLE 5. UNIVARIATE COX REGRESSION ANALYSIS FOR PREDICTORS OF MORTALITY AMONG THE HIV-POSITIVE PARTICIPANTS

Variables	n	HR	95% CI	p-value
<i>Plasma MBL levels</i>				
MBL normal	113	–		
MBL deficient level 100–500 µg/L	41	0.91	0.48-1.70	0.764
MBL deficient level < 100 µg/L	43	0.50	0.22-1.12	0.090
<i>MBL2 exon 1 genotype</i>				
MBL genotype A/A	116	–		
MBL genotype A/O + O/O (combined)	74	0.69	0.39-1.23	0.209
<i>MBL promoter region variants</i>				
MBL haplotypes H/Y	14	–		
MBL haplotypes LY/LX (combined)	176	0.58	0.18-1.86	0.362
<i>MBL2 haplotypes</i>				
YAYA/YAXA (normal MBL levels)	110	–		
XA/XA, YA/YO (intermediate levels)	72	0.69	0.38-1.24	0.215
XA/YO, YO/YO (deficient levels)	8	1.14	0.35-3.75	0.829

Participants were subdivided into groups for each variable listed above. Results are shown as hazard ratios and confidence intervals after controlling for both age and sex. Reference categories were normal plasma MBL levels and wild type *MBL2* genotype.

The possible reasons why our results are different to others who found an association between MBL deficiency and disease progression might be due to differences in characteristics of the different populations studied or might be due to differences in sample sizes used. Our sample size was 198 HIV-positive male and female participants compared to analysis in 96 HIV-positive homosexual men (Garred et al., 1997), 128 HIV positive adult males and females (Vallinoto et al., 2008), 1075 adult males and females (Tan et al., 2009), and only 6 long-term HIV positive non-progressors (Hundt et al., 2000).

Available literature on association between MBL deficiency and HIV disease progression is conflicting, with some reporting faster HIV disease progression among those with MBL deficiency (Garred et al., 1997; Hundt et al., 2000; Tan et al., 2009; Vallinoto et al., 2008) but others have reported no association (Catano et al., 2008; Nielsen et al., 1995; Senaldi et al., 1995). Presence of an association between MBL deficiency and decreased survival in HIV infected people has been reported (Garred et al., 1997), but conflictingly others reported increased survival in those with variant *MBL2* genotypes and MBL deficient individuals (Maas et al., 1998), where MBL deficiency was reported to confer protection.

In contrast to our findings of no association between HIV disease progression and MBL deficiency, other studies showed a higher median plasma MBL concentration in patients who progressed to AIDS compared to those who did not, indicating a protective role of MBL deficiency (Mangano et al., 2008). In addition, in our study population plasma MBL

concentration was not a predictor of HIV-1 status, CD4⁺ T cell count, or viral load, similar to other reports (Catano et al., 2008), but in contrast to other findings (Vallinoto et al., 2008).

Important to note is that even though plasma MBL levels can be categorized into normal, intermediate, and deficient levels according to *MBL2* genotype, evidence in literature shows that there is a wide variation in plasma MBL levels by up to 1000-fold regardless of genetic make-up (Madsen et al., 1995; 1998; Turner, 1996; Ytting et al., 2011). Our earlier results also showed this wide variation in plasma MBL levels despite genetic make-up (Zinyama-Gutsire et al., 2015).

Our results showed a significant decrease in CD4⁺ T cell count and viral load as also reported. The increases in CD4⁺ T cell count and decrease in viral load have been reported in our other publications (Kallestrup et al., 2005) where we reported that there was a significant increase in CD4⁺ T cell count and decrease in viral load after treatment of schistosomiasis. An improvement in the immune system was noted in this study population, even without HAART (Kallestrup et al., 2005).

Survival analysis was done within the HIV-1 infected stratum. Our results showed that MBL deficiency and *MBL2* genetic variants were not associated with increased rate of death, in contrast to findings of faster rate of death among HIV-infected participants with *MBL2* genetic variants (Garred et al., 1997) and among those with XA/XA *MBL2* promoter variant genotype (Catano et al., 2008). The absence of an association in our cohort might be due to analysis in a smaller cohort (379 with only 197 HIV-positive individuals) compared to another report from a significantly larger cohort

TABLE 6. COMPARISON OF MEDIAN CD4⁺ T CELL COUNT AND VIRAL LOAD AMONG THE HIV-1 INFECTED ACCORDING TO *MBL2* GENOTYPE

<i>MBL2</i> genotype	n	Median (IQR) CD4 ⁺ T cell count	p value	Median (IQR) viral load	p value
A/A	116	319 (11–1222)		65000 (20–1250 000)	
A/B	66	325 (52–1893)	0.962	40125 (74–1000 000)	0.675
O/O	8	301 (87–550)		86500 (4115–342 500)	

Differences in median CD4 T cell count and viral load according to *MBL2* genotype were analyzed by the Kruskal-Wallis test, $n=190$.

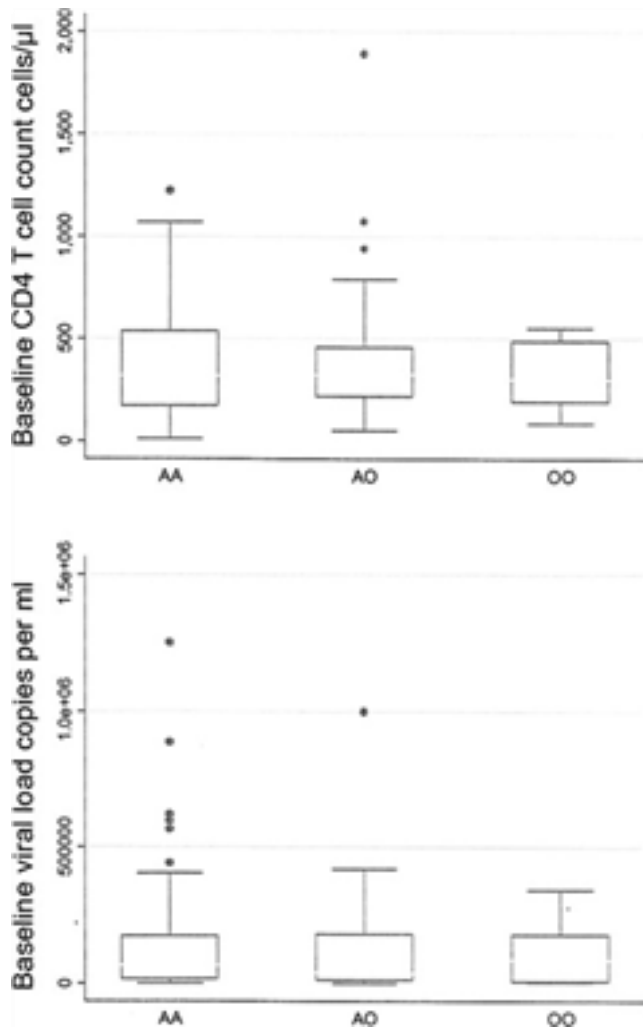


FIG. 5. (A) CD4⁺ T cell count (cells/ μ L) according to genotype. (B) Viral load (copies per mL) according to *MBL2* genotype *A/A*: normal *MBL2* genotype, *A/O*: heterozygous and *O/O*: *MBL2* variant homozygous ($n=190$).

of 3315 with 1102 HIV-positive participants (Catano et al., 2008).

Cox proportional hazards models showed plasma MBL deficiency, *MBL2* exon 1 region variants, and promoter region variants were not predictors of mortality in our study population. We found no differences in *MBL2* levels between CDC HIV categories, CD4⁺ T cell count, and viral load in our study, similar to findings by others (Senaldi et al., 1995; Nielsen et al., 2003; Pastinen et al., 1998), in contrast to other reports of an association (Garred et al., 1997; Hundt et al., 2000; Vallinoto et al., 2008; Tan et al., 2009).

The role of MBL deficiency due to polymorphism in the *MBL2* gene has also been investigated in other infections, and is associated with development of pulmonary TB in younger patients (Hijikata et al., 2014), no role in hepatitis B (Osthoﬀ et al., 2014; Xu, 2013), high MBL levels protective in schistosomiasis (Antony et al., 2013) and malaria (Garred et al., 2003; Luty et al., 1998).

The main limitation of our study was inclusion of few men in the main MUSH study (Kallestrup et al., 2005; 2006; Zinyama-Gutsire et al., 2015). There is a gold panning area in the neighboring district, most men work away from their rural homes, and were not available during our study recruitment phase. This has the potential to bias our findings, but every effort was done to enroll all eligible men into the main MUSH study. The results of our study cannot support recommen-

dations to use *MBL2* polymorphism as a prognostic tool for AIDS progression using CD4⁺ T cell counts and viral load as has been recommended by others (Vallinoto et al., 2008).

Conclusions

Available literature shows that many diseases are influenced by both genetic and social factors results that are relevant to African populations bearing a huge burden of viral, parasitic, and bacterial infections (Dandara et al., 2014). However, our results showed that plasma MBL deficiency, *MBL2* coding region variants, and *MBL2* promoter region variants had no role in change in both CD4⁺ T cell counts and viral load from baseline to 3-year follow up. We therefore cannot recommend use of plasma MBL levels and *MBL2* genetic variants as a prognostic marker in HIV infection, disease progression, and survival in this population.

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Author contributions: RBLZG, HOM, PK, EG, HU, PG, EC, and TM conceived and designed the experiments. RBLZG, PG, and HOM performed the experiments. RBLZG, BN, CC, SR, and TM analyzed the data. RBLZG, HOM, PK, EG, HU, PG, EC, BSP, and TM contributed reagents/materials/analysis tools. RBLZG, BN, CC, SR, MC, and TM drafted the manuscript. All co-authors read the manuscript and approved the final version.

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Author Disclosure Statement

No competing financial interests exist.

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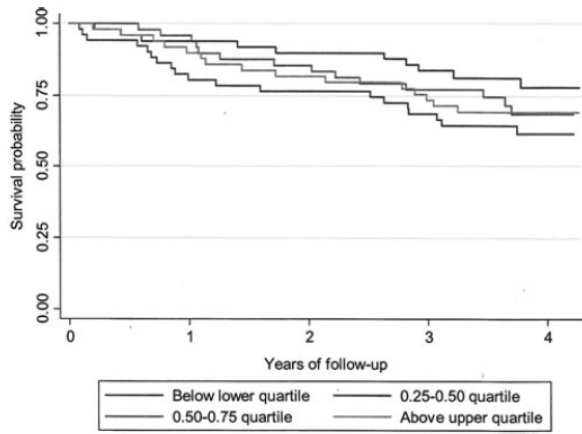
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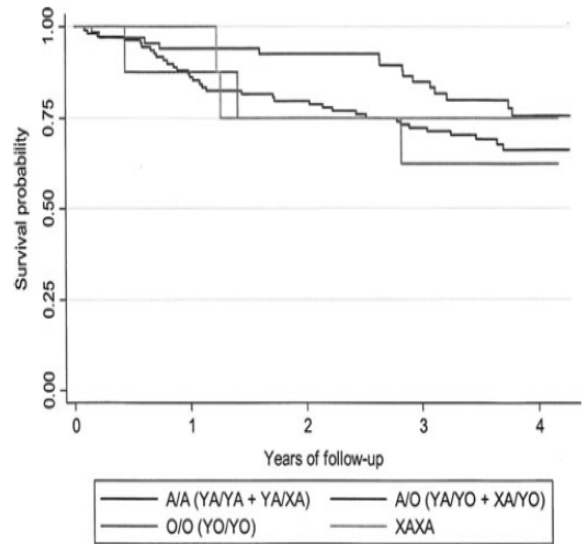
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Supplementary Data



SUPPLEMENTARY FIG. S1. Kaplan Meier plot of the 197 HIV-1 infected participants by plasma MBL concentration. Survival time was calculated from baseline data to death or upon censoring. Participants were stratified into four groups according to plasma MBL concentration: Forty nine (49) participants below the lower quartile ($<147 \mu\text{g/L}$), 51 in the 0.25–0.50 quartile ($147\text{--}687 \mu\text{g/L}$), 48 in the 0.50–0.75 quartile ($688\text{--}1904 \mu\text{g/L}$), and 49 above the upper quartile ($>1904 \mu\text{g/L}$). Log Rank test 0.2916.

