

Filling the gaps: towards improved surveillance and monitoring of immunological status relevant to long term co-morbidities in HIV infection.

by

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DECLARATION OF AUTHORSHIP

I declare that this thesis is my own account of my research, except in the cases where others contributions are acknowledged. It contains, as its main content, work which has not previously been submitted for a degree at any tertiary education institute.

..... Alison S L Castley, student ...

Notes regarding the formatting of this thesis

This PhD thesis is presented as a series of research manuscripts either published or currently being peer reviewed for publication. A general introduction (Chapter 1) will set the tone of the thesis followed by results chapters (Chapters 2-6), then concluded with an overall discussion and final remarks (Chapter 7).

Chapters 2-6 begin with an attribution page followed by the published manuscript (Chapters 2-5) or, as in the case of Chapter 6, the paper currently under peer review. Each includes a literature review, material and methods section and references therefore these sections will not be included separately. The references are in the format required by the journal where the manuscript was submitted.

An additional summary is included at the conclusion of Chapters 2-4 and again for Chapter 5-6. This aims to provide relevant information for the continuity of the thesis and to link the published work and chapters. A final bibliography for Chapters 1, 7, the combined summary for Chapters 2-4 and the combined summary for Chapters 5-6 is included at the end of this dissertation.

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To my father
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19th July 1936 - 28th November 2007

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Look deep into nature, and you will understand everything better. Albert Einstein

Abstract

It is approaching 35 years since human immunodeficiency virus (HIV)-1 was first identified in Western Australia (WA) and 20 years since introducing highly active antiretroviral therapy (HAART) transforming HIV management, in the majority of cases, by maintaining 'undetectable' HIV-1 RNA and restoring CD4 T cell counts to healthy levels. Despite these advances patients are at increased risk of developing age-associated diseases. This thesis therefore investigates whether expanding laboratory approaches to incorporate aspects of the disease process, that are currently 'invisible' in clinical practice but may be prognostically important or which may inform treatment and prevention strategies at a population level, is warranted.

The following aims were addressed; 1) analysing determinants of HIV-1 RNA residual viraemia; 2) investigating monocyte activation during chronic HIV-1 infection and; 3) assessing HIV-1 diversity from large scale sequences throughout WA and Australia, including the collaboration and formation of the Australian Molecular Epidemiology Network (AMEN).

The main findings demonstrated HIV-1 residual viraemia was powerfully associated with the pre-treatment level of viraemia even after 10-15 years of starting HAART. Analysis of monocyte activation revealed multiple pathways involved in chronic immune activation responses to HIV-1, by altering CD16⁺ monocyte expression and elevated levels of sCD14 (previously associated with all-cause mortality), that were not corrected by HAART and therefore prognostically significant. In contrast, levels of other biomarkers (e.g. CXCL10 and sCD163) declined with HAART.

Investigations of HIV-1 genetic diversity within the WA cohort (n=1021) and the Australian cohort (n=4873) revealed new challenges for HIV-1 prevention due to significant increases in HIV-1 non-B-subtypes, consistent with an increasing impact of migration, while local transmissions were predominantly HIV-1 B-subtypes.

Overall, this thesis provides evidence for possible new approaches that may enhance HIV-1 laboratory practice that could aid clinical management, improve long-term health outcomes for those living with HIV-1 infection and guide effective preventative strategies.

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Abbreviations

ACE	angiotensin converting enzyme
ADAM17	a disintegrin and metalloproteinase 17
ADCC	antibody dependant cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
AMEN	Australian Molecular Epidemiology Network
ANOVA	analysis of variance
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ARG	arginine
ART(s)	antiretroviral therapy (therapies)
AZT	zidovudine
BMI	body mass index
BS	bootstrap value
CCR2	MCP-1 receptor C-C chemokine receptor type 2
CCR5	chemokine receptor 5
cDNA	copy DNA
CD	cluster of differentiation (i.e. CD14, CD16, CD143, CD64, CD163, CD62L, CD45)
CD4+T	CD4 positive T cell
CD8+T	CD8 positive T cell
CD4:8	CD4 to CD8 ratio
CHD	coronary heart disease
Cm	centimetres
CpG DNA	cytosine phosphate guanine deoxyribonucleic acid
cpm	copies per millilitre
CRF(s)	circulating recombinant form(s) (i.e. CRF01_AE, CRF02_AG)
CRP	C-reactive protein (hsCRP- highsensitivityCRP)
CST	cytometer setup beads
CVD	cardiovascular disease
CX₃CR1	CX3C chemokine receptor 1
CXCL10	C-X-C motif chemokine 10
CXCR	C-X-C receptor (i.e. CXCR3, CXCR4)
CYP3A4	cytochrome P450 3A4 enzyme inhibitor
DNA	deoxyribonucleic acid

EDTA	ethylenediamine tetraacetic acid
ELISA(s)	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinases (ERK1/2)
FcγR1	high affinity IgG Fc receptor
FcγRIII	Fc fragment of IgG, low affinity III, receptor
FDiaBP	fasting diastolic blood pressure
FI(s)	fusion inhibitor(s)
FMO	fluorescence minus one
FSC	forward-light scatter
FSysBp	fasting systolic blood pressure
GD	genetic distance
GPCR	G protein-coupled receptor
GTR	general time reversible
HAART	highly active antiretroviral therapy
HAND	HIV associated neurocognitive disorders
HCV	hepatitis C virus
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HIV+	human immunodeficiency virus positive
HIV-1	human immunodeficiency virus-1
HIV-1 RNA	human immunodeficiency virus-1 ribonucleic acid
HLA	human leukocyte antigen (i.e. HLA-DR)
IC(s)	isotype control(s)
IDU	injecting drug use(r)
IFN-α	interferon alpha (i.e. IFN α -2b)
IFNAR	interferon alpha receptor (i.e. IFNAR1, IFNAR2)
IFN-β	interferon beta
IFN-ϵ	interferon epsilon
IFN-γ	interferon gamma
IFNGR	interferon gamma receptor (i.e. IFNGR1, IFNGR2)
IFN-λ	interferon lambda (i.e. IFN λ 3)
IFNLR	interferon lambda receptor (i.e. IFNLR1)
IKK	I κ B kinase
IL	interleukin (i.e. IL-1 β , IL-6, IL-10, IL-21, IL-10R)
INI(s)	integrase inhibitor(s)

IP-10	interferon gamma-induced protein 10
IRF(s)	interferon response factors(s) (i.e. IRF3, IRF7)
ISG(s)	interferon stimulatory gene(s)
JAK	janus kinase
kg	kilograms
kg/m²	kilograms per metre squared
LBP	LPS binding protein
lcpm	log copies per millilitre
LDL	low density lipoprotein
LPS	lipopolysaccharide
LTR	long terminal repeat (i.e. 2-LTR)
M1	acute inflammatory activated macrophages
M2	anti-inflammatory activated macrophages
MAM	monocyte activation marker
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral-signalling protein
MCP-1	monocyte chemoattractant protein-1
MEGA-V6	Molecular Evolutionary Genetics Analysis version 6
MHC	major histocompatibility complex
mins	minutes
mL	microlitre
MSM	men who have sex with men
MyD88	myeloid differentiation primary response gene 88
NATA	National Association of Testing Authority
Nef	negative regulatory factor
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng/μl	nanograms/microlitre
NK	natural killer
NNRTI(s)	non-nucleoside reverse transcriptase inhibitor(s)
NRTI(s)	nucleoside reverse transcriptase inhibitor(s)
PAMP(s)	pathogen associated molecular pattern(s)
PBMCs	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cells
peg IFNα-2b	pegylated interferon alfa-2a
pg/μl	pictograms/microlitre

PI(s)	protease inhibitor(s)
PMT	photomultiplier tube
<i>pol</i>	<i>poly</i> merase gene
PR	protease
PRR(s)	pattern recognition receptor(s)
RCPA	Royal College of Pathologists of Australasia
RNA	ribonucleic acid
RPH	Royal Perth Hospital
rpm	revolutions per minute
RT	reverse transcriptase
SAMHD1	SAM domain and HD domain-containing protein 1
sCD14	soluble CD14
sCD163	soluble CD163
SD	standard deviation
SER	serine
SIV	Simian Immunodeficiency Virus
SLE	systemic lupus erythematosus
SMART	Strategies for Management of Antiretroviral Therapy
SNAE(s)	serious non AIDS event(s)
SPSS	Statistical Package for the Social Science
SSC	side-light scatter
ssRNA	single stranded ribonucleic acid
STAT	Signal Transducer and Activator of Transcription (i.e. STAT1, STAT2)
STING	stimulator of interferon genes
Tat	trans-activator of transcription
TB	tuberculosis
TBK1	TANK-binding kinase 1
Th	T helper cells (i.e. Th17, Th22)
TLR(s)	toll like receptor(s) (i.e. TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9)
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor alpha
TREX1	three prime repair exonuclease 1
TRIF	TIR-domain-containing adapter-inducing interferon- β
TRIM	tripartite motif (i.e. TRIM5)

TUR	The United Republic of Tanzania
UK	United Kingdom
UNAIDS	The Joint United Nations Programme on HIV and AIDS
URF(s)	unique recombinant form(s)
US(A)	United States (of America)
US DHHS	United States Department of Health and Human Services
vif	viral infectivity factor
vpr	viral protein R
vpu	viral protein U
vpx	viral protein X
WHO	World Health Organisation

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Academic output during candidature

List of presentations during candidature

(OP= Oral presentations, PP= poster presentations)

Royal Perth Hospital (RPH)-Medical Research Foundation (MRF) research day 2015, Perth, Western Australia September 30th 2015.

Award: *Early Career Scientist Investigator Award*

OP **Castley A** Gizzarelli L, Guelfi G, Gaudieri S, John M, Nolan D. Changing face of HIV: A longitudinal analysis of HIV-1 sequence diversity, transmission networks and the impact of clinical parameters in Western Australia from 2000-2014.

Australian Molecular Epidemiology Network (AMEN) workshop. Brisbane, Australia, September 17th 2015.

OP **Castley A.** Engaging with community groups: Workshop Introduction: A history and timeline into the formation of the Australian Molecular Epidemiology Network (AMEN).

World STI/HIV conference in conjunction with the Australasian Society for HIV Medicine (ASHM), Brisbane Australia, September 13th-18th 2015.

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OP **Castley A,** Sawleshwarkar S, Varma R, Herring B, Thapa K, Chibo D, Nguyen N, Hawke K, Ratcliff R, Dwyer DE, Nolan D: The Australian Molecular Epidemiology Network-HIV (AMEN-HIV). The challenges of diversity: HIV-1 subtype distribution and transmission networks within the Australian Molecular Epidemiology Network-HIV 2005-2012.

OP **Castley A,** Gizzarelli L, Guelfi G, Gaudieri S, John M, Nolan D. HIV-1 sequence diversity and transmission networks in Western Australia from 2000-2014, and their impact on baseline clinical characteristics.

OP **Castley A,** James I, Williams L, Berry C, Nolan D. One profile or many? Plasma biomarkers CXCL10, sCD163 and sCD14 reveal distinct associations

with HIV treatment response, choice of treatment regimen, and cardiovascular risk factors.

Western Australian STI/BBV Quarterly seminar, December 2014.

OP **Alison Castley.** HIV does not require a VISA or does it? Monitoring HIV subtype diversity and mobility in the Western Australia HIV cohort.

AIDS 2014: Victoria, Australia, July 20-25th 2014.

OP+PP **Alison Castley,** Shailendra Sawleshwarkar, Rick Varma, Belinda Herring, Kiran Thapa, Doris Chibo, Nam Nguyen, Karen Hawke, Rodney Ratcliff, Dominic E Dwyer, David Nolan. An epidemic in transition: impacts of migration and local networks on HIV sequence diversity and infection transmission in Australia 2005-2012.

PP Elizabeth McKinnon, **Alison Castley,** Leanne Payne, Stephen Pummer, David Nolan. Determinants of residual viremia during combination HIV treatment: impacts of baseline HIV RNA levels and treatment choice.

Australian Centre for HIV and Hepatitis virology research (ACH²) 2014, Lorne Victoria, June 4-6 2014.

OP **Alison Castley.** Monitoring HIV-1 drug resistance mutations among newly diagnosed Western Australian HIV patients.

Australasian Society for HIV Medicine (ASHM) dinner presentation, Perth WA; February 5th 2014.

OP **Alison Castley.** The changing face of HIV-1 in Western Australia. Monitoring HIV-1 subtype diversity and mobility in the Western Australia HIV cohort.

Department of Clinical Immunology (DCI), Royal Perth Hospital, Western Australia, December 9th 2013.

OP **Alison Castley.** The changing face of HIV in WA! Monitoring HIV-1 subtype diversity and mobility in the Western Australia HIV cohort.

School of Veterinary and Life Sciences Poster Day. Murdoch University, Perth, Western Australia, November 29th 2013.

PP **Alison Castley.** An optimised eight colour flow cytometry protocol for the analysis of monocyte heterogeneity and monocyte activation markers during HIV infection.

Australasian Society for HIV Medicine (ASHM), Darwin Northern Territory, October 20-24th 2013.

OP **Alison Castley.** An optimised eight colour flow cytometry protocol for the analysis of monocyte heterogeneity and monocyte activation markers during HIV infection.

PP **Alison Castley.** An optimised eight colour flow cytometry protocol for the analysis of monocyte heterogeneity and monocyte activation markers during HIV infection.

PP **Alison Castley.** The changing face of HIV-1 in Western Australia

Department of Clinical Immunology Royal Perth Hospital: October 14th 2013.

OP **Alison Castley.** Is Monitoring innate immune cell activation diagnostically important in HIV disease progression?

Murdoch University Postgraduate Student Association (MUPSA) multidisciplinary conference 2013: Murdoch campus, Western Australia, September 23rd 2013.

OP **Alison Castley.** An optimised eight colour flow cytometry protocol for the analysis of monocyte heterogeneity and monocyte activation markers during HIV infection.

Murdoch University Postgraduate Student Association (MUPSA) multidisciplinary conference 2013: Peel campus, Western Australia, September 11th 2013.

OP **Alison Castley.** An optimised eight colour flow cytometry protocol for the analysis of monocyte heterogeneity and monocyte activation markers during HIV infection.

Combined Biological Science Meeting (CBSM), University of Western Australia, Perth Western Australia, August 30th 2013.

OP **Castley, A.** The changing face of HIV-1 in Western Australia.

Institute for Immunology and Infectious Diseases (IID), Murdoch, Western Australia, August 27th 2013.

OP **Alison Castley.** The changing face of HIV in WA! Monitoring HIV-1 subtype diversity and mobility in the Western Australia HIV cohort.

School of Veterinary and Life Science presentation, Murdoch University, Perth, Western Australia, July 5th 2013.

OP **Alison Castley.** Filling the gaps: working towards improved monitoring of immunological status relevant to long-term co-morbidities in HIV-1 infection.

Australian Centre for HIV and Hepatitis virology research (ACH²) 2013: The Carrington Hotel Katoomba New South Wales; June 5-7th 2013.

OP **Alison Castley, Cassandra Berry and David Nolan.** The expression of cell surface monocyte activation markers on CD14^{pos}/CD16^{pos} monocytes show increased activation in HIV patients.

OP **Alison Castley, Silvana Gaudieri, Laila Gizzarelli , George Guelfi, Mina John and David Nolan.** Monitoring HIV-1 subtype diversity and mobility in the Western Australia HIV cohort-Where are HIV infections coming from and are there forward transmissions?

List of publications during candidature

McKinnon E & **Castley A**, Payne L, Pummer S, Nolan D (2016). Determinants of residual viraemia during combination HIV treatment: Impacts of baseline HIV RNA levels and treatment choice. *HIV Medicine* 17(7), 495-504.

Castley A, Berry C, French M, Fernandez S, Krueger R, Nolan D (2014) Elevated plasma soluble CD14 and skewed CD16+ monocyte distribution persist despite normalisation of soluble CD163 and CXCL10 by effective HIV therapy: a changing paradigm for routine HIV laboratory monitoring? *PLoS One* 9(12), e115226.

Castley A, Williams L, James I, Guelfi G, Berry C, Nolan D (2016). Plasma CXCL10, sCD163 and sCD14 Levels Have Distinct Associations with Antiretroviral Treatment and Cardiovascular Disease Risk Factors. *PLoS One* 29;11(6), e0158169.

Castley AS, Gaudieri S, James I, Gizzarelli LS, Guelfi G, John M, Nolan D (2016). Longitudinal Trends in Western Australian HIV-1 Sequence Diversity and Viral Transmission Networks and Their Influence on Clinical Parameters: 2000-2014. *AIDS Research and Human Retroviruses* 32(3), 211-9.

Cha L, Berry CM, Nolan D, **Castley A**, Fernandez S, French MA (2014). Interferon-alpha, immune activation and immune dysfunction in treated HIV infection. *Clinical and Translational Immunology* 3(2):e10.

Ethical considerations throughout candidature

To ensure integrity was attained throughout this thesis, informed written consent was obtained from the patients participating in all investigations. Consent forms were reviewed and accepted by the appropriate ethics committees while written ethics committee approvals were granted and received from Royal Perth Hospital (EC2012/170), Murdoch University (2012/216), the Australian Red Cross Blood Service (11-08WA-15) and for Chapter 6, ethics was granted through each participating state including Royal Perth Hospital (REG-14/112) and Murdoch University (updated; 2012/216).

Patients attending the Royal Perth Hospital (RPH) Immunology clinic and healthy control blood donors attending the Australian Red Cross Blood Service (ARCBS) were recruited for the studies presented in Chapter 2-4.

Confidentiality and anonymity was paramount throughout the studies, therefore all patient information was subsequently de-identified by assigning a unique anonymised assessment number. This system was utilised throughout the study.

For Chapter 5, all viral HIV-1 sequences and clinical parameters were collated and linked through the Royal Perth Hospital routine HIV laboratory service and safely stored at Royal Perth Hospital.

For Chapter 6, all viral HIV-1 sequences were supplied by each state as de-identified samples (including Royal Perth Hospital) and the data was collated and linked through Royal Perth Hospital. The sequences were identified by the “State, identification number, year of sequence and gender” (i.e. VIC_001_2009_M).

Chapter 1:

A general introduction to the history of the Human Immunodeficiency Virus (HIV) in Western Australia and the impact on current era clinical care.



1.1 The worldwide HIV/AIDS epidemic

We are now in the fourth decade since the start of the human immunodeficiency virus 1 (HIV, HIV-1) epidemic, the virus causative of acquired immunodeficiency syndrome (AIDS). To date around 78 million people worldwide have been infected with HIV while 39 million people have reportedly died of AIDS related illnesses, including 1.5 million in 2013. In 2013/2014 there were approximately 35 million people living with HIV worldwide (Figure 1) whilst only 13.6 million HIV positive persons were receiving antiretroviral treatment for their infection [1]. This indicates that over 20 million people were without adequate treatment for their illness and at risk of developing AIDS. Of the 35 million people identified with HIV infection in 2013, 2.1 million were newly identified infections. Together, these numbers put incredible strain on resources especially in resource limited regions where the epidemic is at its highest.

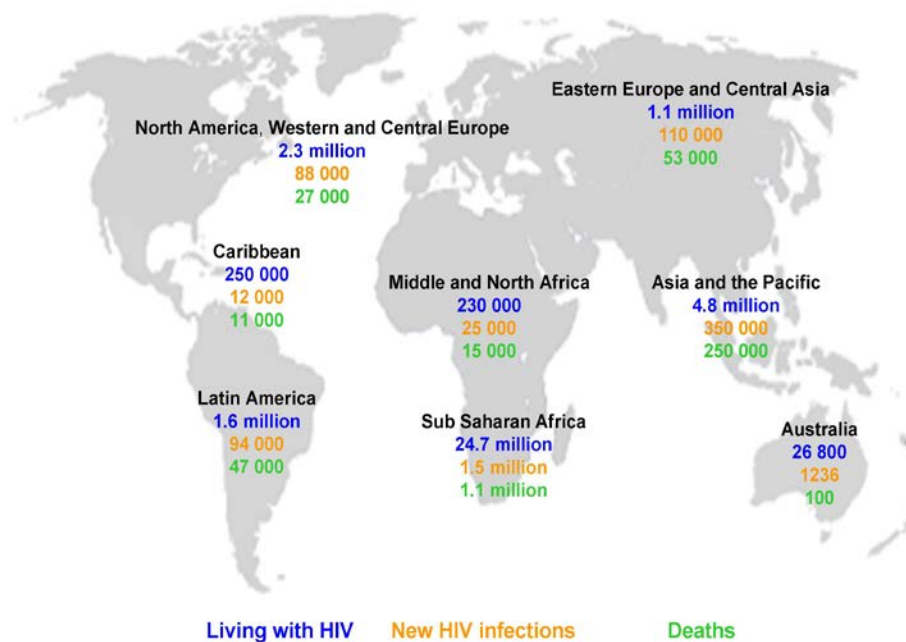


Figure 1: Global map of HIV infections in 2013. Worldwide map showing the number of people living with HIV (blue), the number of new infections (orange) and the number of deaths from AIDS related illnesses (green) per region. *Data was adapted from the World Health Organisation (WHO) 2014 [1] and UNAIDS update 2014 [2].*

Interestingly, 10 countries account for 61% of the current worldwide HIV infections (Figure 2). The country contributing to most worldwide infections in 2013 was South

Africa (18%), however, other African countries also represented in Figure 2 include Zimbabwe (4%), Zambia (3%), eastern African countries; Kenya (5%), Uganda (4%), the United Republic of Tanzania (TUR-4%), Mozambique (4%) and the west African country Nigeria (9%), totalling 51% of worldwide infections in Africa alone. India (6%) and the United States of America (4%) round out the top 10 countries with the “rest of the world” accounting for 39% of worldwide infections.

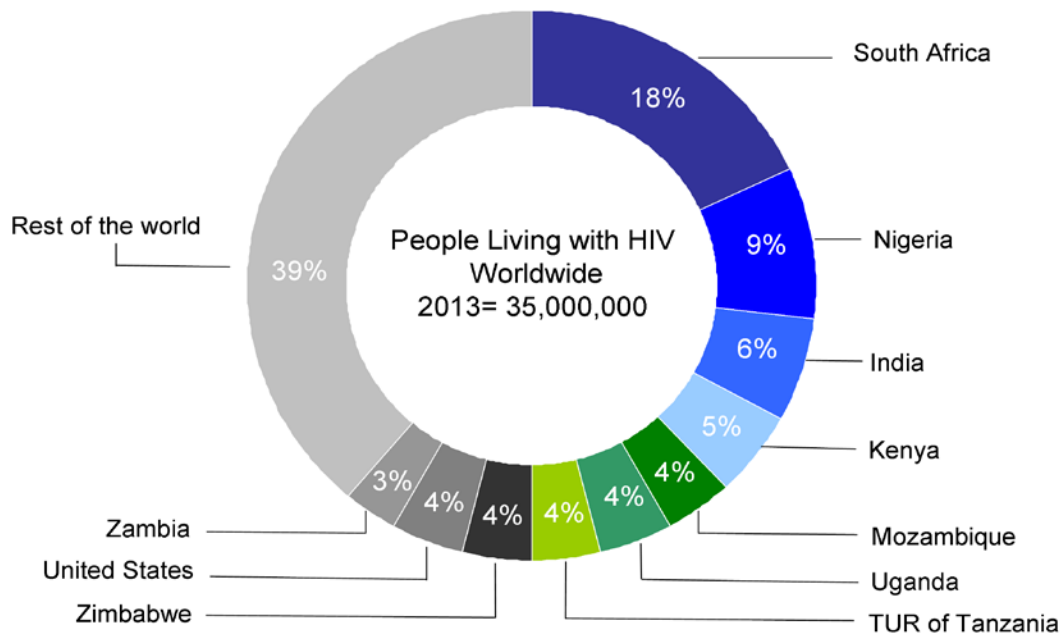


Figure 2: Country distribution of HIV infections in 2013. In 2013 there were an estimated 35 million people living with HIV worldwide. Ten countries account for 61% of the people living with HIV infections. *Adapted from UNAIDS update 2014 [2].*

Furthermore, 10 countries account for 63% of the new HIV infections identified in 2013 (Figure 3). Although new infections were highest in South Africa (16%), five other African countries (30%), India (6%), China (3%), Indonesia (4%) and the Russian Federation (4%) contributed to the high rates of new notifications in 2013. The number of HIV infections identified in eastern Europe and central Asia were on the rise, and rising trends in the Middle East and north Africa (31% increase) have also been recognised. There were also small increases in Europe and North America (6%) as well as Indonesia [2].