SUPPLEMENTARY FIG. S2. Kaplan-Meier plots for time to death for individuals with coding and noncoding variants compared to the *XA/XA* promoter haplotype within the HIV positive stratum. The participants were stratified into four groups *A/A* (*YA/YA* + *YA/XA*), *A/O* (*YA/YO* + *XA/YO*), *O/O* (*YO/YO*), and *XA/XA*. *A/A* is the reference group. Log rank test 0.4175. *A/A* *n*=108, *A/O* *n*=66, *O/O* *n*=8, *XA/XA* *n*=8.



SUPPLEMENTARY TABLE S1. NOMENCLATURE
OF *MBL2* GENOTYPES USED IN THIS ANALYSIS

<i>MBL2</i> variant	Reference SNP ID number (rs#)	Alternative nomenclature
Exon 1 normal		A/A
<i>B</i> (codon 54)	1800450	A/B
<i>C</i> (codon 57)	1800451	A/C
<i>D</i> (codon 52)	1800451	A/D
-550	11003125	H/L
-221	7096206	Y/X
+4	7095891	P/Q
A/O		
O/O		
YA/YA, YA/XA (normal plasma MBL levels)		
XA/XA, YA/YO (intermediate plasma MBL levels)		
XA/YO, YO/YO (deficient plasma MBL levels)		

Appendix C: Published paper 3 and published supplementary materials

HIV-1 Vertical Transmission in Zimbabwe in 622 Mother and Infant Pairs: Rethinking the Contribution of Mannose Binding Lectin Deficiency in Africa

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Abstract

Vertical transmission of human immunodeficiency virus (HIV) remains a major global health problem. We assessed the association of mannose binding lectin (MBL) deficiency and vertical transmission of HIV. Novel diagnostics would be a major breakthrough in this regard. MBL is a liver-derived protein and a key component of the innate immune system. MBL levels may be classified as normal, intermediate, or deficient in the plasma and can use *MBL2* haplotypes as a proxy. These haplotypes comprise polymorphisms in the *MBL2* gene and promoter region and are known to result in varying levels of MBL deficiency. MBL deficiency can be defined as presence of *A/O* and *O/O* genotypes in the mothers and their children. MBL deficiency leads to defective opsonization activities of the innate immune system and increased susceptibility to several infections, including HIV-1. We determined the prevalence of MBL deficiency, using *MBL2* haplotypes among 622 HIV-positive Zimbabwean mothers and their children aged 9–18 months old, in relation to the HIV-1 vertical transmission risk. The median age of the mothers was 30 (26–34, interquartile range [IQR]) years, and the babies' median age was 13 (11–15, IQR) months old at the time of enrollment. From the sample of 622 mothers who were HIV-1 infected, 574 babies were HIV negative and 48 were HIV-1-positive babies, giving a transmission rate of 7.7%. *MBL2* normal structural allele *A* and variants *B* (codon 5A>G), *C* (codon 57A>G), and promoter region SNPs –550(*H/L*) and –221(*X/Y*) were detected. Prevalence of haplotype-predicted MBL deficiency was 34% among the mothers and 32% among the children. We found no association between maternal *MBL2* deficiency and HIV-1 transmission to their children. We found no difference in the distribution of HIV-1 infected and uninfected children between the *MBL2* genotypes of the mothers and those of the children. Taken together, the present study in a large sample of mother–infant pairs in Zimbabwe adds to the emerging literature and the hypothesis that *MBL2* variation as predicted by haplotypes does not influence the vertical transmission risk for HIV. Research from other populations from the African continent is called for to test this hypothesis further.

Introduction

RISK FACTORS FOR HUMAN IMMUNODEFICIENCY VIRUS (HIV) pediatric vertical transmission include viral characteristics, maternal, obstetrical, behavioral, and genetic risk factors (Matt and Roger, 2001). Understanding the interac-

tion between HIV virus, the host immune system, and genetic background has been an interest of many researchers over the past two decades (Singh et al., 2009). Mutations in host immune genes like mannose binding lectin-2 gene (*MBL2*) have been reported to lead to increased susceptibility to HIV infection (Garred et al., 1997, 2003; Madsen et al., 1994, 1995,

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1998). MBL is a pattern recognition molecule, which plays an important role as a constituent of the innate immune system (Turner, 1996). MBL deficiency, characterized by decreased plasma/serum MBL concentration, leads to defective opsonization activities of the innate immune system and increased susceptibility to infections (Soothill and Harvey, 1976; Sumiya et al., 1991; Super et al., 1989). MBL deficiency has been associated with various diseases, including HIV (Garred et al., 1997), tuberculosis (Garred et al., 1997), lung diseases (Garred et al., 1999), and parasitic diseases (Antony et al., 2013; Garred et al., 2003). The inherited MBL deficiency is not itself pathogenic, but it predisposes individuals to infections and faster disease progression (Madsen et al., 1994, 1998). MBL deficiency has been identified as a very common opsonic defect in both adults and children (Super et al., 1989).

In children between the ages 6 and 24 months, MBL plays an important role in the control of infectious microorganisms as it is during this period in which the maternal protective IgG antibodies have waned and the child's immune system is still very immature (Dommert et al., 2006). The normal *MBL2* allele is designated as *A*, and point mutations in the *MBL2* gene result in presence of variant alleles, which encode three different structural variants designated *B* (codon 54, *rs1800450 A>G*), *C* (codon 57, *rs1800451 A>G*), and *D* (codon 52, *rs5030737 T>C*) (Madsen et al., 1995, 1998). These mutations affect the structural integrity of MBL protein, resulting in a 2-fold to 100-fold decrease in circulating MBL serum/plasma levels (Madsen et al., 1995, 1998). In addition to mutations in the *MBL2* gene, point mutations in the *MBL2* promoter region at positions *H/L* (−550, *rs11003125 G>C*), *X/Y* (−221, *rs7096206 C>G*), and in the 5' untranslated region of exon 1 at position *PQ* (+4, *rs7095891 T>C*) have been well documented (Madsen et al., 1995, 1998). These variants lead to a 10% reduction of functional MBL in individuals that are heterozygous for defective alleles compared with the functional MBL found in individuals with two functional alleles (Summerfield et al., 1995). The SNPs at the promoter region are reported to be in strong linkage disequilibrium with SNPs at the exon 1 position, giving rise to different haplotypes classified into three groups as high or normal serum MBL producers (*YA/YA*, *YA/XA*), intermediate levels (*XA/XA*, *YA/YO*), and deficient levels (*XA/YO*, *YO/YO*) (Garred et al., 2003; Vengen et al., 2012).

Several studies have investigated the role of *MBL2* genetic variants in HIV transmission from mothers to their children (Amoroso et al., 1999; Arraes et al., 2006; Boniotto et al., 2000, 2003; Kuhn et al., 2006; Mangano et al., 2008; Mhandire et al., 2014; Zupin et al., 2016); as reviewed by Israels et al. (2012), the findings are conflicting (Israels et al., 2012). Some have reported MBL deficiency assessed by the presence of *MBL2* genetic variants to be a risk factor for HIV vertical transmission (Arraes et al., 2006; Kuhn et al., 2006; Mangano et al., 2008), but others reported no association (Boniotto et al., 2000; Mhandire et al., 2014; Zupin et al., 2016). Zimbabwe is one of the countries in Southern Africa bearing a huge burden of mother to child HIV transmission (5–15%) (UNAIDS, 2013); it is therefore of public health importance to determine host genetic factors contributing to mother to child transmission (MTCT). The translation of knowledge and multiomics research are critical and urgently needed within the African context in view of the high diseases burden (Ahmed et al., 2014; Bosch et al., 2014;

Dandara et al., 2014; EIRakaiby et al., 2014). This study aimed to determine the role of *MBL2* genetic variants, promoter region variants, and *MBL2* haplotypes in HIV-1 infection and vertical transmission in a subset of the blood samples collected during the national prevention of mother to child transmission (PMTCT) survey. The survey was carried out in 2012 by the Centre for Sexual Health and HIV/AIDS Research (CESHAAR) in Zimbabwe.

Materials and Methods

Study design and study population

This was a laboratory-based study and used dried blood spot samples that were collected as part of a national cross-sectional survey (McCoy et al., 2015a, 2015b) on PMTCT carried out by the CESHAAR Research Centre, Harare Zimbabwe. The primary study was entitled Evaluation of Zimbabwe's Accelerated PMTCT of HIV Program (Buzdugan et al., 2012; McCoy et al., 2015a, 2015b). This article reports the investigations on *MBL2* genotyping on a subset of the dried blood samples collected during this national PMTCT survey (Fig. 1). Details of the PMTCT national survey, including blood sample collection, screening procedures, and the study population, have been described in detail elsewhere (Buzdugan et al., 2012). Briefly, the PMTCT survey was undertaken in September 2012 and targeted women aged 16 years and above who had children between 9 and 15 months old (McCoy et al., 2015a, 2015b). The survey examined the uptake of services and behaviors in the PMTCT in Zimbabwe and determined the factors associated with MTCT and maternal antiretroviral therapy (ART) or antiretroviral (ARV) prophylaxis. Dried blood spots were collected from 8800 babies and their biological mothers (McCoy et al., 2015a, 2015b).

Sample size calculation

The exposure of interest was MBL deficiency (using *MBL2* haplotype as a proxy) among HIV-infected mothers, and the primary outcome was infant HIV status. Transmission rates range from 5% to 20% for mother–infant pairs on ART (UNAIDS, 2013). Assuming an alpha of 0.05 and beta of 0.90, 95% confidence interval, and 10% infant HIV status among the infants born from mothers with normal *MBL2* genotype, we needed a sample size, among the exposed population, of 572 mother–infant pairs, 1144 individual blood spots in total. Fifty-eight (10%) more samples were selected to allow for dried blood spots that would not give adequate DNA for PCR, to give a final sample size of 632 mother baby pairs calculated using Epi Info 7 program (CDC, 2015). DNA was extracted from all 632 mother–baby pairs (Fig. 1). Complete data set on HIV status and *MBL2* genotype was available on 622 mother–baby pairs used in the final analysis.