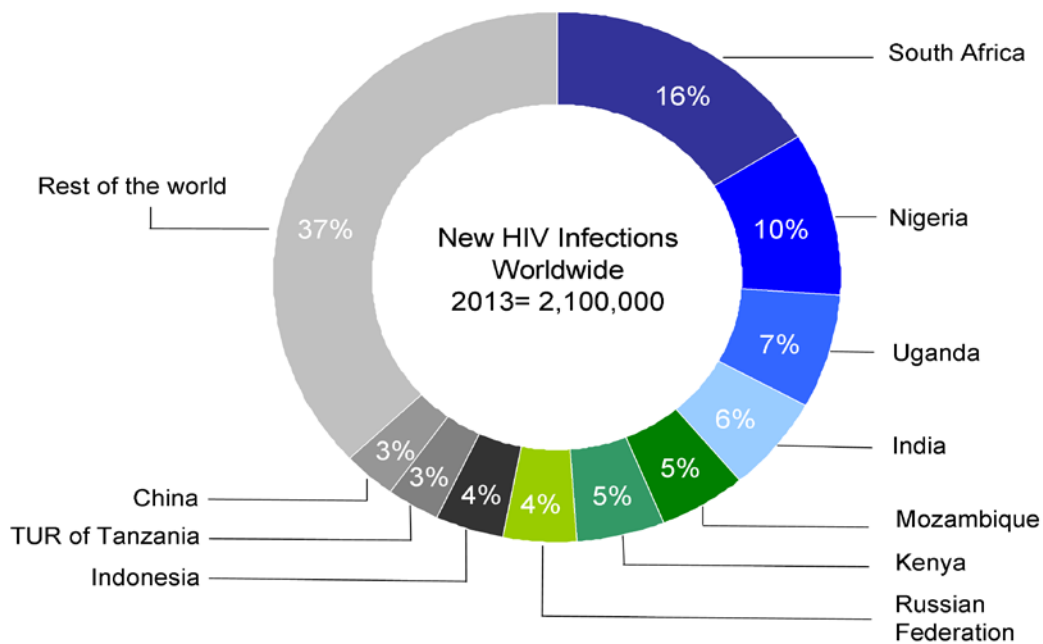


Figure 3: Country distribution of new HIV infections in 2013. In 2013 there were an estimated 2.1 million people with new HIV infections worldwide. Ten countries account for 63% of the new infections. *Adapted from UNAIDS update 2014 [2].*

In Australia there have been 35287 cases of HIV diagnosed since 1982, of which around 10000 people have died from AIDS related illnesses. At the end of 2013, there were an estimated 27000 people living with HIV infection (Figure 1) with the prediction of approximately 14% of all HIV cases being undiagnosed [3]. This increase is set against an overall downward global trend in new HIV diagnoses, most notably in sub-Saharan Africa and also evident in the overall Asia Pacific region [2]. Recent data from the Asia/Pacific region for 2013 is remarkable. According to the current UNAIDS report (represented in Figure 1), people living with HIV in the Asia/Pacific region accounted for 4.8 million infections in 2013 which is the second largest region worldwide with HIV infections [2]. Four countries within this region account for 83% of the infections (India- 44%, China-17%, Indonesia-13% and Thailand- 9%), while a total of 11 countries account for 97% of infections (Figure 4). In India alone the UNAIDS gap report [2] concluded “India has the third largest number of people living with HIV in the world — 2.1 million at the end of 2013 — and accounts for about 4 out of 10 people living with HIV in the region”. In 2013 the number of new HIV notifications in the Asia/Pacific region was 350,000 (Figure 1). India (38%), Indonesia (23%) and China (20%) account for 81% of new notifications

within this region (Figure 5). Although reports suggest a decline in new HIV notifications in India, this country still accounts for 38% of new infections in 2013. It has been recognized that new HIV infections in Thailand, Vietnam and Papua New Guinea have also reportedly declined while the number of new HIV infections has increased in Indonesia, Pakistan and Australia. Indonesia has been identified as the country in this region with the second highest number of new infections. Also of relevance is that sex workers in Indonesia and Pakistan have the lowest rate of using preventative coverage [2].

The prevalence of HIV among men who have sex with men (MSM) in 2013 was high in Malaysia (12%), Myanmar, Mongolia and Australia (10% each), Indonesia (8%), Thailand (6.5%) and China (6%).

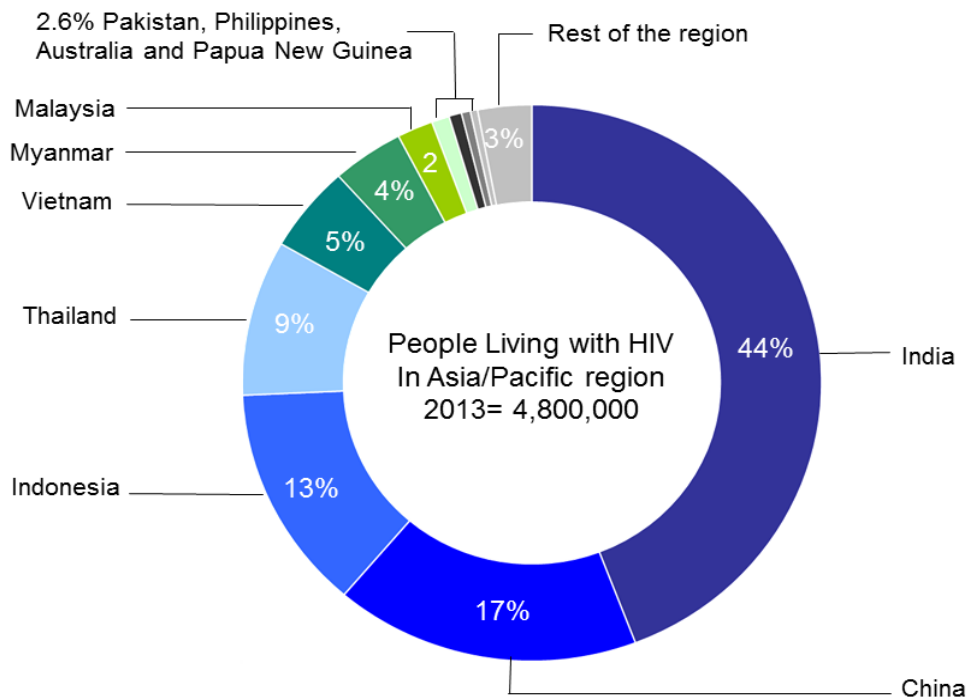


Figure 4: HIV infections in Asia/Pacific region in 2013. In 2013 there were 4,800,000 people living with HIV in the Asian/Pacific region many of whom live in India, China and Indonesia. Adapted from UNAIDS [2]

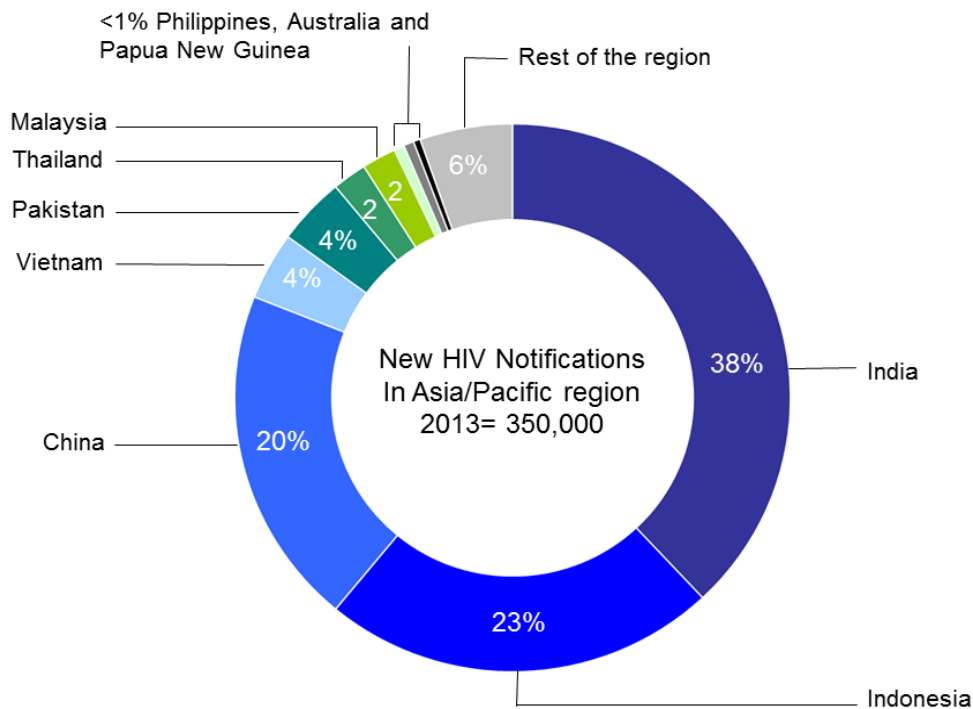


Figure 5: New HIV notification in the Asian/Pacific region reached 350,000 in 2013. Three countries in this region (India, Indonesia and China) accounted for 283,500 of the new notifications. *Adapted from UNAIDS [2]*

1.2 Epidemiology and phylogenetic analysis of HIV

To understand the impact of changes to HIV notifications worldwide it is important to comprehend the diversity of HIV subtypes present around the world.

There are known characteristics of HIV-1 genetic diversity including historical associations between geographical location and distinct HIV-1 subtype groups M, N and O (and a more recently identified fourth group, the P group) [4]. Within this phylogenetic structure, the M group is responsible for the majority (90%) of worldwide infections. The M group comprises nine defined subtypes (A, B, C, D, F, G, H, J, and K) of which the C subtype accounts for the majority (50%) of HIV infections worldwide and is prevalent in sub-Saharan and eastern Africa as well as India, while the B subtype accounts for infections within Caucasian populations (mainly in Europe, United States of America and Australia). Together the B and C subtypes account for approximately 60% of HIV-1 infections worldwide [5]. The B subtype is typically characterized by transmission via male-to-male sexual contact, as previously documented in 2002 for the Western Australian HIV population [6].

Genetic diversification of HIV-1 is also promoted by high rates of recombination events [7] that generate stable circulating recombinant forms (CRFs) as well as unique recombinant forms (URFs) of HIV, which together account for 20% of global HIV-1 infections [5]. A recombinant form of the HIV virus results from recombination events between two or more HIV strains within a host, however, to be classified as a new CRF three unlinked viruses of the same genetic makeup must be identified. Until this occurs the term URF is used to address the recombination forms of HIV.

While CRFs are increasing in number within the Los Alamos National Laboratory repository (as at 1 October 2015, 72 CRFs were identified compared with 10 in 2003), it is also relevant to identify the more common form of recombinant virus such as the CRF01_AE subtype. This is thought to have originated in Africa (as the HIV-1 E subtype) then progressed to south-east Asia where it established itself as a major circulating form by recombining with the HIV-1 A subtype. The CRF01_AE epidemic exploded and more recently dispersed to other areas worldwide [8], while the HIV CRF02_AG virus originated in west and central-west Africa [7,9]. World maps showing the HIV epidemic and epidemiological studies before 2010 indicate HIV-1 diversity was widespread in central regions of Africa [9-11] but little subtype diversification existed in other regions of the world. Recent studies, however, show increasing HIV-1 diversification existing elsewhere in the world [12-14]. These results suggest there has been an increase in the pervasiveness of non-B subtypes in populations previously defined as predominantly B subtype, potentially introduced through migration or travel and it is almost certain new strains of the HIV virus, including new CRFs, will be discovered in the future.

1.3 The early years of HIV-1 in Western Australia and current trends

The first case of HIV-1 was recognised in Western Australia in 1983 after a male presented with an opportunistic infection that was otherwise unexplained. He was admitted to Royal Perth Hospital where doctors recognised his acquired immune deficiency syndromes but, as this preceded the availability of antiretroviral therapy, they were unable to treat him in his dying days. Nurses wore multiple gowns, gloves and masks, simply because of the lack of information relating to HIV and AIDS. Little was known about its potential impact on society except to acknowledge that it was a contractible disease, identified in the early years in homosexual men, which attacked the immune system.

By the mid-1980s there was a dramatic rise in new HIV notifications in Western Australia and by 1986 around 200 people were infected with HIV (Figure 6). By this time clinicians and researchers worldwide were employing considerable effort to search for the causes, to find ways to stop the virus from being transmitted and to reduce HIV replication after infection, while providing patients with the best possible care in an era where HIV was quickly becoming an epidemic.

These efforts lead to the introduction of antiretroviral therapy (ART) in Australia. A nucleoside reverse transcriptase inhibitor (NRTI) was initially introduced as a clinical trial in 1986 and became more widely available in 1988-9. This NTRI interfered with the action of HIV reverse transcriptase, which the virus needs to make new copies of itself. The first available treatment was called zidovudine (also known as AZT), which was initially used as monotherapy and then combined with other NRTI drugs such as didanosine, zalcitabine and lamivudine. These treatment regimens were not well tolerated by patients and were largely ineffective in producing sustained treatment responses due to the emergence of drug resistance. In this context, the introduction in 1996 of highly active antiretroviral therapy (HAART) as a treatment option offered much hope. As will be discussed further (see section 1.4), this approach combined a 'backbone' of two NRTI drugs with a third antiretroviral agent derived from a different drug class, either non-nucleoside reverse transcriptase inhibitors (NNRTIs) or HIV protease inhibitors (PIs). It was clear very soon after the initiation of HAART that this combination treatment strategy was successful in controlling HIV-1 replication in many patients, and that virological suppression could then be maintained for long periods of time. This is in contrast with the previous experience of virtually inevitable emergence of drug resistance and treatment failure with NRTI monotherapy and dual therapy. Not only did the clinical parameters of patients improve ($CD4^+$ T cell counts were rising) but, as shown in Figure 6, there was a dramatic decrease in the number of AIDS related cases being notified in Western Australia and importantly, the number of AIDS related deaths dropped to very low levels. There was also a decline in the number of new HIV notifications to a nadir of 34 cases in 1999 indicating that forward transmission of HIV was being successfully reduced with HAART treatment and marked changes in risk behaviour in the MSM population.

This was followed by a period of stability from 2000-2004 where HIV notifications remained constant (at around 50/year), but this steady state changed after 2004, when

the number of new HIV notifications in Western Australia began to increase (blue line, Figure 6) despite effective HIV therapy being used (as shown by the decline in AIDS cases, yellow line, and AIDS related deaths, green line). In 2014 Western Australia recorded its highest number of HIV notifications (n=141) since records began. This indicates there may have been other reasons why new HIV notifications were on the increase. The gap between HIV notifications and AIDS related deaths has become dramatically larger over recent times representing a growing population of people infected with HIV and suggesting the HIV epidemic in Western Australia is not contained or under control.

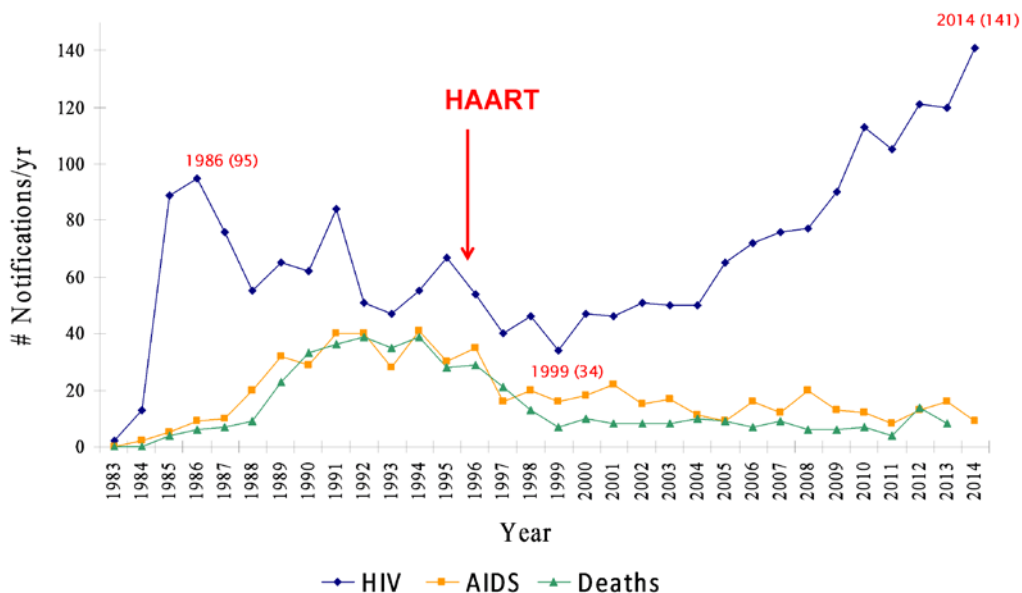


Figure 6: Trends in notification of HIV-1 infection, AIDS and Deaths from AIDS in Western Australia from 1983 to 2014. Adapted from; *The Government of Western Australia, Department of Health 2014 [15]*.

The increase in HIV notifications could be explained in part by increases in overseas migration from countries where HIV is highly prevalent. Figure 7 shows the close proximity of Australia to south and eastern Africa, Thailand, Indonesia, India and China. The adult HIV prevalence rate differs among these countries (19.1%, 1.1%, 0.5%, 0.3%, 0.05% respectively in 2013) although interestingly, all (excluding Thailand) are listed in the top 10 countries accounting for new HIV infections worldwide (Figure 3).

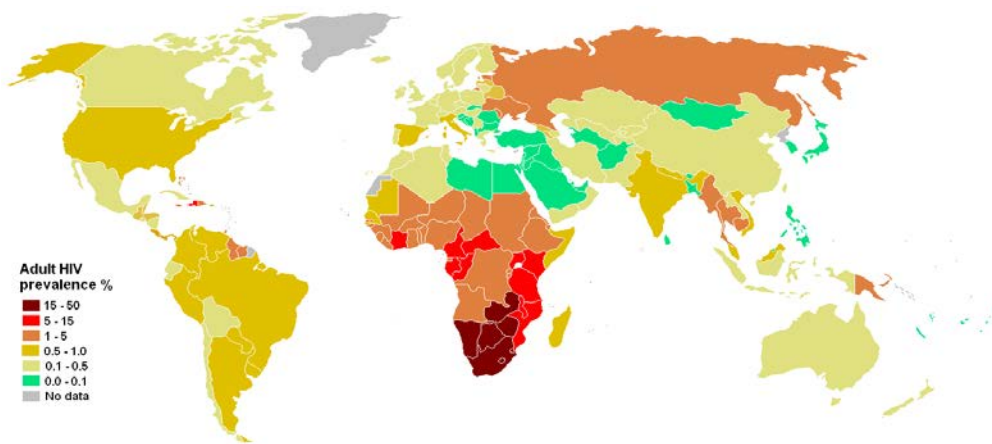


Figure 7: Prevalence of HIV infections worldwide in Adults 18-45. *Based on UNAIDS data [2].*

Data collected between 2006 and 2011, on birth countries for settlers in Western Australia when compared to national data, shows a number of these countries are proportionally larger contributors to the growing number of overseas settlers in Western Australia (Figure 8). This is especially so for people settling in Western Australia from South Africa, India and China (5%-10%).

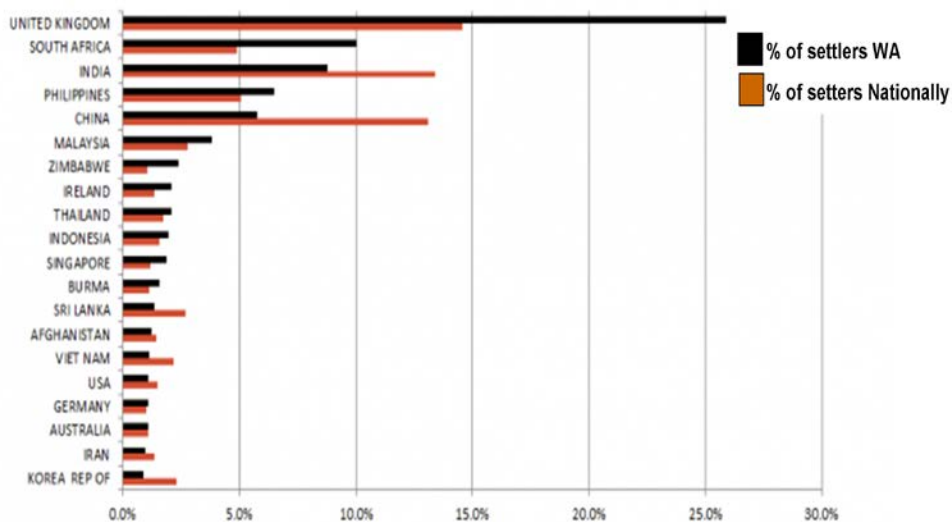


Figure 8: Comparisons of the birth countries of settlers into Australia (orange bars) and into Western Australia (black bars) from 2006 to 2011 [16].

Furthermore, Figure 9 illustrates the top 10 countries where short term visitor arrivals to Australia originate from. Some of these countries have a higher prevalence of people living with HIV or are countries where new HIV infections were highest in 2013 (i.e. China, Malaysia and India), while Figure 10 reflects the top 10 countries Australian residents visit on overseas short term stays. This indicates Australians are travelling to countries where the number of people living with HIV is high or where new HIV infections are high. From 2013 to 2014, 8,940,000 Australians travelled overseas for short term trips, including 66.8% to Indonesia, Thailand, China and Malaysia.

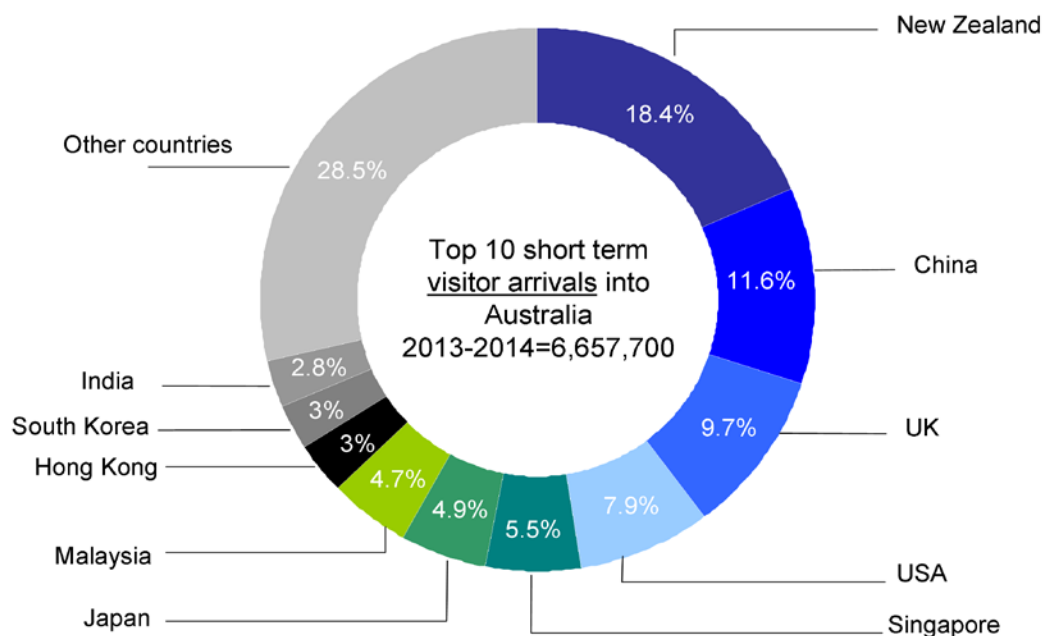


Figure 9: HIV infection and short term visitors to Australia. Ten countries accounted for 71.5% of short term visitor arrivals into Australia from 2013-2014 including arrivals from countries in the Asia/Pacific region where new HIV notifications were high (China, Malaysia and India). *Compiled from the Australian Bureau of Statistics [17].*

It is therefore relevant to assess the impact short term overseas travellers, short term arrivals and long term settlers have on the increase in HIV notifications seen in Western Australia and Australia. Since the subtypes of HIV have been shown to be associated with distinct geographical locations, HIV genotyping and subtype assignment can aid in determining if HIV subtype diversity has changed in Western

Australia and Australia more generally; and if there is a change, what impact it may have on transmission patterns, laboratory monitoring and on public health surveillance especially if eradicating HIV infections in Australia is going to be an achievable goal.