Ethics statement

The CESHAAR PMTCT study was approved by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/1655), University of California and University of London Human Research Ethics Committees. The *MBL2* substudy was also approved by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/1770) and University of the

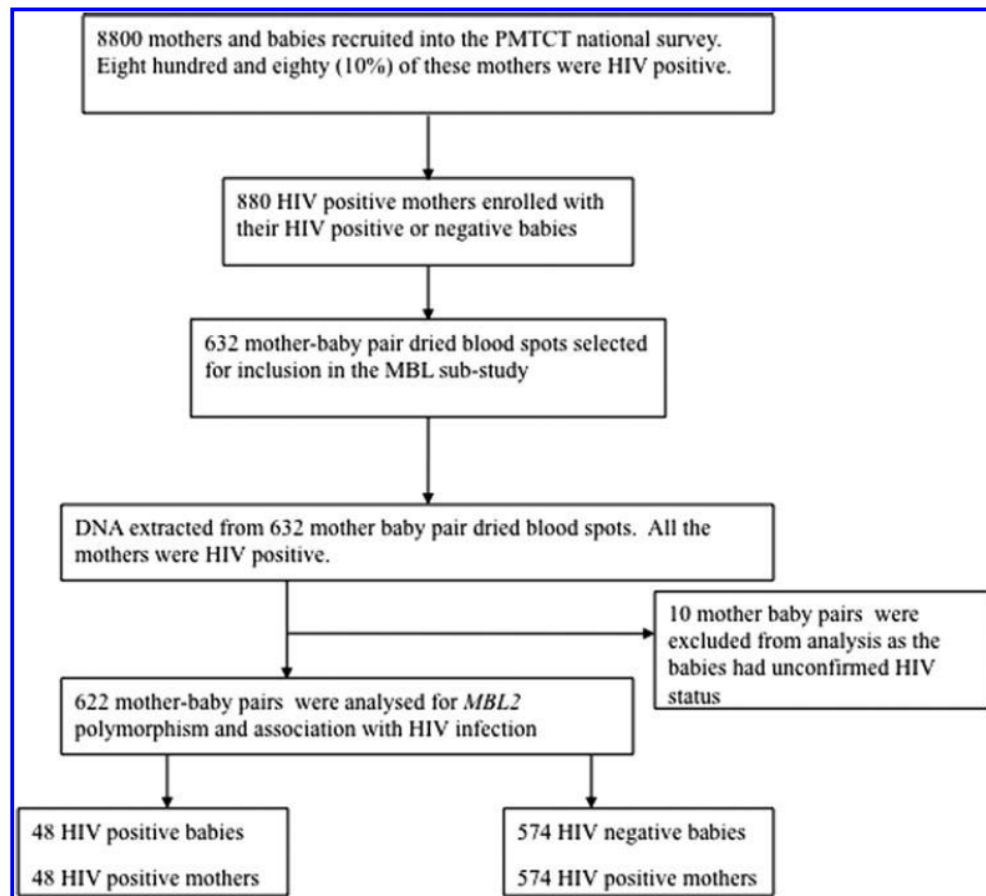


FIG. 1. Participant Flow Chart showing the recruitment procedures for the PMTCT survey. Eight thousand eight hundred mother–baby pairs were enrolled in the national PMTCT survey, and 880 mothers were HIV positive. The 632 mother–baby pair dried blood spots selected for inclusion in the MBL substudy had adequate *left over* sample for DNA analysis. DNA was extracted from 632 mother–baby pair dried blood spots for *MBL2* genotype analysis by the pyrosequencing method. Ten mother–baby pairs were excluded from analysis because of incomplete information. HIV, human immunodeficiency virus; MBL, mannose binding lectin; PMTCT, prevention of mother to child transmission.

Witwatersrand Human Research Ethics Committee (M130348). All the mothers who took part in the main PMTCT National Survey gave written informed consent for specimen collection, storage, and future laboratory studies.

Laboratory procedures

Collection of dried blood spots. Venous blood from the heel of the child and a finger of the mother was collected on filter papers (PerkinElmer 226, Health Sciences). Blood was collected on five designated spots on the filter papers. The filter papers were air-dried overnight and then packed the next day in zip lock bags with desiccant granules to prevent humid formation and keep the spots dry. The zip lock bags were kept at room temperature until analysis of the spots.

HIV testing. All the mothers were initially tested for HIV antibodies using EIA HIV test kits (OyToilette 3, FIN-01720; AniLabsystems Ltd). All the mothers that tested HIV positive on the initial screening tests were confirmed using Enzygnost Anti-HIV ½ Plus ELISA (Dade Behring), and indeterminate results were resolved by Western Blot. All the children as they were below 2 years of age were tested using HIV polymerase chain reaction test kits (Roche Amplicor HIV-1 DNA Test, version1.5) (McCoy et al., 2015b).

DNA extraction. Genomic DNA was extracted from dried blood spots using SIGMA Extract and Amp kit. Briefly, two 3 mm punches were made for each dried blood sample and incubated in 20 μ L Lysis solution (SIGMA) at 75°C for 30 min. Subsequently, 180 μ L of Neutralizing solution (SIGMA) was added and mixed by pipetting in the plate wells and centrifuged for 1 min at 1000 rpm. The extracted DNA quantified using the Qubit kit according to the manufacturer's instructions and stored at 4°C.

Pyrosequencing method

Amplification of MBL2 genes by Initial general PCR. DNA was amplified by PCR using the following reaction mixture (per sample): 41.125 μ L Milli-Q water, 0.625 μ L dNTPs at 10 mM each (from Pharmacia Biotech), 0.25 μ L of 5U/ μ L HotStar DNA Taq Polymerase Enzyme (QIAGEN), 1 μ L of 20 pmol of *MBL2* Exon 1, *H/L* and *Y/X* labeled forward and reverse PCR primer (DNA Technology), 5 μ L of 10 \times HotStar PCR buffer, and a 2 μ L sample DNA extract. A positive control (a Danish DNA sample of known *MBL2* genotype) and a negative control (no DNA added) were included in each PCR run. The oligonucleotide primer sequences used for *MBL2* genotyping are as shown (Supplementary Table S1). The following thermocycling program was used: an initial

denaturation step at 95°C for 15 min, followed by 38 cycles of 95°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec; the reaction was completed by a final elongation/extension step at 72°C for 10 min. The PCR was expected to produce one specific amplicon of ~240 bp; this was evaluated by agarose gel electrophoresis. This PCR product was used for the *MBL2* pyrosequencing reaction.

Visualization of PCR products by agarose gel electrophoresis. The PCR products were separated by a 2% agarose gel (Invitrogen) electrophoresis run in an electrophoresis chamber (Sub-cell Biorad) at 150V, 400 mA for 32 min, including the positive and negative control samples. The gels were then carefully transferred to the UV Transilluminator (Syngene Synoptics Ltd) where a photograph of the gel was taken. Samples that showed the required *MBL2* band were used for pyrosequencing (Fakruddin et al., 2012; Nyren 2007; Ronaghi 2001; Roos et al., 2006; Vengen et al., 2012)

Pyrosequencing reaction. In preparation for the pyrosequencing reaction, 25 μ L of biotinylated PCR product was mixed with 3 μ L of streptavidin-coated magnetic beads (Streptavidin Sepharose High performance; GE Healthcare Life Sciences), 37 μ L of binding buffer (Qiagen), and 15 μ L Milli-Q water. This mixture was agitated for 10–20 min at 1000 rpm on a plate shaker to allow the biotinylated PCR product to bind to the streptavidin sepharose beads. Subsequently, immobilized DNA was transferred, using a PSQ 9 magnetic sample prep tool (Pyrosequencing; Biotage), through a series of washing steps and finally released into a primer plate (Pyromark Q96; Qiagen) containing 39 μ L annealing buffer (Qiagen), 1 μ L of 16 pmol/ μ L of each specific pyrosequencing primer that is, Exon1, *HL*, and *YX* (DNA Technology) (Supplementary Table S1). This plate was incubated for 2 min at 80°C followed by cooling in the pyrosequencing machine (Pyromark Q96; Qiagen) for 10 min. Enzymes (DNA polymerase, ATP sulfurylase, luciferase, and apyrase), luciferine, and adenosine-5-phosphosulphate (APS, Pyromark Q96 cartridge reagents kit; Qiagen) were added to the cartridge wells according to instructions from the installed pyro program.

***MBL2* genotypes and haplotype functional groups.** Participants were classified into the different *MBL2* Exon 1 genotypes, normal *MBL2* allele designated as *A* and point mutations in the *MBL2* gene and the variants designated as *B* (codon 54, rs1800450 A>G), *C* (codon 57, rs1800451 A>G), and *D* (codon 52, rs5030737 T>C) and the different combinations of variants (Madsen et al., 1995, 1998). Participants were classified into six *MBL2* haplotype functional groups, which were combined into three groups for statistical analysis: haplotypes that give normal plasma MBL levels (*YA/YA*, *YA/XA*), intermediate levels (*XA/XA*, *YA/YO*), and deficient levels (*XA/YO*, *YO/YO*) (Garred et al., 2003; Vengen et al., 2012). We neither had plasma nor serum samples to determine the plasma MBL concentration for our study population, but used *MBL2* genotypes as a proxy for plasma MBL levels (Amoroso et al., 1999; Arraes et al., 2006; Boniotto et al., 2000, 2003; Crovella et al., 2005; Dzwonek et al., 2006; Kuhn et al., 2006; Mangano et al., 2008; Singh et al., 2008) and to classify individuals into normal plasma MBL levels, intermediate levels, and deficient levels (Amoroso et al.,

1999; Arraes et al., 2006; Boniotto et al., 2000, 2003; Crovella et al., 2005; Dzwonek et al., 2006; Kuhn et al. 2006; Mangano et al. 2008; Singh et al., 2008).

Statistical analyses. All statistical analyses were done using Stata 11 statistical package (STATA Corp, Timberlake Consultants). For *MBL2* genotype analysis, normal homozygous *MBL2* is denoted as *A/A*. The heterozygous variant *MBL2* genotypes were grouped together as *A/O* heterozygous *MBL2* as they all give low plasma MBL levels, and all homozygous and compound homozygotes were grouped together as *O/O*. Conformation to the Hardy–Weinberg equilibrium (HWE) was determined using the Chi Square test in the SHEsis online program (Li et al., 2009; Shi and He, 2005). The frequencies of the *MBL2* alleles were obtained by direct gene counting. To determine prevalence of MBL deficiency, *MBL2* genotypes were used as proxy for MBL levels, *MBL2 A/A* genotype was considered as normal, and those with *A/O* and *O/O* categorized as deficient MBL. Participants were classified into six *MBL2* haplotype functional groups, which were combined into three groups for statistical analysis: haplotypes that give normal plasma MBL levels (*YA/YA*, *YA/XA*), intermediate levels (*XA/XA*, *YA/YO*), and deficient levels (*XA/YO*, *YO/YO*), and promoter region haplotypes (Garred et al., 2003; Vengen et al., 2012). Differences in proportions of MBL deficiency were determined using Chi Square or Fisher's exact tests. The differences in distribution frequency between HIV-1 infected and uninfected children, of the *MBL2* genotypes, promoter region variants, and promoter haplotypes were determined by the Chi Square test or Fisher's exact tests. The effect of maternal MBL deficiency on HIV status of the children was determined in a logistic regression model after controlling for use of ART. The level of statistical significance used was $p < 0.05$.

Results

Demographic characteristics

The national PMTCT survey collected 8800 dried blood spots from mother baby pairs for HIV testing; among these, 880 mothers (10%) were HIV positive (Fig 1). Dried blood spots with an adequate sample were selected from 632 HIV-positive mothers and their children for inclusion in this substudy, that is, 1264 dried blood spots were selected for MBL analysis (Fig 1). The mothers' median age (interquartile range) was 30 (26–34) years old and the children were 12 (11–15) months old at the time of enrollment. Some of the HIV-positive mothers reported that they and their children were receiving ART (McCoy et al., 2015a, 2015b).

Frequency of *MBL2* alleles and haplotypes of mothers and children

DNA was extracted from all 632 mother–child pair dried blood spots. Ten mother–baby pairs were excluded from analysis because the children had unconfirmed HIV status by the time of data analysis. We report here *MBL2* analysis of 622 mother–baby pairs with complete information on *MBL2* genotyping and HIV status. All 622 mothers were HIV-1 infected; there were 574 HIV-negative babies and 48 HIV-1-positive babies. The *MBL2* exon 1 alleles *A/A*, *A/C*, *C/C*, *–550(H/L)*, and *–221(Y/X)* of the HIV-negative babies conformed to HWE (Supplementary Table S2). The *MBL2* exon

1 alleles *A/A*, *A/C*, *C/C*, and *-550 (H/L)* of the 622 HIV-positive mothers conformed to HWE, but allele *-221(Y/X)* deviated from HWE ($p < 0.05$) (Supplementary Table S2). *MBL2* alleles *B (codon 54, rs1800450A>G)* and *C (codon 57A>G)* are the variant alleles detected in this study population. Prevalence of MBL deficiency was 34% among the mothers and 32% among the children. Frequency of heterozygous exon 1 variants among the mothers was *AB* (0.3%) and *A/C* (31%) and among the infants was *AB* (0.8%) and *A/C* (29%). The homozygous *MBL2* variant *C/C* was 2% in both mothers and their babies (Table 1).