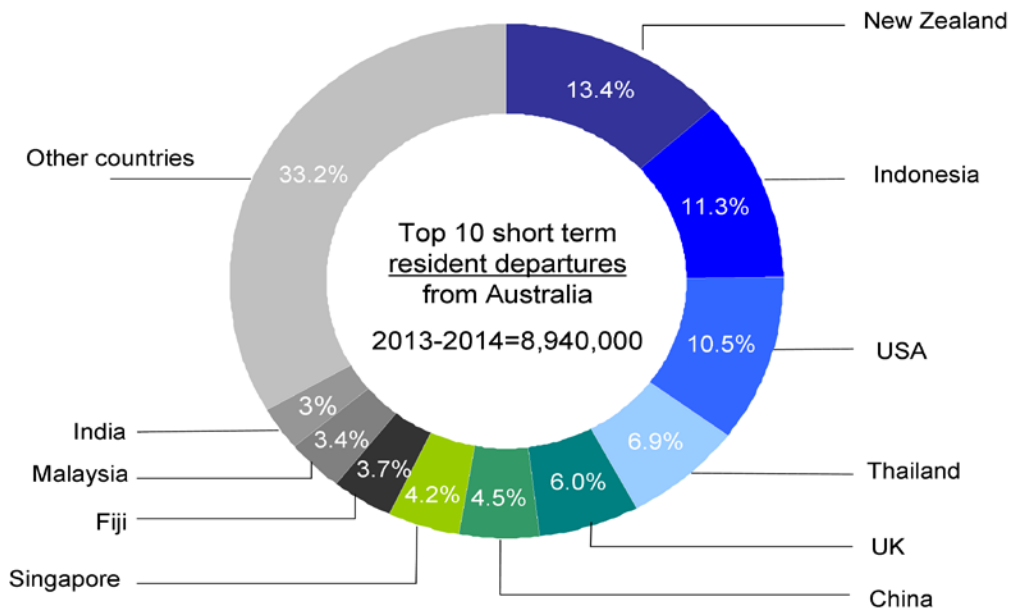


Figure 10: HIV infection and short term resident departures from Australia. Ten countries accounted for 66.8% of short term resident departures from Australia during 2013-2014 including departures to countries in the Asia/Pacific region where new HIV notifications were high (Indonesia, China, India and Malaysia). *Compiled from the Australian Bureau of Statistics [17].*

1.4 HIV clinical progression and the current treatment era

The clinical progression of untreated HIV infection has been well documented, particularly with respect to CD4⁺ T cell counts and HIV-1 RNA levels, from primary HIV infection to the acute infection stage, progressing to the clinical latency stage and to the impact of opportunistic diseases that define AIDS and death (Figure 11A). Those infected with HIV progress through the infection at different rates depending on a variety of factors including how early the infection is diagnosed and treated, HIV-1 subtype, co-morbidities (e.g. cardiovascular disease (CVD)) and genetic influences (chemokine receptor 5 (CCR5) and human leukocyte antigens (HLA)). The

implementation of antiretroviral therapy in Australia in the late 1980s and early 1990s was the beginning of successful HIV treatment but not eradication. These regimens disrupt various points of the HIV replication cycle to halt replication and to reduce the virus to undetectable levels while establishing and maintaining near to normal healthy levels of CD4⁺ T cell counts in the majority of patients (Figure 11B). Interestingly, there are a small percentage of virally suppressed patients and immune non responders, where CD4⁺ T cell counts do not adequately recover, that are at a higher risk of CVD.

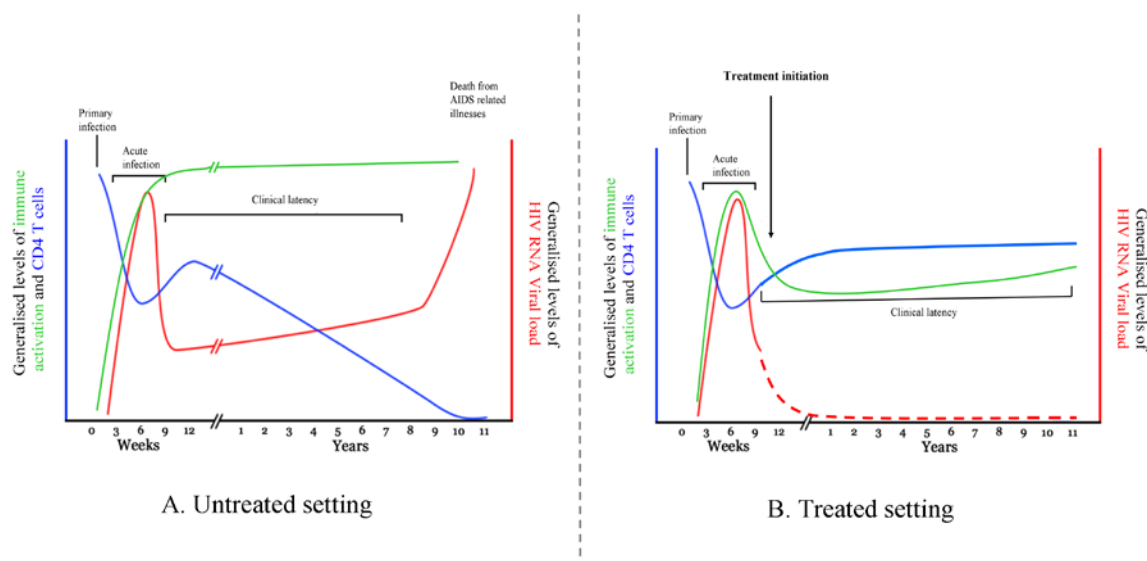


Figure 11: The natural progression of HIV infection. Generalised timeline showing the impact on CD4⁺ T cell counts, HIV RNA viral load levels and immune activation during the natural progression of HIV from initial exposure in an untreated setting (A) and in a treated setting (B). *Modified from Pantaleo G [18].*

Zidovudine (also known as AZT), a nucleoside reverse transcriptase inhibitor (NRTI) that targeted viral replication, was used in clinical trials in 1986 however it became widely available in Australia in 1991 (Figure 12). Other NRTIs were introduced a short time later, followed by the first generation of protease inhibitors (PIs) in 1996 (not shown in Figure 12 but included indinavir, nelfinavir, saquinavir and high-dose ritonavir), first non-nucleoside reverse transcriptase inhibitors (NNRTIs) in 1997, 2nd generation PIs (ritonavir boosted regimens) in 2004, and finally integrase inhibitors (INIs) and fusion inhibitors (FIs) in 2008. The NNRTIs also block HIV replication within a cell by inhibition of the reverse transcriptase protein, while PIs inhibit the HIV protease involved in the later stages of the viral replication cycle including virion

assembly. Integrase inhibitors interfere with the integrase enzyme, minimising viral integration into the deoxyribonucleic acid (DNA) of the host cell, while fusion inhibitors prevent HIV binding to T cells and therefore limit HIV entry into the cell.

The choice of ART for HIV treatment is somewhat dependent on individual case factors. Some ARTs are preferentially used during pregnancy (Combivir: combination of zidovudine and lamivudine) while others such as abacavir and nevirapine have been associated with hypersensitivity reactions in some persons [19]. Recent years has seen a swing from prescribing patients with multiple antiretroviral tablets, to the use of single multi-class tablets. Atripla, combining tenofovir and emtricitabine (NRTIs) with efavirenz (NNRTI), was the first multi-class tablet available and used in Australia in 2009. This has been followed by Eviplera (tenofovir, emtricitabine and rilpivirine (NNRTI), Stribild (tenofovir, emtricitabine and elvitegravir (INI) with cobicistat added as a cytochrome P450 3A4 (CYP3A4) enzyme inhibitor, and most recently Triumeq tablet which combines dolutegravir (INI) with abacavir and lamivudine (NRTIs).

In the current treatment era it is now clear that HIV infection can be durably controlled with suppressive antiretroviral medication regimens that are both effective and mostly well tolerated, to delay the onset of AIDS and to extend the life expectancy of those infected [20-22].

There are limitations when using antiretroviral therapies as their clinical utility can be limited due to drug resistance, toxicity and adherence. Antiretroviral regimens cannot prevent HIV from infecting some cells leading to persistent chronic HIV infection. This is due to immune evasion that exists during early HIV infection via the integration of proviral DNA into the host genome. This allows for the production of the provirus that can lead to the expression of antigens and the activation of the cell, however, the infected cell evades immune surveillance if the expression levels are low thus allowing HIV to persist regardless of successful ART. There are a number of cellular reservoirs that exist within a host that can avoid the challenge by the innate immune system. Enormous effort has recently concentrated on eradicating latent HIV in CD4⁺ T cells, however, CD16⁺ monocytes are another source of latent HIV [23-25], primarily due to the cells being more resistant to apoptosis induced by HIV or due to poor drug penetration. Adding to the interest in this area, proviral HIV can be isolated from monocytes, despite patients being on successful ART [23-25], indicating HIV can maintain viral fitness even in the current era of sophisticated

antiviral therapy. Furthermore, ART successfully reduces HIV infection to below detectable levels however treatment interruptions result in detectable viraemia in HIV patients thus indicating resumption of viral replication.

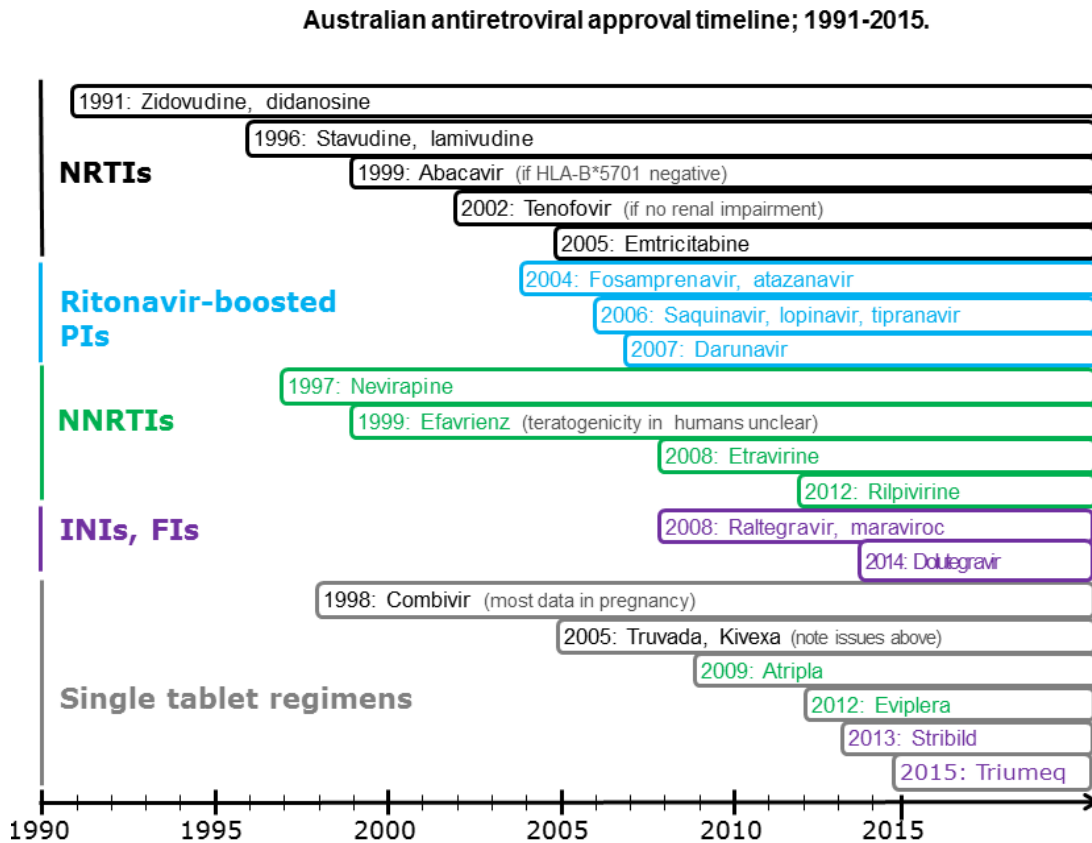


Figure 12: Australian antiretroviral regimes for the treatment of HIV. A timeline showing when HIV antiretroviral therapies became widely available for use in Australia. Treatments groups are represented in colour: NRTIs - represented in black; PIs - represented in light blue; NNRTIs - represented in green; INIs and FIs – represented in purple; and single tablet regimens represented in grey.

As the life expectancy of HIV patients increase so to do the possibilities of experiencing a serious non AIDS event (SNAE) including bone disorders, renal disease and cardiovascular events. These SNAEs are the major causes of mortality in the current ART era (SMART study).The pathogenesis and development of SNAEs is thought to be multifaceted and warrants ongoing research however immune activation and inflammation are thought to play a significant role therefore the focus of this

thesis is largely on immune activation, particularly monocyte activation and CVD risk in an HIV cohort and will be introduced further in section 1.7.

It is now clear that HIV-associated mortality is more commonly associated with prevalent diseases of ageing, rather than traditional ‘AIDS-defining’ illnesses and that chronic HIV infection may have an association with a greater risk of these co-morbid illnesses. Despite their sophistication, current strategies for monitoring HIV infection in the clinical setting (focusing on CD4⁺ T cell counts and HIV-1 RNA levels, as well as the T cell immune activation levels being monitored in Western Australia) do not appear to predict these outcomes. On the other hand, recent studies have identified novel markers of systemic inflammation that appear to be robust and predictive of adverse events including serious morbidity and mortality [26-29]. These markers appear to reflect activation of monocyte/macrophage cell populations, indicating that HIV infection may contribute to a chronic inflammatory state that is only partially reversed by HIV treatment. At this stage, however, it is not possible to assess relationships between systemic inflammation and HIV management strategies (e.g. decisions about when to commence treatment, or which antiretroviral medications to use), as potential ‘biomarkers’ have not been fully assessed and validated in a routine laboratory setting.

A proposed cause of immune activation is through circulating microbial products derived in the gastrointestinal tract. It is crucial these bacteria avoid contact with the immune system hence there are structural barriers (epithelial cells) and immunological host factors (monocytes, T cells, dendritic cells) that exist to prevent microbial products translocating across the intestine barrier to the systemic circulation. During the early phase of HIV exposure and infection however, damage to the gastrointestinal tract occurs and microbial products do transverse the epithelial barrier. Lipopolysaccharide (LPS) is a component of the cell wall of gram negative bacteria. It has previously been shown plasma levels of LPS and to a lesser extent bacterial DNA correlate with immune activation [30]. Added to this, the monocytes, T cells and dendritic cells express the viral co-receptor CCR5 providing a setting for HIV to replicate leading to the depletion of CD4 + T lymphocytes in the gastrointestinal tract. Together, there is an increased production of inflammatory cytokines including IFN- α , CXCL10 and TNF- α .

Classical monocytes (CD14⁺) secrete sCD14 which binds LPS and although sCD14 can be released from monocytes in response to LPS it can also be released by other

stimuli that are not solely specific to the gut translocating pathway. A recent study has shown inflammatory cytokines can induce the release of sCD14, and TLR ligands (flagellin and CpG oligodeoxynucleotides) can decrease the cell surface expression of CD14 therefore showing sCD14 as a marker of monocyte activation that is not restricted to activation by LPS [31].

1.5 Innate immunity and the role monocytes play to combat disease

The host immune system comprises innate and adaptive arms of immunity. The innate immune response is triggered by the recognition of determinants that may be shared by a range of pathogens, distinguishing “self” from “non-self” through pattern recognition receptors (PRRs), while the adaptive immune response is antigen-specific and generates an immunological memory. Generally, the adaptive response will not provide an antigen-specific response until after HIV infection has established itself. Toll like receptors (TLRs) are PRRs that recognise unique pathogen associated molecular patterns (PAMPs) present on the infecting pathogen [32, 33]. There are 10 TLRs found in humans, many of which recognise a specific PAMP (e.g. TLR4 recognises lipopolysaccharide (LPS) [34]; TLR7 recognises single stranded RNA [32] while TLR9 recognises CpG DNA [35]). The extracellular TLRs (TLR1, 2, 4-6, 10) recognise molecules unique to microbes but not produced by the host, and the endosomal TLRs (TLR3, 7-9) specialise in the recognition of nucleic acids.

The innate immune response to HIV infection is driven primarily through interactions with endosomal TLR7, 8 and 9 [36, 37] although some studies show interactions with TLR2 and 4 are also relevant [38]. Activation of TLRs by PAMPs results in triggering of either an antiviral cascade provoked by interferon response factors (IRFs) or an inflammatory cascade mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [36]. It is thought that the magnitude of the inflammatory response is dependent on the level of TLR expression, either extracellular or intracellular. This diverse array of TLR expression, found on and within monocytes, may be critical in mediating a response during HIV infection both in the acute and chronic phases of infection and may provide the key in immune activation and co-infections with HIV.

Monocytes are derived from the myeloid lineage, originating from precursor stem cells in the bone marrow. The monocytes undergo three main cycles of differentiation from hematopoietic stem cells to monoblasts then to pro-monocytes and finally to

monocytes where they are released into circulation into the blood stream accounting for 2-10% of circulating leukocytes [39-41]. As a part of normal immune surveillance, monocytes leave the bone marrow to circulate in the blood for 3-5 days where they express relatively high levels of TLRs, cytokines and chemokines [37, 40-42]. Monocytes migrate into the endothelium and body's tissues where they proceed to differentiate further into M1 (acute inflammatory phenotype) and M2 (anti-inflammatory phenotype) activated macrophages and dendritic cells (Figure 13).

Macrophages play a key role in the transition from innate immunity to adaptive immunity and the activation of macrophages is also pivotal in the pathogenesis of HIV infection. When monocytes migrate into the tissue compartments, TLR expression becomes refined [40-42]. They are considered to be resistant to the cytopathic effects of HIV [43, 44] and therefore the macrophages can survive for long periods enabling the establishment of a long-lived reservoir of HIV-infected cells capable of releasing HIV particles into the plasma compartment. It is now clear monocytes and macrophages are both critical in the pathogenesis of HIV infection by providing three key roles including early viral transmission, dissemination within the host and as a reservoir for viral persistence.

Conventionally, monocytes (CD14⁺) were divided into two subsets based on their CD16⁺ and CD16⁻ expression [39], however, it is now evident that monocytes are a heterogeneous population of cells that can be classified into at least three phenotypically independent populations, identified by the surface expression of CD14 and CD16 [45].

CD14 is the monocyte differentiation marker and a monocyte activation marker that mediates the innate immune response to bacterial LPS, with help from TLR4. CD14 is a receptor for the complex of LPS and LPS binding protein (LBP), resulting in the cleavage of CD14 from the cell, into circulation as soluble CD14 (sCD14).

CD16, however, is expressed on many cells including neutrophils, natural killer (NK) cells, monocytes and macrophages. It is a component of the low affinity Fc receptor, FcγRIII, mediating phagocytosis, cytokine production and antibody dependent cellular cytotoxicity (ADCC).

This more recent classification of monocyte sub-populations suggests the development of unique specialised functions within the three monocyte subsets, which has now been investigated further through genome-wide analyses that helped to define these cellular phenotypes further [46-48].

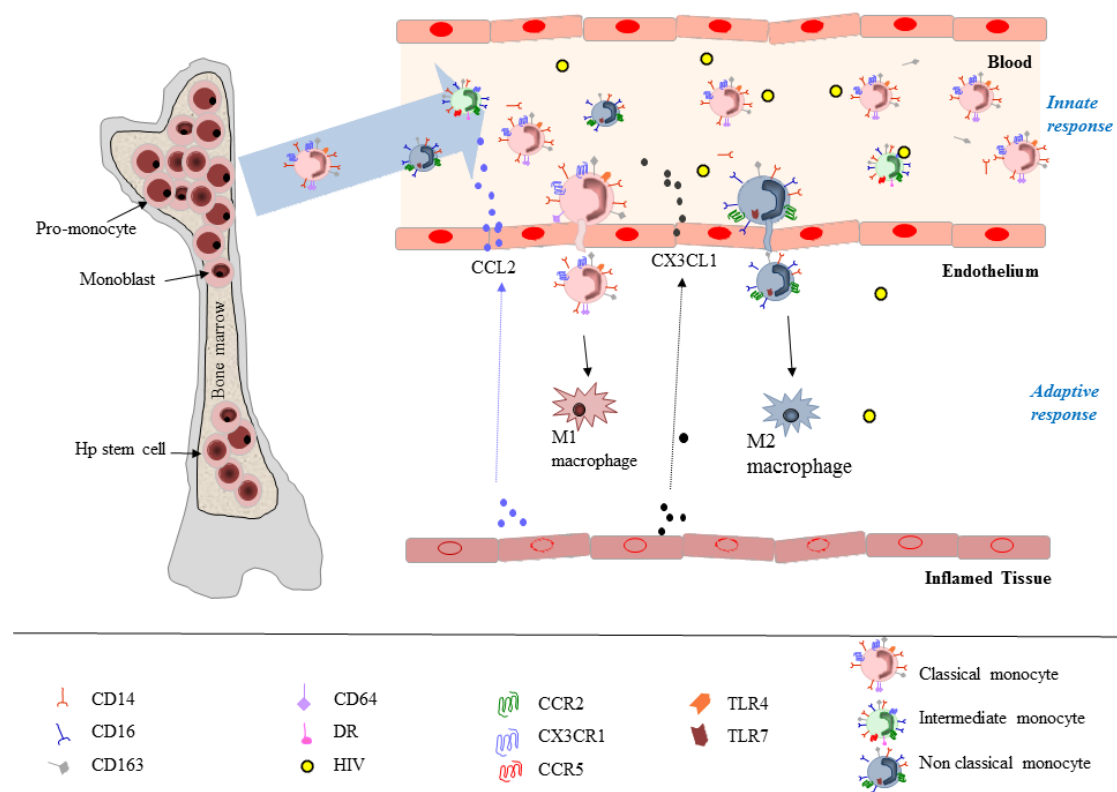


Figure 13: Development and migration of differentiating monocytes from bone marrow into the blood and to tissue compartments.

Under a steady state, classical monocytes account for approximately 85%-90% of all monocytes, expressing CD14 but not CD16 ($CD14^{++} CD16^{-}$). Classical monocytes also express high levels of Fc γ R1 (CD64), monocyte chemoattractant protein-1 (MCP-1) receptor also known as C-C chemokine receptor type 2 (CCR2), CD163 and release cytokines (IL-10 and IFN- β) while expressing low levels of CX $_3$ CR1 (receptor for fractalkine and a minor co-receptor for HIV) [39, 46, 49]. Upon activation, monocytes actively shed CD163 from the cell surface into circulation as soluble CD163 (sCD163) [50].

The percentage of CD16 $^{+}$ expressing monocytes is low under normal conditions however they expand in response to inflammatory conditions [41, 51]. Phagocytosis, the process used by cells to engulf foreign matter, is a primary role of the CD16 $^{+}$ monocytes and therefore important in primary defence to control the entrance of microbes and viral particles. The intermediate monocytes are the smallest set of monocytes only occurring at around 5% of the total monocyte population. They are

known as the inflammatory monocytes and express high levels of both CD14 and CD16 (CD14⁺⁺ CD16⁺), HLA-DR, CCR5 and CD163 and are thought to play a role in major histocompatibility complex (MHC) Class II processing and antigen presentation. Due to the expression of CCR5 it is thought that the intermediate monocytes are more susceptible to viral infection [52].

The third monocyte subset is known as the non-classical monocytes. They are highly mobile, mature monocytes, whose functions include cytoskeletal rearrangement and patrolling vessel walls. They account for approximately 5%-10% of the total monocyte population. Non classical monocytes co-express CD16 and a low level of CD14 (CD14⁺ CD16⁺) while strongly expressing CX₃CR1, expressing CCR2 and CD62L at low levels and producing high levels of pro-inflammatory cytokines (IFN γ , IL-6, IL-1 β and TNF α) to RNA/DNA stimuli [46, 53]. Non-classical monocytes have shown to be more effective against viral infections as they produce higher amounts of pro-inflammatory cytokines via the activated TLR7 and TLR8 dependant pathways [54]. They have also been linked to the increased expression of a large number of genes required for maintaining effective cytoskeletal dynamics and structure integrity suggesting they play a more migratory role than the classical monocytes [46, 55] and may provide a molecular basis for the patrolling behaviour observed *in vivo* [54].

Generally, a balance between the monocyte subsets is required to maintain homeostasis by regulating inflammation and tissue repair. Failure of these processes may result in aberrant inflammatory responses potentially leading to chronic inflammation and ongoing immune activation.