Relationship between mothers' and infants' MBL2 genotype, haplotypes, and HIV status

We determined the relationship between *MBL2* genotype in the mothers and the HIV status of their children. We found no statistically significant difference in the distribution of the HIV infected and uninfected children between the *MBL2* exon 1 genotypes of the mothers ($p = 0.546$) (Table 2). We also found no association between HIV infection and all the promoter genotypes detected and the three *MBL2* functional classes (Table 2). We also found no statistically significant difference in the distribution of HIV infected and uninfected children in relation to the children's *MBL2* genotypes, all promoter genotypes detected and the three *MBL2* functional classes (Table 3). Participants were classified into six *MBL2* haplotype functional groups, which were combined into three groups for statistical analysis: haplotypes that give normal plasma MBL levels (*YA/YA*, *YA/XA*), intermediate levels (*XA/XA*, *YA/YO*), and deficient levels (*XA/YO*, *YO/YO*) (Garred et al., 2003; Vengen et al., 2012). We found no statistically significant difference in distribution of HIV infected and uninfected children between these *MBL2* functional groups of the mothers (Tables 2) and of the children (Table 3).

TABLE 1. FREQUENCY OF *MBL2* GENOTYPES OF THE MOTHERS AND THEIR CHILDREN DETERMINED USING THE PYROSEQUENCING TECHNIQUE

<i>MBL2</i> genotypes, promoter region alleles and haplotypes	Mothers n (%)	Children n (%)
<i>AA</i>	411 (66)	423 (68)
<i>AB</i>	2 (0.32)	5 (0.8)
<i>AC</i>	196 (31)	181 (29)
<i>CC</i>	13 (2)	13 (2)
Total	622	622
<i>HH</i>	0 (0)	1(0.2)
<i>HL</i>	59 (9)	66 (11)
<i>LL</i>	563 (91)	555 (89)
Total	622	622
<i>YY</i>	440 (71)	450 (72)
<i>XY</i>	178 (29)	163 (26)
<i>XX</i>	4 (0.6)	9 (9)
Total	622	622
Functional <i>MBL2</i> genotype classification		
Normal MBL levels <i>YA/YA</i> , <i>YA/XA</i>	413 (66)	416 (67)
Intermediate levels <i>XA/XA</i> , <i>YA/YO</i>	136 (22)	147 (24)
Deficient levels <i>XA/YO</i> , <i>YO/YO</i>	73 (12)	59 (9)
Total	622	622

This analysis was done using direct gene counting, $n = 622$ mother-baby pairs, HIV-positive children $n = 48$, HIV-negative children $n = 574$. HIV, human immunodeficiency virus; MBL, mannose binding lectin.

The effect of MBL deficiency on HIV status of children after controlling for ART

All the mothers were HIV positive, 72.3% (450 out of 622 mothers) were on ART, Nevirapine, AZT, and Nevirapine and AZT. Of the 622 perinatally exposed children, 71.5% (445 out of 622) of the children were on ART during the time of the survey using such drugs as Nevirapine only, Nevirapine and AZT syrup, and AZT only. The effect of MBL deficiency of the mothers on HIV status of the children was determined in a logistic regression model after controlling for use of ART by the mothers and was found not statistically significant ($p = 0.624$), OR 1.12 (95% CI), and the test of the overall model was also not statistically different ($p = 0.421$). The effect of MBL deficiency of the babies on their HIV status after controlling for use of ART by the children in a logistic model was not significant ($p = 0.423$), OR 1.18 (95% CI), and the test of the overall model was also not statistically different ($p = 0.564$).

The effect of MBL deficiency on HIV status of infants after controlling for type of ART used

Out of 622 mothers included in our analysis, 456 mothers were reported to be on maternal ARVs; additional information about what drugs they used is known for 306 mothers: 116 were on NVP only, 39 were on AZT only, 126 were on NVP and AZT, 7 were on HAART, and for 18 others the ARVs were not specified. The effect of MBL deficiency of the mothers on HIV status of the children was additionally determined in a logistic regression model after controlling for type of ART used by the mothers and was found not statistically significant ($p = 0.633$), OR 0.83, and the test of the overall model was also not statistically different ($p = 0.877$).

Out of 622 children included in this analysis, 449 children were reported to be on infant ARVs: 262 were on NVP & AZT syrup, 132 were on NVP only, 13 were on AZT only, and 42 children had unspecified ARVs. The effect of MBL deficiency of the babies on their HIV status was additionally determined in a logistic regression model after controlling for type of ART used by the children was not significant ($p = 0.567$), OR 1.18, and the test of the overall model was also not statistically different ($p = 0.402$).

Discussion

The main aim of our study was to determine prevalence and role of polymorphic *MBL2* genotypes in vertical transmission among HIV-1-positive mothers and their HIV-1 infected or uninfected children enrolled in a national PMTCT survey. The prevalence of MBL deficiency as determined by presence of variant *MBL2* genotypes *A/O* and *O/O* was high, at 34% among the mothers and 32% among the children, similar to other reports in children (Kuhn et al., 2006). We found no association between MBL deficiency of the mothers and of the children, HIV infection in the children and vertical transmission. We used *MBL2* genotyping as a proxy to determine MBL deficiency in children, similar to reports by others (Amoroso et al., 1999; Arraes et al., 2006; Boniotto et al., 2000, 2003; Crovella et al., 2005; Dzwonek et al., 2006; Kuhn et al., 2006; Mangano et al., 2008; Singh et al., 2008; Zupin et al., 2016).

We detected currently known *MBL2* Exon 1 coding and promoter region variants (Madsen et al., 1994, 1995, 1998) in

TABLE 2. DISTRIBUTION OF HIV-1 INFECTED AND UNINFECTED CHILDREN ACCORDING TO THE MOTHERS' *MBL2* GENOTYPE AND *MBL2* HAPLOTYPE COMBINATIONS AND PROMOTER REGION ALLELES

<i>Mothers' MBL2 genotypes, promoter region allele, and haplotypes</i>	n	<i>HIV-positive children, n (%)</i>	<i>HIV-negative children, n (%)</i>	χ^2 or Fisher's exact test	p
AA	411	30 (63)	381 (66)	χ^2 test	0.546
AB/AC	198	16 (33)	182 (32)		
CC	13	2 (4)	11 (2)		
Total	622	48	574		
HL	59	7 (15)	52 (9)	χ^2 test	0.210
LL	563	41 (85)	522 (91)		
Total	622	48	574		
YY	440	32 (66)	408 (71)	Fisher's exact test	0.280
YX	178	15 (31)	163 (28)		
XX	4	1 (2)	3 (0.5)		
Total	622	48	574		
Functional <i>MBL2</i> genotype classification					
Normal MBL levels YA/YA, YA/XA	413	30 (62.5)	383 (67)	Fisher's exact test	0.827
Intermediate levels XA/XA, YA/YO	136	12 (25)	124 (21)		
Deficient levels XA/YO, YO/YO	73	6 (12.5)	67 (12)		
Total	622	48	574		

This analysis was done using the Chi Square and Fisher's exact tests, $n=622$ mother-baby pairs, HIV-positive children $n=48$, HIV-negative children $n=574$.

our study population, in accordance with reports from other African populations (Garred et al., 1997; Lipscombe et al., 1992; Madsen et al., 1998; Madsen et al., 1995). We detected *MBL2* variants *B* (codon 5A>G) and *C* (codon 57, *rs1800451 A>G*) in our study population. The available evidence on association between HIV infection and MBL deficiency in children is conflicting (Eisen et al., 2008; Israels et al., 2012). Our results showed no association between mothers' *MBL2* Exon 1 variants and HIV vertical transmission, in accordance with another published study on Zimbabwean children (Mhandire et al., 2014) and among Zambian mothers and their children (Zupin et al., 2016). In contrast to our findings, other studies reported an association between HIV vertical transmission and MBL deficiency, showing a

statistically significant higher frequency of *MBL2* Exon 1 variants among HIV-infected children (Amoroso et al., 1999; Arraes et al., 2006; Boniotto et al., 2000, 2003; Crovella et al., 2005; Dzwonek et al., 2006; Kuhn et al., 2006; Mangano et al., 2008; Singh et al., 2008).

Promoter region variants have been reported to significantly affect plasma/serum MBL concentration (Madsen et al., 1995). The *H/L* promoter alleles are associated with high (*H/H*), medium (*H/L*), and low (*L/L*) plasma/serum MBL expression; the *MBL2* promoter region genotypes in combination with Exon 1 region genotypes result in *MBL2* haplotypes with varying levels of serum/plasma MBL concentrations (Boldt and Petzl-Erler, 2002; Boldt et al., 2006, 2006, 2010; Madsen et al., 1995, 1998; Steffensen et al.,

TABLE 3. DISTRIBUTION OF HIV-1 INFECTED AND UNINFECTED CHILDREN ACCORDING TO THE *MBL2* GENOTYPE, *MBL2* HAPLOTYPE COMBINATIONS, AND PROMOTER REGION ALLELES OF THE CHILDREN

<i>Children MBL2 genotypes, promoter region allele, and haplotypes</i>	n	<i>HIV-positive children, n (%)</i>	<i>HIV-negative children, n (%)</i>	χ^2 or Fisher's exact test	p
AA	423	31 (65)	392 (68)	χ^2 test	0.863
AB/AC	186	16 (33)	170 (30)		
CC	13	1 (2)	12 (2)		
Total	622	48	574		
HH/HL	67	4 (8)	63 (11)	χ^2 test	0.571
LL	555	44 (92)	511(89)		
Total	622	48	574		
YY	450	34 (71)	416 (72)	Fisher's exact test	0.915
YX	163	13 (27)	150 (26)		
XX	9	1 (2)	8 (1.4)		
Total	622	48	574		
Functional <i>MBL2</i> genotype classification					
Normal MBL levels YA/YA, YA/XA	416	31 (65)	385 (67)	χ^2 test	0.441
Intermediate levels XA/XA, YA/YO	147	10 (21)	137 (24)		
Deficient levels XA/YO, YO/YO	59	7 (15)	52 (9)		
Total	622	48	574		

This analysis was done using the Chi Square and Fisher's exact tests, $n=622$ mother-baby pairs, HIV-positive children $n=48$, HIV-negative children $n=574$.

2000). In our study, the *L/L* allele had the highest frequency among both mothers and children, and *H/H* allele had the lowest frequency among both mothers and their infants, in accord with reports on African populations (Madsen et al., 1995). The *H* allele is found predominantly in white populations who also have very low *L* and *X* alleles (Madsen et al., 1995). Our results showed that the *MBL2* variant haplotypes were not associated with HIV infection in the mothers, the children, or with MTCT. Mangano et al. (2008) also reported no difference in frequency of *XY* and *H/L* alleles between HIV infected and uninfected children (Mangano et al., 2008); others have also reported no association with *MBL2* haplotypes (Mhandire et al., 2014; Zupin et al., 2016).

Studies done on African populations which reported results similar to ours include (Dzwonek et al., 2006; Kuhn et al., 2006; Mhandire et al., 2014; Zupin et al., 2016). We report here the frequency of *A/O MBL2* variants among the children to be 35%, comparable to other reports of 24% (Mhandire et al., 2014) and 32.4% (Kuhn et al., 2006) on African children. Elsewhere frequencies of *A/O MBL2* variants are reported as 41% among Hispanic children and 35% among white children (Singh et al., 2008). We found that a very low frequency of *C/C* homozygous *MBL2* variant was 2% among HIV infected children, but others have reported frequencies of 20% among black children, 7% among Hispanics, and 5% among white children (Singh et al., 2008). We used a sample size of 622 mother and baby pairs with a 90% power to detect presence of any association between MBL deficiency, HIV infection in the children, and MTCT.