1.6 The dynamics of ageing, monocytes and HIV infection

Alterations in the phenotype and functionality of monocytes have been an increasing focus of attention in healthy ageing population studies with emphasis on the impact of these changes on immune dysfunction and specific inflammatory responses. Recent studies have found monocyte frequency and phenotype are altered in the aged and frail [56] and that these changes correlated with chronic diseases such as dementia [57]. Of interest, CD16⁺ monocytes have been shown to be increased in an ageing population and monocyte impairment has been identified with cytokine production and expression of TLR [56]. Added to this, a lower frequency of monocytes expressing CX₃CR1 in the elderly was identified when compared to young adults, however, CD16⁺ monocytes expressed more CX₃CR1 in patients with dementia than

in controls [57]. In addition, CD16⁺ intermediate monocytes showed increased HLA-DR and CX₃CR1 expression than CD16⁻ monocytes, although the expression on CD16⁺ monocytes declined in the elderly [58]. These findings could be functionally important given CX₃CR1 has been linked to monocyte survival in atherosclerotic conditions [59]. Finally although these results appear to indicate that the ageing populations have immune dysfunction, the mechanisms associated with this dysfunction remain largely unclear. This warrants further investigation, particularly in relation to the role of monocyte subpopulations in promoting (and/or protecting against) chronic age-related diseases.

This interest in altered monocyte phenotypes in healthy populations also extends to changes occurring during HIV infection. In 2013-2014, an estimated 36.3 million people were living with HIV and an estimated 3.6 million were older than fifty. Furthermore, in high income countries alone, around one third of adults living with HIV were over fifty [2] and as previously suggested, it will soon be the case that the majority of HIV infected individuals in the developed setting will be older than 50 years of age [60]. In the context of HIV infection, the increased uptake of antiretroviral therapy throughout the world enables those living with HIV to have increased life expectancy [20-22]. For this reason, ageing with HIV infection presents a number of challenges as HIV not only reduces CD4⁺ T cell numbers but also causes immune dysfunction of cells responsible for combating infection that persists even in individuals on successful HIV suppressing therapy. HIV infection is associated with increased age-related changes to the immune system causing it to be less likely to adequately respond to foreign pathogens and therefore increases the level of damaging inflammation. There is now strong evidence of premature physiological ageing with HIV infection, with markers of physical frailty [60, 61], cerebral function [62] and atherosclerotic burden [63] noted to be 10-15 years more advanced than normally expected.

It is not known whether accelerated ageing continues throughout the course of HIV infection and treatment, however, proposed associations with persistent immune activation [64] along with evidence from the SMART study [65] and numerous sub-studies that have arisen from it [26, 27] have demonstrated increased rates of adverse events, including all-cause mortality, among patients with untreated HIV infection. Importantly, these studies have shown that markers of innate immune activation and coagulation such as C-reactive protein (CRP), sCD14, IL-6, CXCL10 and D-dimer

levels, were strongly associated with both infectious and non-infectious complications including mortality [28, 29, 66]. Interestingly a recent study confirmed higher rates of multiple morbidities in patients with long term-ART treated HIV when compared to patients with shorter term-ART treated HIV but acquiring HIV at an older age (>45yo) [67], indicating that HIV itself may contribute to increased risk of serious non AIDS illnesses even in the setting of HIV treatment.

The link between HIV infection and monocyte activation is gaining growing attention. During HIV infection, monocyte responses occur early through the production of interferon (IFN) along with other cytokines. Type 1 IFNs however are produced primarily by plasmacytoid dendritic cells (pDC), monocytes and T cells playing a central role in mediating persistent inflammation during HIV infection and many studies focus on the role of pDC and T cells in these settings. Monocytes have been gaining increased attention in HIV studies as they have many functions particularly in immune defence by driving innate immunity as described in section 1.5. Briefly recapping, monocytes limit the access of a pathogen into cellular components by raising innate immune responses, aiding in tissue repair and responding to foreign invasions by promoting phagocytosis. Damage to monocytes, however, leads to a loss of the innate barrier thus allowing HIV to establish systemic infection [32, 33, 36]. One study, in an HIV cohort, suggests that low CD4⁺ T cell counts correlate with higher levels of intermediate monocytes (CD14⁺⁺/CD16⁺) in particular those expressing CD163 [68]. This fits with the concept that higher sCD14 levels, resulting from being cleaved off monocytes, was deemed to be detrimental in the SMART study [28]. Several studies suggest that ageing and HIV infection have interrelated effects on monocyte function, including observations that CD16⁺ monocytes in young males with untreated or virologically suppressed HIV infection are similar in frequency to older HIV negative males [69], while two studies focusing on HIV in women have shown plasma levels of monocyte activation markers in HIV infected women were equivalent to HIV negative women 14 years older. Additionally, biomarkers of CVD and impaired cognitive function were elevated in HIV infected women and correlated with immune activation [70, 71].

Overall the monocyte phenotype during HIV infection shares the characteristics of those found in the elderly population, suggesting a prominent contribution of monocyte activation (and of interferon signalling pathways) towards adverse events during long-term HIV outcomes. Despite their predictive value and emerging

biological importance in HIV infection, in other infections (e.g. tuberculosis (TB), hepatitis C virus (HCV) infection) and in other diseases (e.g. CVD, diabetes, renal failure), these biomarkers are not monitored in routine HIV clinical practice. For this reason, the diagnostic value of measuring monocyte expression and soluble levels of monocyte activation in HIV infection will be investigated in Chapter 3 and Chapter 4.

1.7 Co-morbidity: HIV-1/cardiovascular disease in an ageing HIV population

The challenge of HIV care in an era where plasma HIV-1 RNA levels are well controlled by HAART has increasingly focused on clinical problems associated with both infectious and non-infectious co-morbidities in an ageing HIV population. Among the most important clinical challenges facing HIV carers is the rising rate of CVD which has been found to occur earlier and at a higher rate in HIV patients than in the HIV negative population [63, 72]. Considering that the World Health Organisation (WHO) forecast shows ischaemic heart disease being one of the leading causes of death in the general population globally by 2030 [73], including in regions where HIV-1 prevalence rates are high, this signals an increasing need to better understand the mechanisms involved in co-morbidities during HIV infection, and to develop effective monitoring and treatment strategies based on this improved understanding of disease pathogenesis.

In the early years of ART the interaction between antiretroviral exposure and increased risk of myocardial infarction was shown in large study cohorts [74-76]. Along with these results, other studies relate chronic inflammation to atherosclerosis and mortality in HIV patients [77-79] and T cell activation in HIV patients to early vascular damage even when on successful therapy [80, 81]. There has also been a great deal of interest in the potential role of monocytes in HIV infection, particularly CD14⁺⁺/CD16⁺ intermediate monocytes, in disease pathogenesis given that these populations (which co-express CCR5) [82] are permissive to HIV infection [23] and are capable of transferring HIV infection across the genital mucosal barrier [83] as well as into the central nervous system [84]. Expansion of this intermediate monocyte population also appears to be associated with cardiovascular events in subjects referred for elective coronary angiography [85] and has been noted in acute coronary syndromes in the general population as well as in chronic HIV infection [86].

In addition cell surface markers may further characterise CD16⁺ monocyte function, particularly in the context of HIV infection and/or inflammation. These include the

angiotensin converting enzyme (CD143), expressed predominantly on CD14⁺⁺/CD16⁺ intermediate monocytes, which has been linked to mortality and cardiovascular disease in haemodialysis patients [87]; as well as the scavenger receptor CD163 which has been shown to be significantly elevated in the context of HIV infection [88, 89]. In contrast, the high affinity FcγR1 (CD64) is principally expressed on CD14⁺⁺/CD16⁻ classical monocytes, where it may serve as a biomarker of type I interferon activation in autoimmune diseases [49]. Monocyte CD64 expression may restrict productive HIV-1 infection by facilitating viral phagocytosis and degradation [90].

Besides cell surface markers, several plasma biomarkers of monocyte activation have also been linked to associations between HIV and CVD, including sCD14 which has been shown to be associated with artery calcification [91], atherosclerosis [92] and all-cause mortality in HIV patients [28], even in the setting of undetectable plasma HIV-1 RNA levels that would generally define effective HIV therapy [93]. Elevated levels of sCD163 have also been shown to be associated with arterial inflammation and cardiovascular disease in HIV-infected patients [94] although sCD163 levels appear to be responsive to HIV therapy [68]. Similarly, plasma levels of CXCL10 (also known as interferon gamma-induced protein 10 [IP-10]) are induced by HIV infection [95] but are also readily reduced by effective therapy [96]. The role of CXCL10 in predicting CVD in an HIV setting is largely unknown however CXCL10 is likely to play a role in coronary heart disease (CHD) [97, 98].

It is therefore clear that identifying HIV patients at risk of CVD has become a focus in many clinics over recent years, being partially achieved by monitoring traditional risk factors. In recent studies, markers of immune activation promoting the increased risk of CVD in HIV have been identified however the mechanisms driving the immune activation (particularly monocyte activation) and causing serious non AIDS related conditions are likely to be multifactorial. In this thesis I will investigate the determinants of monocyte phenotypes and plasma biomarkers of monocyte activation in a large and well-characterised HIV cohort, considering a range of variables including plasma HIV-1 RNA levels, CD4⁺ T cell immune deficiency, HIV treatment choice, as well as traditional cardiovascular risk factors and other demographic variables. These studies will seek to address the clinical utility of measuring monocytes – both quantitatively and functionally – in the HIV laboratory setting, and

to explore the relationships between different ‘biomarkers’ of monocyte function in this setting.

1.8 In with the new and out with the old: A shift in the HIV monitoring paradigm

After HIV infection has been diagnosed, monitoring systems, both laboratory and clinical, are required to provide information regarding the stage of HIV infection, likelihood of co-infections and to determine when ART should start. We have now entered a period where the long-term success of ART has created new challenges relating to the potential effects of HIV infection and its treatment on the onset and severity of prevalent age-associated diseases. This is becoming increasingly relevant now that the average age of male patients in the Royal Perth Hospital HIV cohort is 50 years and preliminary data from 530 patients surveyed over 2 years found 25% with high CVD risk (>10%; 5-year risk), with 45% of the cohort currently smoking, suggesting that there is a significant risk of long-term illness in this population, as has been found in other HIV cohorts internationally [64, 94, 99, 100]. As previously mentioned sCD14, IL-6, CXCL10 and D-dimer levels have been shown to be strongly associated with both infectious and non-infectious complications including mortality. These biomarkers appear to indicate a prominent contribution of monocyte activation towards adverse events during long-term HIV outcomes however they are not monitored in routine clinical practice. A recent study supported the idea of testing CD4⁺ T cell counts less frequently in clinically stable settings as HIV patients who maintained HIV viral suppression below <200cpm and had CD4 counts >300cells/μl were highly unlikely to experience a CD4 count <200cells/μl [101, 102].

It would therefore be valuable to gain a better understanding of factors that contribute to immunological activation during routine management of HIV infection, particularly in long-term care when ‘traditional’ monitoring tests for virological and immunological response to treatment (i.e. HIV-1 RNA and CD4⁺ T cell count) indicate that treatment cannot be further optimised.

1.9 Structure and goals of this thesis

The hypothesis of this study is that chronic immune activation may be a feature of long-term HIV infection, even in the context of effective antiretroviral treatment with ongoing undetectable plasma HIV-1 RNA levels and normal-range CD4⁺ T cell

counts. This immunological phenotype is likely to involve monocyte/macrophage activation and cytokine and chemokine production that are not currently assessed in routine HIV management, and are therefore 'invisible' from a clinical management (or therapeutic) perspective. An improved understanding of this immunological phenotype, and an ability to monitor it in a clinical setting, should allow for a better understanding of many non-infectious co-morbidities of ageing (e.g. cardiovascular disease, cognitive decline) that are clearly more common among HIV-infected individuals who are otherwise clinically stable.

This thesis seeks to address four key aspects in HIV monitoring and care in Western Australia (Figure 14). Chapter 2 describes the investigation of determinants of HIV-1 RNA suppression below the threshold of the routinely used method. Chapter 3 explains the concept of monocyte activation during HIV infection while Chapter 4 explores co-morbidities with HIV (specifically HIV and CVD) in relation to circulating monocyte activation markers and the level of effects from three activation markers. Improving diagnostic approaches is likely to have significant implications for developing more effective management strategies during chronic HIV infection and may also contribute to an improved understanding of other prevalent illnesses.