Most of the studies reporting effect of MBL deficiency on pediatric HIV infection were done during the pre-ART era, but the children in our study were on ART or were exposed to ART through treatment given to their mothers. We found MBL deficiency was not associated with HIV infection in a logistic regression model after controlling for ART in the children and their mothers. The only available study that has determined association between HIV paediatric infections and MBL deficiency in the context of ART (Dzwonek et al., 2006) found that despite the complications of performing this analysis in the era of HAART, *MBL2* variants were associated with HIV infection and disease progression in children. The only study available in literature that showed association between *MBL2* haplotypes *XAXA* and HIV infection and faster disease progression was done in Argentina (Mangano et al., 2008).

In our study, distribution of the *MBL2* allele $-221(Y/X)$ for the HIV-positive mothers deviated from HWE. Deviation of genotypes from HWE has been attributed to stratification of study populations or genetic selection pressure (Das and Panda, 2015; Hosking et al., 2004; Panda et al., 2013). In our study, we selected HIV-positive mothers for inclusion so the possible reason for the deviation of allele $-221(Y/X)$ could be stratification. HIV-negative mothers were not included in this study as our main aim was to determine the role of *MBL2* genotype on HIV vertical transmission. Deviation from HWE has been reported from a study carried out in Argentina, which showed deviation from HWE among HIV-positive children (Mangano et al., 2008), similar to our findings among HIV-positive mothers.

We carried out similar research in an adult population in Zimbabwe and found that MBL deficiency neither played a role in HIV infection (Zinyama-Gutsire et al., 2015a,b) nor in disease progression and survival (Zinyama-Gutsire et al., 2015a).

A limitation in our study was that we did not have serum/plasma samples to determine MBL concentration. However, *MBL2* exon 1 region and promoter region polymorphisms can be used as proxy to classify participants into normal plasma/serum MBL levels, intermediate, and deficient (Boldt and Petzl-Erler, 2002; Boldt et al., 2006, 2006, 2010; Madsen et al., 1994, 1995, 1998; Steffensen et al., 2000; Zupin et al., 2016).

Conclusion

In conclusion, our results showed a high prevalence of *MBL2* genetic variant *AC* among the mothers and their children, in accordance with other studies of *MBL2* genetic variation in African populations (Madsen et al., 1995; Mhandire et al., 2014; Zupin et al., 2016). This *MBL2* variant form is associated with MBL deficiency; however, the high prevalence of MBL deficiency among the mothers and their children in our study was not associated with HIV-1 vertical transmission. It is important to explore the genetic makeup of the Zimbabwean population in view of the high prevalence of several viral, parasitic, and bacterial infections considering the conflicting evidence associating MBL deficiency and protection against some infections and increased susceptibility in others. It is our hope that more OMICS research in African countries will provide more knowledge on the hypothesis role of the MBL protein in HIV infection and vertical transmission, a current major health problem in African populations.

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Authors' Contributions

All authors have met the ICMJE criteria for authorship and have read the article and approved the final version.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Abbreviations Used

- ART = antiretroviral therapy
- CESHAAR = Centre for Sexual Health and HIV/AIDS Research
- HIV = human immunodeficiency virus
- IQR = interquartile range
- MBL = mannose binding lectin
- MTCT = mother to child transmission
- PMTCT = prevention of mother to child transmission

Supplementary Data

SUPPLEMENTARY TABLE S1. THE OLIGONUCLEOTIDE PRIMER SEQUENCES THAT WERE USED IN THIS STUDY FOR DETECTION OF *MBL2* CODING AND PROMOTER NORMAL AND VARIANT ALLELES

<i>Oligonucleotide name</i>	<i>Primer name</i>	<i>Specific primer sequence</i>		<i>Primer modification</i>
		<i>5'-3'</i>		
MBL exon 1 codons 52, 54 and 57	Forward	5' AGT ATG GTG GCA GCG TCT TAC TCA G 3'		5' biotin
	Reverse	5'/5Bio/GGC AGT TTC CTC TGG AAG GTA AAG A 3'		
	Sequencing	5' CAG GCA AAG ATG GG 3'		
<i>H/L</i>	Forward	5'/5Bio/GCC AGG GCC AAC GTA GTA A 3'		5' biotin
	Reverse	5' GAG TTT GCT TCC CCT TGG T 3'		
	Sequencing	5' TCC CCT TGG TGT TTT 3'		
<i>Y/X</i>	Forward	5'/5Bio/TCC CCT AAG CTA ACA GCC ATA AG 3'		5' biotin
	Reverse	5' TGA TGA GCA GTG GGG ATC CTA 3'		
	Sequencing	5' AAG ACT ATA AAC ATG CTT TC 3'		

SUPPLEMENTARY TABLE S2. *MBL2* GENOTYPES AND PROMOTER SNPs DETERMINED BY THE PYROSEQUENCING TECHNIQUE AND CONFORMATION TO THE HARDY–WEINBERG EQUILIBRIUM

<i>MBL2 variant</i>	<i>Reference SNP ID number (rs#)</i>	<i>Alleles</i>	<i>Alternative nomenclature</i>	<i>Location</i>	<i>HWE children</i>	<i>HWE mothers</i>
-550	11003125	G/C	H/L	Promoter	0.505	0.214
-221	7096206	C/G	X/Y	Promoter	0.178	0.002
Exon 1						
B (codon 54)	1800450	A/G	A/B			
C (codon 57)	1800451	G/A	A/C			
D (codon 52)	5030737	C/T	A/D	Exon 1	0.455	0.264

The HWE for the above *MBL2* SNPs was determined among the 622 mothers and their children separately. HWE, Hardy–Weinberg equilibrium.

Appendix D: Manuscript under review

Optimization of *MBL2* genotyping assay using dried blood spots: Experiences from the PMTCT Survey in Zimbabwe.

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Abstract

Background

Use of dried blood spots in detection of infections and molecular testing has increased over the years because of ease of collection and storage especially for developing countries where storage of blood samples is a challenge. But there are still lessons to be learnt in use of dried blood spots for laboratory assays and areas for improvement. We share our experiences using stored dried blood spot samples for *MBL2* genotyping to determine prevalence of MBL deficiency in mother–baby pairs. Mannose Binding Lectin (MBL) is a normal plasma protein produced in the liver and is a key component of the innate immune system, MBL deficiency leads to increased susceptibility to infections like HIV.

Objectives: To share our experiences optimising use of stored dried blood spot samples for determination of MBL deficiency using *MBL2* genotyping pyrosequencing technique.

Methods: We assessed *MBL2* polymorphism in Zimbabwean mothers and their children enrolled in a national PMTCT survey carried out in 2012. The dried blood spots were collected on Perkin Elmer 226 filter papers and had been stored for 1 year 8 months at room temperature before *MBL2* genotyping was done. We used two methods to optimise DNA extraction, initially Sigma Generation kit and later used Gene Extract and Amp kit to extract DNA from dried blood spots of 632 mothers-infant pairs. *MBL2* genotyping of the 632 mother-baby pair samples was done using the pyrosequencing technique.

Results: We optimized DNA extraction from 632 mother-baby pair dried blood spots. We could not amplify by PCR the DNA extracted using the Sigma Generation kit so we changed to Gene Extract and Amp kit. We initially used Taq DNA Polymerase enzyme and changed

to TEMPaseHot Start DNA Polymerase enzyme which gave clearer bands during PCR amplification. DNA extraction using the Gene Extract and Amp kit and amplification using the TEMPaseHot Start DNA Polymerase enzyme gave the best and optimal results.

Conclusion

DNA extraction from dried blood spots was optimal and gave the best PCR bands using the Gene Extract and Amp kit and amplification using the TEMPaseHot Start DNA Polymerase enzyme.

Key words: Mannose Binding Lectin (MBL), *MBL2* genetic polymorphism, Perkin Elmer 226 filter paper, Gene Extract and Amp kit, pyrosequencing.

Short Title: Optimization of *MBL2* genotyping by pyrosequencing using dried blood spots

Introduction

Use of dried blood spots in detection of infections has increased over the years because of ease of collection and storage (1) especially for developing countries where storage of blood samples is a challenge (2). Available literature shows an increase in use of dried blood spots for detection of infections, genetic testing 3, 4 and for *MBL2* genotyping (5, 6), but there are still lessons to be learnt and areas for improvement (2). There are several types of filter paper brands available made of cellulose and the filter papers vary in their thickness and pore size. Only two brands, Whatman 903 also called Guthrie cards and 226 Perkin Elmer filter papers are approved for use by WHO and the US Food and Drug Administration for collection and storage of human whole blood (7, 8). There are reports of use of Whatman 903 or Guthrie

cards (1, 3, 5, 7, 9-14) and others have used 226 Perkin Elmer filter paper (7) for collection and storage of dried blood spots for analysis of infections. Mannose Binding Lectin (MBL) is a normal plasma protein produced in the liver and is a key component of the innate immune system. In the absence of plasma samples, *MBL2* genotyping can be used as proxy to determine MBL deficiency. Currently available literature shows that *MBL2* genotyping can be done on stored dried blood spots collected on Guthrie cards (3, 5, 6, 12, 14, 15). We share our experiences using stored dried blood samples collected on 226 Perkin Elmer filter paper for determination of MBL deficiency using *MBL2* genotyping techniques, using the pyrosequencing method on mother-baby dried blood spot samples collected in Zimbabwe.

Materials and Methods

Study design

This was a laboratory based study where we optimized DNA extraction and *MBL2* genotyping (16). The dried blood spot samples used for this sub-study were collected during a national survey on prevention of mother to child transmission (PMTCT) carried out in 2012 by the Centre for Sexual Health And HIV/AIDS Research (CESHAAR), Harare Zimbabwe, entitled Evaluation of Zimbabwe's Accelerated prevention of Mother-to-Child Transmission of HIV Program (17-19). This paper reports our experiences and processes in more detail as we optimised the DNA extraction methods and PCR amplification on 632 dried blood spots of mother-baby pairs collected and stored during the survey.

Study population

The PMTCT survey carried out by the CESHAAR Research team in September 2012 targeted women aged 16 years and above who had children between 9 and 18 months old.

Details including blood sample collection, screening procedures, the setting, the study

population have been described in detail elsewhere(17-19). Briefly, recruitment into the CESHAAAR study, sample collection and HIV laboratory assays took place between September 2012 and September 2013.

Ethics Statement

The CESHAAAR PMTCT study was approved by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/1655), University of California and University of London Human Research Ethics Committees. The *MBL2* sub-study was also approved by the National Research Ethics Committee of the Medical Research Council of Zimbabwe (MRCZ/A/1770) and University of the Witwatersrand Human Research Ethics Committee (M130348) (16). All the mothers who took part in the main PMTCT National Survey gave written informed consent for specimen collection, storage and future laboratory studies. Permission to ship the dried blood spot samples to Denmark was granted by the Research Council of Zimbabwe, certificate No. 02338 of 2016.

Collection of dried blood spots

Venous blood was collected on filter papers (Perkin Elmer 226, Health Sciences, USA) after punching the heel of the child or finger of the mother. Blood was collected on five designated spots on the filter papers. The filter papers were air dried overnight and then packed the next day in zip lock bags with desiccant granules to prevent humid formation and keep the spots dry. The zip lock bags were kept at room temperature until analysis of the spots.

DNA extraction

For routine *MBL2* genotyping at Serum State Institute DNA extracted from whole blood samples is normally used. To optimise DNA extraction from the PMTCT dried blood spots for our study, two different methods were therefore selected to be used, the Sigma Generation kit and SIGMA Extract and Amp kit, compared amplification of the DNA extracts by PCR, as fully described below:

1. Sigma Generation kit

Initially DNA was extracted using the Sigma Generation kit. Two 3mm punches [5] were made for each dried blood sample and put in a 200 μ l 96 well PCR plate (DNA/NDase/RNase/PCR inhibitor free, SARSTEDT, UK). One hundred and forty (140 μ l) of assay buffer were added and the plates vortexed for 45 minutes to clean the dried blood spots. Supernatant was discarded and another 140 μ l assay buffer added, vortexed for 45 minutes and supernatant discarded. Ninety (90 μ l) Generation DNA Solution 1 added and allowed to stand for 1 minute and supernatant discarded, this step repeated once more. Ninety (90 μ l) Generation DNA Solution 2 added and allowed to stand for 1 minute and supernatant discarded. Sixty (60 μ l) of filtered milli-Q water was added and extract vortexed at -20°C and transferred to a PCR machine set for 1 cycle at 99°C for 25 minutes to stabilize the DNA. The extracted DNA dissolved in the water was stored in a freezer at -20°C until ready for use in PCR.

2. SIGMA Extract and Amp kit

Genomic DNA was extracted from dried blood spots using SIGMA Extract and Amp kit. Two 3mm punches were made for each dried blood sample and put in a 96 well PCR plate (DNA/NDase/RNase/PCR inhibitor free, SARSTEDT, UK). Plates were covered with

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8 well PCR plate cover strips (SARSTEDT, UK). Twenty (20µl) Lysis solution (SIGMA) was added per well using sterile pipette tips (DNA/NDase/RNase/PCR inhibitor free, SARSTEDT, UK). Plates were incubated at 75°C for 30 minutes and cooled at 10 °C in a programmed PCR thermocycler (Applied Biosystems, 2720 thermal cycler version 2.09). 180µl of Neutralizing solution (SIGMA) stored at room temperature, mixed by pipetting in the plate wells and spun down in a plate centrifuge for 1 min at 1000rpm and the extracted DNA was stored at 4°C. DNA was quantified using the Qubit kit according to manufacturer's instructions. Amplification of *MBL2* genes by Initial general PCR, Visualisation of PCR products by agarose gel electrophoresis, Pyrosequencing and Sanger Direct Genetic Screening were carried out according to manufacturer's instructions

Optimisation of DNA extraction

Initially DNA extraction was done using Sigma Generation kit. We could not amplify by PCR the DNA from this method. Then extraction was done using the second kit SIGMA Extract and Amp kit and DNA extracts from this method could be amplified by PCR. The initial protocol for the lysis step during DNA extraction was 55°C for 15 minutes this was optimised to 75°C for 30 minutes. This change in the conditions for the lysis step gave the best bands in *MBL2* PCR.

Optimization of *MBL2* PCR

The *MBL* initial PCR method was optimised by initially using the enzyme Taq DNA Polymerase for amplification. We obtained very weak amplification bands using the Taq DNA Polymerase enzyme and the standard *MBL2* genotyping method used at Serum State Institute. We then changed the amplification enzyme to TEMPase Hot Start DNA Polymerase (VWR, Belgium) which gave us better bands for all the *MBL2* SNPS, *HL*, *YX*,
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PQ and *Exon 1*. The amount of template DNA to be used for the PCR reaction was also optimised. We used 2µl, 5µl and 10µl and obtained the best clear bands by using 2µl of the template DNA, 2µl was therefore used throughout all the *MBL2* genotyping assays (16).

Statistical analysis

All statistical analysis were done using Stata 11 statistical package (STATA Corp, Timberlake Consultants). For *MBL2* genotype analysis, normal homozygous *MBL2* was denoted as *A/A*. The heterozygous variant *MBL2* genotypes were grouped together as *A/O* heterozygous *MBL2* as they all give low *MBL2* plasma levels and all homozygous and compound homozygotes were grouped together as *O/O*. The frequencies of the *MBL2* alleles were obtained by direct gene counting. To determine prevalence of MBL deficiency, *MBL2* genotypes were used as proxy for MBL levels, *MBL2A/A* genotype was considered as normal and those with *A/O* and *O/O* categorised as deficient MBL, as reported earlier (16).

RESULTS

Baseline characteristics

Mothers aged above 16 years old and their babies aged between 9 and 18 months old were enrolled into the national PMTCT survey. Six hundred and thirty two (632) dried blood spot samples from mother-baby pairs were selected for DNA extraction and *MBL2* genotyping (16).

Optimization of DNA extraction

Initially DNA extraction was done using Sigma Generation kit. We could not amplify the DNA extracts obtained from this method by PCR. Then extraction was done using the second kit SIGMA Extract and Amp kit and DNA extracts from this method could be amplified by PCR.

Optimization of *MBL2* PCR

The *MBL2* initial PCR method was optimised by initially using the Taq DNA Polymerase enzyme for amplification of *MBL2* SNPS. The PCR products produced very weak bands after gel electrophoresis. The PCR enzyme was then changed to TEMPase Hot Start DNA Polymerase and the PCR products produced clear bands after gel electrophoresis .

***MBL2* genotyping by pyrosequencing technique**

MBL2 genotyping was initially done on all the available 632 mother-baby pair DNA extracts using pyrosequencing. Ten mother-baby pairs were dropped from this analysis because of the incomplete data. The frequencies of all the *MBL2* SNPS for the mothers and their children using the pyrosequencing method were reported earlier (16).

Discussion

We share here our experiences optimising DNA extraction from dried blood spots collected on Perkin Elmer 226 filter papers and *MBL2* genotyping results using the pyrosequencing technique. We optimized DNA extraction by initially using the Sigma Generation kit but the DNA could not be amplified. Then we used the SIGMA Extract and Amp kit and the extracts from this method could be amplified by PCR. We then optimized initial *MBL2* PCR by using

Taq DNA Polymerase enzyme. The amplification process was then improved by using TEMPase Hot Start DNA Polymerase enzyme.

We obtained the best PCR amplification bands after substituting the Taq DNA Polymerase PCR enzyme with TEMPase Hot Start DNA polymerase enzyme. TEMPase Hot Start DNA polymerase enzyme is a modified form of Taq DNA Polymerase PCR enzyme which is activated by heat treatment. A chemical moiety is attached to the enzyme at the active site, which renders the enzyme inactive at room temperature. Thus, during setup and the first ramp of thermal cycling, the enzyme is not active and misprimed primers are not extended. This results in higher specificity and greater yields when compared to standard DNA polymerases. Once the reaction reaches optimal activating temperature, the chemical moiety is cleaved during a 15 minute heat activation step, releasing the active TEMPase Hot Start DNA Polymerase into the reaction.

Available literature has shown *MBL2* genotyping can be done on stored dried blood spots collected on Guthrie cards (3, 5, 6, 12, 14, 15). The reasons for the poor quality DNA from our samples might have been the long storage time before analysis. The dried blood spots we used for *MBL2* genotyping were collected on Perkin Elmer 226 filter papers and had been stored for 1 year 8 months at room temperature before *MBL2* genotyping was done. The other possibility could be the type of filter paper that was used for collection and storage of the dried blood spots. The Perkin Elmer 226 Filter paper was used for collection of the dried blood spots. Important also to note is that these PMTCT dried blood spots were not initially intended for *MBL2* genotyping but for HIV testing of the mothers and their babies for a different programme, the national PMTCT programme (17-19). Available literature shows that studies that carried out *MBL2* genotyping using dried blood spots collected on Guthrie

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cards which were stored at room temperature before analysis and these reported no challenges with DNA extraction and amplification (3, 5, 12, 14).

In conclusion, DNA extraction from dried blood spots was optimal using the Gene Extract and Amp kit and PCR amplification was optimal using TEMPase Hot Start DNA polymerase enzyme. We therefore recommend use of the Gene Extract and Amp kit for DNA extraction from dried blood spots and use of the enzyme TEMPase Hot Start DNA polymerase enzyme for genotyping by pyrosequencing using dried blood spots collected on Perkin Elmer 226 filter papers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions conducted the field work. RBLGZ, CSC, SR, BSP, MC, FC, RB designed and set the study objectives. FC and RB provided the PMTCT dried blood spots from the national survey carried out in 2012. CM, BSP provided the reagents and funding logistics, RBLZG, CH, PH, MC optimized and carried out the immunoassays. RBLZG, CSC, CH, PH, MC, SR analyzed the data. RBLZG and CSC drafted the manuscript. All authors read the manuscript and approved the final version.

Financial support

Sample collection was funded by UNFPA Zimbabwe, Soul City and Children's Investment Fund Foundation (CIFF). Dr. McCoy is supported by Award Number K01MH094246 from the National Institute of Mental Health. Funding was also provided by the Letten Research Foundation, University of Oslo, Norway/Zimbabwe Collaborative PMTCT BHAMC

Programme special mention Professor Letten F. Saugstad. Laboratory space and reagents for *MBL2* genotyping were provided by Professor Michael Christiansen at Serum State Institut Copenhagen, Denmark. An abstract on this study was accepted for presentation as a poster at the AIDS 2016: 21st International AIDS Conference, 18–22 July 2016, Durban, South Africa funded by the IAS, the 21st International AIDS Conference abstract number TUPEA016, University of the Witwatersrand travel grant 2016, the Letten Research Foundation, University of Oslo Norway/Zimbabwe Collaborative PMTCT BHAMC Programme, the Medical Research Council of Zimbabwe. Funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements:

Serum State Institute for providing accommodation, many thanks to SSI technical staff namely Denise Schmidt, Karina Liebmann Madsen, for expert technical assistance on *MBL2* genotyping assays in Denmark, the Director and the Executive Committee of the Medical Research Council of Zimbabwe (MRCZ) for permission and time to do this research project. Finally special gratitude to all the women and children who participated in the national PMTCT survey carried out by the CESHAAAR Research team in Zimbabwe in September 2012 (17-19).

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Appendices E TO R



M130348

R14/49 Mrs R Zinyama-Gutsire et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130348

NAME: Mrs R Zinyama-Gutsire et al
(Principal Investigator)

DEPARTMENT: School of Public Health
 Medical Research Council of Zimbabwe

PROJECT TITLE: Mannose Binding Lectin Genetic Polymorphism
 : Association with HIV-1 Infection in Adults and
 Children in Zimbabwe

DATE CONSIDERED: 05/04/2013

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr Charles S Chasela

APPROVED BY:

Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 10/07/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**

Principal Investigator Signature

Date 10/7/2013

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES



Faculty of Health Sciences
Private Bag 3 Wits, 2050
Fax: 0117172119
Tel: 011 7172040

Reference: Ms Mpumi Mngapu
E-mail: mpumi.mngapu@wits.ac.za

12 August 2013
Person No: 681272
PAG

Mrs RBL Zinyama-Gutsire
Rosebank Lodge and Badeparker
219 Jan Smuts Avenue
Parktown North
South Africa

Dear Mrs Zinyama-Gutsire

Doctor of Philosophy: Approval of Title

We have pleasure in advising that your proposal entitled *Mannose binding lectin genetic polymorphism: Association with HIV-1 infection in adults and children in Zimbabwe* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

A handwritten signature in cursive script, appearing to read 'Sandra Benn'.

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

Telephone: 791792/791193
Telefax: (263) - 4 - 790715
E-mail: mrcz@mrcz.org.zw
Website: <http://www.mrcz.org.zw>



Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

APPROVAL

Ref: MRCZ/A/1770

12 September, 2013

Rutendo B.L. Zinyama-Gutsire
University of Witwatersrand
School of Public Health
7 York Road, Parktown 2193
South Africa

RE:- Mannose Binding Lectin Genetic Polymorphism: Association with HIV-1 Infection in Adults and Children in Zimbabwe

Thank you for the above titled proposal that you submitted to the Medical Research Council of Zimbabwe (MRCZ) for review. Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to conduct the above titled study. This is based on the following documents that were submitted to the MRCZ for review:

a) Research Protocol

• **APPROVAL NUMBER** : MRCZ/A/1770

This number should be used on all correspondence, consent forms and documents as appropriate.

• **TYPE OF REVIEW** : Full Board

• **EFFECTIVE APPROVAL DATE** : 12 September 2013

• **EXPIRATION DATE** : 11 September 2014

After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Website should be submitted three months before the expiration date for continuing review.

• **SERIOUS ADVERSE EVENT REPORTING:** All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Website.

• **MODIFICATIONS:** Prior MRCZ and IERC approval using standard forms obtainable from the MRCZ Website is required before implementing any changes in the Protocol (including changes in the consent documents).

• **TERMINATION OF STUDY:** On termination of a study, a report has to be submitted to the MRCZ using standard forms obtainable from the MRCZ Website.

• **QUESTIONS:** Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrcz.org.zw

• **Other**

• Please be reminded to send in copies of your research results for our records as well as for Health Research Database.

• You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE

MEDICAL RESEARCH COUNCIL OF ZIMBABWE

2013 -09- 12

APPROVED

P.O. BOX CY 573 CAUSEWAY, HARARE

Telephone: 791792/791193
Telefax: (263) - 4 - 790715
E-mail: mrcz@mrcz.org.zw
Website: www.mrcz.org.zw



Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

Ref: MRCZ/A/1770

19 December, 2013

Rutendo B.L Zinyama-Gutsire
University of Witwatersrand
School of Public Health
7 York Road, Parktown 2193
South Africa

RE: Request for an amendment: Mannose Binding Lectin Genetic Polymorphism : Association with HIV-1 Infection in adults and children in Zimbabwe.

We refer to your correspondence received on the 18th of December, 2013 on the above mentioned subject.

Please be advised that the Medical Research Council of Zimbabwe has reviewed and **approved** the following:-

- Change of laboratory to carry out MBL PCR assays from AiBST laboratories in Harare to Statens Serum Institute in Copenhagen, Denmark.

Also, be advised or reminded to apply for shipment of specimen from the Research Council of Zimbabwe.

Yours Faithfully

MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE





CONTINUING APPROVAL

Ref: MRCZ/A/1770

1 September 2014

Rutendo B.L Zinyama-Gutsire
University of Witwatersrand
School of Public Health
7 York Road, Parktown 2193
South Africa

RE: Mannose Binding Lectin Genetic Polymorphism: Association with HIV-1 Infection in Adults and Children in Zimbabwe

Thank you for the application for review of Research Activity that you submitted to the Medical Research Council of Zimbabwe (MRCZ). Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to continue conducting the above titled study.

This approval is based on the review and approval of the following documents that were submitted to MRCZ for review:-

- Continuing Renewal Form
- Research Protocol

- **APPROVAL NUMBER** : MRCZ/A/1770
This number should be used on all correspondence, consent forms and documents as appropriate.
- **TYPE OF MEETING** : Expedited
- **EFFECTIVE APPROVAL DATE** : 12 September 2014
- **EXPIRATION DATE** : 11 September 2015

After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Offices should be submitted three months before the expiration date for continuing review.

- **SERIOUS ADVERSE EVENT REPORTING:** All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Offices.
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 - **QUESTIONS:** Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrcz.org.zw
- Other**
- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
 - You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

.....
**MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE**





CONTINUING APPROVAL

Ref: MRCZ/A/1770

10 September 2015

Rutendo B.L. Zinyama-Gutsire
University of Witwatersrand
School of Public Health
7 York Road, Parktown 2193
South Africa

RE: Mannose Binding Lectin Genetic Polymorphism: Association with HIV-1 Infection in Adults and Children in Zimbabwe

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Other

- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
- You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

.....
**MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE**

MEDICAL RESEARCH COUNCIL OF ZIMBABWE

2015 -09- 10

APPROVED

P.O. BOX CY 573 CAUSEWAY, HARARE



CONTINUING APPROVAL

Ref: MRCZ/A/1770

5 September 2016

Rutendo B.L. Zinyama-Gutsire
University of Witwatersrand
School of Public Health
7 York Road, Parktown 2193
South Africa

RE: Mannose Binding Lectin Genetic Polymorphism: Association with HIV-1 Infection in Adults and Children in Zimbabwe

Thank you for the application for review of Research Activity that you submitted to the Medical Research Council of Zimbabwe (MRCZ). Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to continue conducting the above titled study.

This approval is based on the review and approval of the following documents that were submitted to MRCZ for review:-

- Continuing Renewal Form
- Research Protocol

• **APPROVAL NUMBER** : MRCZ/A/1770

This number should be used on all correspondence, consent forms and documents as appropriate.

- **TYPE OF MEETING** : Expedited
- **EFFECTIVE APPROVAL DATE** : 12 September 2016
- **EXPIRATION DATE** : 11 September 2017

After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Offices should be submitted three months before the expiration date for continuing review.

- **SERIOUS ADVERSE EVENT REPORTING:** All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Offices.
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 - **QUESTIONS:** Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrcz.org.zw
- Other**
- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
 - You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

.....
**MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE**



Letten

FOUNDATION

RESEARCH CENTRE TRUST

No 3 Everett Close Avondale

Harare Zimbabwe



7 March 2014

To whom it may concern

Dear Sir/Madam

Re: Shipment of human dried blood samples to Denmark, Copenhagen for Rutendo B.L Zinyama-Gutsire for research purposes.

I confirm that Rutendo Zinyama-Gutsire is registered as a PhD student at the University of Witwatersrand, Johannesburg South Africa. She is funded for her studies by the Letten Foundation Research Centre, a collaborative project between University of Zimbabwe and University of Oslo.

She has been invited by Dr Michael Christiansen of Serum State Institute Copenhagen Denmark to carry out her PhD research project in his laboratory using the dried blood spots collected from mothers and children in Zimbabwe.

Rutendo has received approval and clearance from relevant Zimbabwean research organisations, namely, the Medical Research Council of Zimbabwe (MRCZ) to do this research and from the Research Council of Zimbabwe to ship the samples to Denmark.

I do confirm that the samples being shipped are of no commercial value.

Yours Sincerely



Mrs Auxillia Mazhambe

Letten Foundation Research Centre Office Manager



Nº 02338

RESEARCH ACT, 1986
RESEARCH COUNCIL OF ZIMBABWE
CERTIFICATE OF REGISTRATION

Name MRS RUTENDO B.L. ZINYAMA - GUTSIRE
Nationality: ZIMBABWEAN Passport No.: CN832726
Institution of Affiliation in Zimbabwe: MEDICAL RESEARCH COUNCIL OF ZIMBABWE
J. TONGOGARA AVE / MAZOWE STREET
HARARE
Residential Address in Zimbabwe: 3115 NEW MARLBOROUGH
RED ROOFS
HARARE

The bearer has been registered to conduct research in the field of PUBLIC HEALTH
in terms of section 26A of the Research Act, 1986.

Expiry date: 24 FEBRUARY 2015

MINISTRY OF HEALTH & C. WELFARE
PORT HEALTH OFFICER
HARARE INTERNATIONAL AIRPORT
07 MAR 2014
P.O. BOX 176, HARARE
ZIMBABWE

Issuing Officer
Research Council of Zimbabwe

Date: 25 FEB 2014
TECHNICAL OFFICE
P.O. BOX CY 294
CAUSEWAY, HARARE

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TITLE : MANNOSE BINDING LECTIN POLYMORPHISM : ASSOCIATED WITH HIV-1 INFECTION IN ADULTS AND CHILDREN IN ZIMBABWE : MRC2/A/1770

BIOSPECIMENS FOR SHIPMENT :

SEE ATTACHED STAMPED AND SIGNED SCHEDULE

ZIMBABWE REVENUE AUTHORITY
500 07 MAR 2014 500 HARARE

NB : EXTRA TERRITORIAL STORAGE OF BIOSPECIMENS BEYOND THE RCZ STUDY APPROVED PERIOD IS ILLEGAL

ANNEXURE 1: Details of intended research

1. Summary of intended use of the specimens including methods specifying the volume/quantity of specimens

Two dried blood spots for each mother and two for the baby will be obtained from CESHAAAR and packed. The sample size for my study is 572 mother-baby pairs but 10% more blood spots (630) will be selected to allow for samples that do not yield adequate DNA for genotyping. Therefore a total of $630 \times 4 = 2520$ individual dried blood spots have been requested from CESHAAAR. The dried blood spots will be used to carry out MBL genotyping of the mother and baby blood samples using the pyrosequencing technology. (Detailed method on next page).

2. Justification of the shipment of the specimens

Initially I intended to carry out the MBL genotyping assays locally at AIBST laboratories using traditional PCR methods and gel electrophoresis. Dr Christiansen, a Medical Doctor –Scientist at Serum State Institute in Denmark was appointed my second co-supervisor by the University of the Witwatersrand and he has been carrying out MBL genotyping for more than 10 years. He indicated that there has been many improvements in MBL genotyping work over the last decade and they no longer use traditional PCR and gel electrophoresis methods. The pyrosequencing machine that Dr Christiansen currently uses in Denmark is not available in Zimbabwe. He has therefore invited me to travel to Denmark and carry out the required MBL genotyping assays using the currently available latest technology, under his expert supervision.

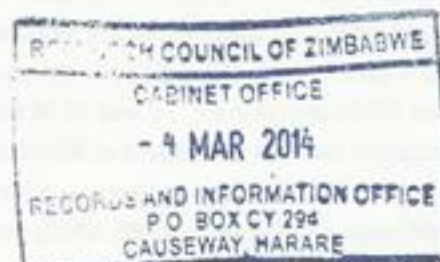
This is an important opportunity for technology transfer for Zimbabwean researchers as our long term plans are to continue this collaboration, apply for research grants and be able to buy a pyrosequencing machine to be stationed at AIBST and continue with this type of relevant molecular research work.

ANNEXURE 2: description of the specimens

Dried blood spots which are currently being kept at room temperature and ziplock backs with desiccant granules, the recommended method for storage of dried blood spots. The CESHAAAR study team carried out a national PMTCT survey in September 2012, collected and stored five dried blood for each mother-baby pair. They used two blood spots to carry out HIV tests and permission was granted to use the remaining blood spots.

ANNEXURE 3: plans for left over specimens:

All left over blood samples will be destroyed on 31 December 2014.



UNIVERSITY OF CALIFORNIA, BERKELEY



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PADIAN RESEARCH GROUP
1918 UNIVERSITY AVENUE, SUITE 3B
BERKELEY, CALIFORNIA 94704

April 24, 2013

To Whom It May Concern:

On April 3, 2013 Rutendo B. L. Zinyama-Gutsire requested access to dried blood spot samples collected from mothers-infant pairs enrolled in the baseline survey of the impact evaluation of Zimbabwe's National Program for the Prevention of Mother-to-Child Transmission of HIV, to conduct MBL genotyping assay testing in connection with her PhD thesis at the Univeristy of the Witwatersrand, Johannesburg South Africa. Rutendo's request has been approved.

Should there be any further questions, please contact me at the below email address.

Sincerely,

A handwritten signature in blue ink, appearing to read 'Raluca Buzdugan'.

Raluca Buzdugan, PhD
Project Director
Padian Research Group
University of California, Berkeley
rbuzdugan@berkeley.edu





9th December, 2013

TO WHOM IT MAY CONCERN

Invitation for Mrs Rutendo B. L Zinyama-Gutsire (Zimbabwean passport nr CN832726) to carry out Laboratory Assays at Serum State Institute, Copenhagen Denmark from 1 March to 31 May 2014

I am inviting the above named student to be attached at Serum State Institute, Copenhagen, Denmark. Rutendo is employed as a Research and Training Officer at the Medical Research Council of Zimbabwe and she is registered as a PhD student at the University of the Witwatersrand, Johannesburg South Africa since July 2012 to December 2014.

The title of her PhD research project is: **Mannose Binding Lectin Genetic Polymorphism: Association with HIV-1 Infection in Adults and Children in Zimbabwe.**

I confirm that I was appointed as one of her co-supervisors by the University of the Witwatersrand. Rutendo will be attached at Serum State Institute from 1 March to 31 May 2014 to carry out MBL ELISA and MBL genotyping assays. A pyrosequencing machine needed for the MBL genotyping is not available in Zimbabwe hence the need for her to ship samples to Denmark and travel to carry out her laboratory work under my supervision.

Yours Sincerely

Dr Michael Christiansen
Serum State institute
Copenhagen, Denmark



Nhamba:

GWARO RINORATIDZA IRO KUTI MANZWISISA
ZVAKARE MABVUMIRANA NEZVATSANANGURWA

URWERE HWEMUNHU ANEBILHARZIA HWAKAKONDANA NEHIV

Chirongwa ichi ndechekuongorora Schistosomiasis (bilharzia) neHIV muropa, neweti netsvina zvaticatora kubva mamuri. Vose avo vanenge vachirwara neBilharzia vacharapiwa zviripachena.

Kana mukabvuma kuva umwe wevanhu vachapinda muhurongwa uhwu tichange tichitora weti, neropa netsvina kubva kwamuri mushure memwedzi mitatu yega yega kusvikira gore rapera. Hapana achamanikidzwa kupinda muhurongwa uhu zvakare hapana mari yamuchasungirwa kubvisa.

Zvose hazvo zvichange zvawanikwa pane munhu upi neupi hazvizoshambadzwi. Ikodzero yenyu, zvakare makasununguka kubvunza pamusoro pezvose zvinenge zvawanikwa pamuri.

Makasununguka zvakare kubvunza mibvunzo ipi neipi zvayo pamusoro pazvamunenge musina kunzwisisa. Ikodzero yenyu kubuda muhurongwa uhu pane ipi nguva kana musingachazvidi. Nyangwe muka sarudza kubuda muhurongwa uhu, hazvirevi izvo kuti munobva maguma kurapiwa.

Tsananguro yakakwana nezvechirongwa chino munochiwana pamapepa amuchapiwa. Musati mabvuma kupinda mune uhu hurongwa, tinozvikoshesa chaizvo kuti munge mavana nguva yokunyatso fungisisa nezvazvo. Zvakare, munokurudzirwa kuti munyatso tanga mataura nehama kana kuti shamwari musati mazvipira kuve nhengo yehurongwa uhu.

Makawana here gwaro rinotsanangura nezvehurongwa uhu? YES/NO

Manzwisisa here zvatsanangurwa? YES/NO

Mawana mukana wokubvunza here? YES/NO

Munoziva here kuti ropa renyu richaongororwa kuti zvionekwe kuti mune hutachiwana hweHIV? YES/NO

Muri kubvuma here kupinda muhurongwa uhu? YES/NO

Ini ndanyatsonzwisisa maitirwo ehurongwa uhu sezvakanyorwa mugwarwo ratapihwa rokuverenga kuti tinzwisise pamwe nefomu rinozivisa ani zvake kuti ndanzwisisa zvakare ndinobvuma kupinda muhurongwa uhu.

Naizvozvo ini ndabvuma pasina kumanikidzwa, kupinda muhurongwa uhu.

Date _____

Signature _____ (ini pachezvangu)

Signature _____ (chapupu/hwitinesi)

Other Papers on the MUSH study co-authored by the candidate related to my PhD work, but not included in this thesis:

- 1 HIV and schistosomiasis in rural Zimbabwe: the association of retinol-binding protein with disease progression, inflammation and mortality. Kotzé SR, **Zinyama-Gutsire R**, Kallestrup P, Benn CS, Gomo E, Gerstoft J, van Dam G, Mortensen OH, Ullum H, Erikstrup C. *Int J Infect Dis.* 2015 Apr;33:159-64. doi: 10.1016/j.ijid.2015.01.015. Epub 2015 Jan 22.

2. Association of HIV and ART with cardiometabolic traits in sub-Saharan Africa: a systematic review and meta-analysis. Dillon DG, Gurdasani D, Riha J, Ekoru K, Asiki G, Mayanja BN, Levitt NS, Crowther NJ, Nyirenda M, Njelekela M, Ramaiya K, Nyan O, Adewole OO, Anastos K, Azzoni L, Boom WH, Compostella C, Dave JA, Dawood H, Erikstrup C, Fourie CM, Friis H, Kruger A, Idoko JA, Longenecker CT, Mboni S, Mukaya JE, Mutimura E, Ndhlovu CE, Praygod G, Pefura Yone EW, Pujades-Rodriguez M, Range N, Sani MU, Schutte AE, Sliwa K, Tien PC, Vorster EH, Walsh C, **Zinyama R**, Mashili F, Sobngwi E, Adebamowo C, Kamali A, Seeley J, Young EH, Smeeth L, Motala AA, Kaleebu P, Sandhu MS; African Partnership for Chronic Disease Research (APCDR). *Int J Epidemiol.* 2013 Dec;42(6):1754-71. doi: 10.1093/ije/dyt198.

3. Polymorphism in the interleukin-7 receptor α gene and mortality in untreated HIV-infected individuals. AIDS. Hans Hartling, Lise Thorner, Christian Erikstrup, Rutendo Zinyama, Per Kallestrup, Exnevia Gomo, Susanne Nielsen and Henrik Ullum. 2013. 1615-1620.

4. Longitudinal Analysis of CCR5 and CXCR4 Usage in a Cohort of Antiretroviral Therapy-Naïve Subjects with Progressive HIV-1 Subtype C Infection. Jakobsen MR, Cashin K, Roche M, Sterjovski J, Ellett A, Borm K, Flynn J, Erikstrup C, Gouillou M, Gray LR, Saksena NK, Wang B, Purcell DF, Kallestrup P, **Zinyama-Gutsire R**, Gomo E, Ullum H, Ostergaard L, Lee B, Ramsland PA, Churchill MJ, Gorry PR. PLoS One. 2013 Jun 18;8(6):e65950.
5. HLA-G3 untranslated region 14-base pair deletion: Association with poor survival in an HIV infected Zimbabwean population. Margit Larsen, Christian Erikstrup, Lise Thorner, **Rutendo Zinyama**, Per Kallestrup, Exnevia Gomo, Susanne Nielsen, Henrik Ullum et al. 2013. J Infect Dis. 207 : 903-906. 2013.
6. CCL3 gene copy number and survival in an HIV infected Zimbabwean population. Infection Genetics and Evolution. Margit Larsen, Christian Erikstrup, Lise Thorner, **Rutendo Zinyama**, Per kallestrup, Exnevia Gomo, Susanne Nielsen, Henrik Ullum et al. 2012. (12) 1087-1093.
7. Downregulation of MIP-1 α /CCL3 with praziquantel treatment in *Schistosoma haematobium* and HIV-1 co-infected individuals in a rural Zimbabwean community. **Zinyama-Gutsire R**, Kallestrup P, Ullum H Butterworth A, Erikstrup C, Gomo E Vennervald B.J Mduzuza TBMC Infectious Diseases. 2009. 9: 174-185.
8. Schistosomiasis and infection with HIV-1 in rural Zimbabwe: systemic Inflammation during Co-infection and after treatment for schistosomiasis. Erikstrup C, Kallestrup P, **Zinyama-Gutsire R**, Gomo E, Govert J van -Dam, Andre Deelder, Butterworth

- A, Bente K Pedersen, Sisse R Ostrowski, Jan Gerstoft, Ullum H. *Am. J Trop. Med.* 2008. 79(3) 331-337.
9. p24 as a predictor of mortality in a cohort of HIV-1-infected adults in rural Africa. Erikstrup C, Kallestrup P, **Zinyama-Gutsire RB**, Gomo E, Lüneborg-Nielsen M, Gerstoft J, Schüpbach J, Ullum H, Katzenstein TL. *J Acquir Immune Defic Syndr.* 2008 Jul 1;48(3):345-9. doi: 10.1097/QAI.0b013e31817dc3d1.
 10. Reduced mortality and CD4 cell loss among carriers of the interleukin 10-1082G allele in a Zimbabwean cohort of HIV-1 infected adults. Erikstrup C, Kallestrup P, **Zinyama-Gutsire R**, Gomo E, Butterworth A, Bente K Pedersen, Sisse R Ostrowski, Jan Gerstoft, Ullum H. *AIDS.* 2007. 21: 2283-2291.
 11. Predictors of mortality in a cohort of HIV-1-infected adults in rural Africa. Erikstrup C, Kallestrup P, **Zinyama R**, Gomo E, Mudenge B, Gerstoft J, Ullum H. *J Acquir Immune Defic Syndr.* 2007 Apr 1;44(4):478-83.
 12. Schistosomiasis and HIV in rural Zimbabwe: efficacy of treatment of schistosomiasis in individuals with HIV coinfection. Kallestrup P, **Zinyama R**, Gomo E, Butterworth AE, van Dam GJ, Gerstoft J, Erikstrup C, Ullum H. *Clin Infect Dis.* 2006 Jun 15;42(12):1781-9.
 13. Schistosomiasis and HIV-1 infection in rural Zimbabwe: implications of coinfection for excretion of eggs. Kallestrup P, **Zinyama R**, Gomo E, Butterworth AE, van Dam GJ, Erikstrup C, Ullum H. *J Infect Dis.* 2005 Apr 15;191(8):1311-20.

14. Schistosomiasis and HIV-1 infection in rural Zimbabwe: effect of treatment of schistosomiasis on CD4 cell count and plasma HIV-1 RNA load. Kallestrup P, **Zinyama R**, Gomo E, Butterworth AE, Mudenge B, van Dam GJ, Gerstoft J, Erikstrup C, Ullum H. J Infect Dis. 2005 Dec 1;192(11):1956-61
15. HIV in Africa - still a major matter of unsafe sex. Kallestrup P, Zinyama R, Gomo E, Gerstoft J, Ullum H.Int J STD AIDS. 2004 Oct;15(10):709-10
16. Low prevalence of anti-HCV antibodies in an HIV endemic area of Zimbabwe support sexual transmission as the major route of HIV transmission in Africa. Per Kallestrup, **Rutendo Zinyama**, Exnevia Gomo, Ebbe Dickmeiss, Per Platz, Jan Gerstoft, Henrik Ullum. AIDS. 2003. Jun 13;17(9):1400-2.



AIDS 2016
21ST INTERNATIONAL
AIDS CONFERENCE
DURBAN, SOUTH AFRICA, JULY 18-22, 2016
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CERTIFICATE OF ATTENDANCE

The Conference Organizers hereby certify that

RUTENDOB.LZINYAMA-
GUTSIREZimbabwe

attended the 21st International AIDS Conference
held in Durban, South Africa
18 - 22 July 2016

We thank you for your participation

Sincerely,

Chris Beyrer, MD, MPH.
International Chair
President, International AIDS Society
Desmond M. Tutu Professor of Public Health
and Human Rights
Departments of Epidemiology, International
Health, Health Behavior and Society, and
Nursing Associate Director,
Bloomberg School of Public Health,
The Johns Hopkins University,
Baltimore, Maryland, United States

Dr. Olive Shisana
Local Co-Chair
Honorary Professor at the University
of Cape Town, South Africa
President & CEO, Evidence Based
Solutions

ORIGINALITY REPORT

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INTERNET SOURCES

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PUBLICATIONS

% **3**

STUDENT PAPERS

PRIMARY SOURCES

1 Zinyama-Gutsire, Rutendo B. L., Charles Chasela, Hans O. Madsen, Simbarashe Rusakaniko, Per Kallestrup, Michael Christiansen, Exnevia Gomo, Henrik Ullum, Christian Erikstrup, Shungu Munyati, Edith N. Kurewa, Babill Stray-Pedersen, Peter Garred, and Takafira Mduluza. "Role of Mannose-Binding Lectin Deficiency in HIV-1 and Schistosoma Infections in a Rural Adult Population in Zimbabwe", PLoS ONE, 2015. % **9**
Publication

2 Zinyama-Gutsire, Rutendo B.L., Michael Christiansen, Paula L. Hedley, Simbarashe Rusakaniko, Christian Hagen, Babill Stray-Pedersen, Raluca Buzdugan, Frances Cowan, and Charles Chasela. "HIV-1 Vertical Transmission in Zimbabwe in 622 Mother and Infant Pairs: Rethinking the Contribution of Mannose Binding Lectin Deficiency in Africa", OMICS A Journal of Integrative Biology, 2016. % **7**
Publication

3 Zinyama-Gutsire, Rutendo B.L, Charles