The next phase of the study presented in Chapter 5 will focus on monitoring HIV in WA by employing a phylogenetic approach to map HIV subtypes and assess networks by including clinical parameters into the analysis ($CD4^+$ T cell counts and HIV-1 RNA levels). This will lead into the reasoning behind the formation and collaboration of the Australian Molecular Epidemiology Network (AMEN). This network involves experts in the field of HIV and epidemiology and was formed to establish an ongoing connection throughout Australia with the aim of assessing HIV phylogenetic characteristics within Australia as detailed in Chapter 6.

The final discussion (Chapter 7) highlights the importance of developing, validating and implementing cost-effective assays pertaining to immune activation and HIV phylogeny as a way forward for routine HIV care thus allowing for improved clinical assessment and targeted therapeutic strategies.

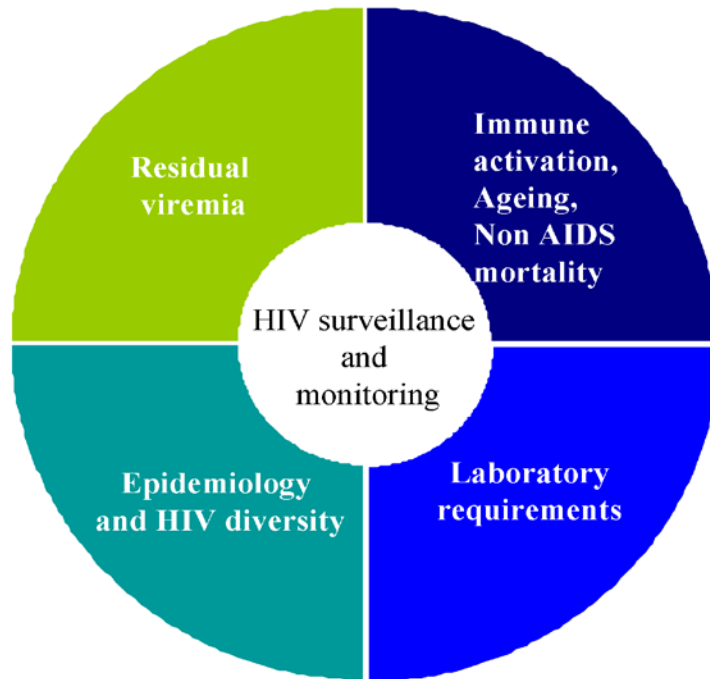


Figure 14: Improving HIV surveillance and monitoring in Western Australia by focusing on four key points throughout this citation including an analysis of HIV-1 epidemiology and HIV-1 diversity throughout Australia.

Throughout the following chapters I aim to bridge this gap through:

- 1) The development of monitoring approaches and routine diagnostic assays that may ‘reveal’ underlying systemic immune activation when current analytes like CD4⁺ T cell counts are within normal health range and HIV-1 RNA is successfully suppressed, while immune activation persists.
- 2) Surveying the HIV epidemic at an epidemiological level by investigating HIV subtypes, HIV transmission and network characteristics in the current era in Western Australia and Australia.

This project is translational in nature, seeking to establish improved clinical laboratory methods for the routine assessment of immune function in ageing patients with HIV infection. This approach fits well with several established frameworks and declarations including; 1) the Western Australian HIV strategy 2015-2018; 2) the 7th National HIV Strategy for 2014-2017; and 3) the United Nations declaration on HIV and AIDS 2011, and directly addresses several priority actions identified in these documents (see exerts below).

Box 1 WA HIV Strategy 2015–2018

“Goals

- *to work towards achieving the virtual elimination of HIV transmission in Western Australia by 2020*
- *to work towards achieving the virtual elimination of HIV transmission in Western Australia by 2020*
- *to reduce the morbidity and mortality caused by HIV”*

“Prevention, training and addressing gaps

- *increase knowledge of HIV and transmission risks among priority populations*
- *developing and implementing approaches that prepare for an ageing cohort of people living with HIV*
- *identifying areas and regions, based on epidemiological data, where primary healthcare professionals may require enhanced support*
- *use epidemiological data in service planning and implementation*
- *work with local and national research centres on epidemiological research, including longitudinal studies”*

Box 2 The 7th National HIV Strategy for 2014-2017

“Goals

- *work towards achieving the virtual elimination of HIV transmission in Australia by 2020*
- *reduce the morbidity and mortality caused by HIV”*

“Management, care and support

The ageing of the population of people living with HIV is an important feature of the changing epidemiology of HIV. Advances in HIV antiretroviral therapy has decreased HIV and AIDS-associated mortality and morbidity. The increasing numbers of people living with HIV and the ageing nature of this population have important implications for the healthcare system

The effectiveness of antiretroviral therapy means there are more people with HIV living longer and surviving into old age. There is a growing body of evidence that HIV can substantially impact on people as they age; for example, diseases normally associated with ageing can occur at younger ages. HIV models of care need to facilitate the monitoring, care and support of people living with HIV to manage the comorbidities associated with living with HIV infection long-term and the natural ageing process [103].”

Box 3 United Nations declaration on HIV and AIDS 2011

“Treatment, care and support: eliminating AIDS-related illness and death

Pledge to intensify efforts that will help to increase the life expectancy and quality of life of all people living with HIV.

Commit to investing in accelerated basic research on the development of sustainable and affordable HIV and tuberculosis diagnostics and treatments for HIV and its associated co-infections and ensuring that it is based on the highest ethical and scientific standards.”

Chapter 2:

**Determinants of residual viraemia during combination HIV
treatment: Impacts of baseline HIV RNA levels and
treatment choice.**



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Attributions:

AC proposed and helped design the study, accumulated results, reviewed literature, designed the figures and critically reviewed the manuscript. EM performed the statistical analysis of the data along with manuscript preparation. LP helped compile Figure 1 and reviewed the manuscript. SP supervised LP and AC in assay performance and reviewed the manuscript. DN proposed and developed the design of the study, supervised and critically reviewed the manuscript.

AC: 60%

Determinants of residual viraemia during combination HIV treatment: Impacts of baseline HIV RNA levels and treatment choice

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Objectives

Effective HIV therapy reflects suppression of plasma HIV RNA levels below assay detection thresholds, although lower levels of “residual viraemia” have also been demonstrated over extended periods of effective antiretroviral treatment. Here we examine the determinants of HIV RNA suppression below the standard assay threshold (40 HIV-1 RNA copies/mL) as well as factors associated with detectable HIV RNA below this reported detection limit.

Methods

Between 2007 and 2010, 11 575 consecutive viral load (VL) tests were obtained from 1540 patients, including 356 on effective antiretroviral therapy followed since initiation (1996–2001: $n = 165$; 2002–2009: $n = 191$). Analyses modelled the probability of an undetectable VL given successful suppression to < 200 copies/mL, and the probability of residual viraemia given an undetectable result.

Results

Detectable HIV RNA amplification was demonstrated in 20% of samples with a VL result < 40 copies/mL. Longitudinal analyses from 356 patients revealed that the likelihood of achieving results < 40 copies/mL was increased with current nonnucleoside reverse transcriptase inhibitor (NNRTI) therapy [odds ratio (OR) 2.0; $P < 0.05$] and reduced with prior virological rebound (OR 0.5; $P < 0.05$). In contrast, the presence of detectable HIV RNA < 40 copies/mL was strongly associated with pretreatment HIV RNA levels among those on current protease inhibitor (PI) treatment (OR 1.5 per \log_{10} copies/mL increase; $P = 0.02$) as well as those on NNRTIs (OR 1.7; $P = 0.002$).

Conclusions

While HIV treatment history was associated with plasma HIV RNA levels below the detection limit, residual viraemia results were dominantly determined by pretreatment VL. These findings support the concept of a stable, long-lived reservoir of latently infected cells as a source of residual viraemia despite effective HIV treatment.

Keywords: determinants of virological control, HIV, residual viraemia

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Introduction

The use of combination antiretroviral therapy (cART) regimens is associated with durable virological response in

the majority of HIV-infected patients, reflected by the laboratory assessment of an “undetectable viral load” on monitoring blood tests. While the maintenance of viral load levels at < 40 HIV-1 RNA copies/mL remains the standard of care, as measured by current assays [1], there is now increasing evidence that many patients have “residual viraemia” with low-level viral replication below this detection limit [2]. The long-term consequences of persistent low-level viraemia are not known, although there is growing interest in the chronic inflammatory

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component of HIV infection, which can be observed among those who have residual viraemia [3,4]. For these patients, there is no evidence that treatment intensification with additional antiretroviral agents, [5] including integrase inhibitor treatment [6], leads to better virological outcomes. Previous studies have provided evidence that better viral load outcomes are associated with lower pre-therapy HIV RNA levels and higher CD4 T-cell count [2,3], as well as with choice of treatment regimen [7,8], although these data are difficult to interpret or to incorporate into clinical management guidelines [9].

In this study, we have investigated likely determinants of virological response to treatment within the Western Australian (WA) HIV Cohort study population, including disease-specific factors (e.g. pretreatment viral load and CD4 T-cell count [2,3]) as well as choice of antiretroviral treatment regimen. In particular, we have assessed the likelihood of achieving and maintaining a viral load result where there is no detectable RNA target (“no target detected”, as defined below) in the context of successful suppression of HIV RNA levels to below the prescribed assay detection limit. This cohort-based analysis has the advantage of large sample size, single-site laboratory evaluation using a standardized and quality-assured viral load assay, and availability of comprehensive longitudinal demographic, clinical and laboratory data for participants in the WA HIV Cohort study. An additional advantage of this analysis is that management decisions regarding HIV treatment could not be influenced by knowledge of target detection in HIV RNA results below the reported assay limit of 40 copies/mL, as this information was not included in laboratory reports to clinicians.

Methods

The study analysed data from consecutive viral load measurements collected between 2007 and 2010 from patients attending the HIV clinic at Royal Perth Hospital. All viral load tests were performed at the hospital Department of Immunology, which provides a state-wide HIV laboratory service for Western Australia, using the Cobas Ampli-prep/Cobas TaqMan HIV-1 test (version 1: Roche Diagnostics, Mannheim, Germany). All assay runs were conducted according to National Association of Testing Authority, Australia (NATA) accreditation standards, subject to quality control procedures and an ongoing quality assurance programme. Although plasma HIV RNA measurements were determined during routine laboratory testing, results below the prescribed assay detection limit were simply reported to clinicians as “< 40 copies/mL”; there was no reported differentiation between having detectable HIV RNA levels in the range 0–40 copies/mL

and a “no target detected” result which required no detection of target polymerase chain reaction (PCR) amplification for > 15 cycles beyond the expected crossing threshold of 32 cycles (Fig. S1).

Formal analyses were conducted on viral load measurements of patients who had been followed for at least 1 year from commencement of cART based on either a protease inhibitor (PI) or a nonnucleoside reverse transcriptase inhibitor (NNRTI), who had a CD4 count at baseline < 1000 cells/ μ L, and who had a viral load measurement at baseline > 1000 copies/mL.

Binary analyses of the viral load measurements modelled two conditional probabilities: the probability of a patient having a viral load below the assay lower limit of 40 copies/mL, given that the viral load was successfully suppressed by treatment to at least < 200 copies/mL; and the probability of a patient having “detectable target” results given the achievement of a viral load < 40 copies/mL. These analyses were undertaken within a random effects logistic regression framework to take account of within-subject correlation attributable to the multiple viral load measurements per patient. The logistic regression models the probability of an event on the log odds scale, so in the analyses presented here the relative odds [i.e. odds ratio (OR)] pertaining to two values of a specific variable is calculated as $\exp(\text{difference in predicted values})$. The 95% confidence intervals (CIs) are then obtained by the appropriate transformation of the 95% CI on the log odds scale.

The analyses examined the impact of both treatment-related factors (class of cART regimen, current regimen, duration of cART, and prior viral rebound to > 400 copies/mL), clinical factors (CD4 count and viral load at commencement of cART) and demographic factors (sex, age and race). The demographic variables and the clinical measurements obtained at commencement of cART were considered as baseline variables.

Results

A schematic of the available data is presented in Fig. 1. In total, 11 575 viral load measurements were collected from 1540 patients [median (interquartile range (IQR)) 7 (2–12) visits per person]. Of these, 74.1% ($n = 8575$) were measured at < 200 copies/mL and 64.4% ($n = 7451$) were suppressed to < 40 copies/mL, with 51.4% ($n = 5954$) having no target detectable. Hence, the overall proportion of results < 40 copies/mL in which HIV RNA target remained persistently detectable (i.e. residual viraemia) was 20.1% ($n = 1497$). Of the 636 patients included in the clinical database with demographic information available and documented treatment data during this study

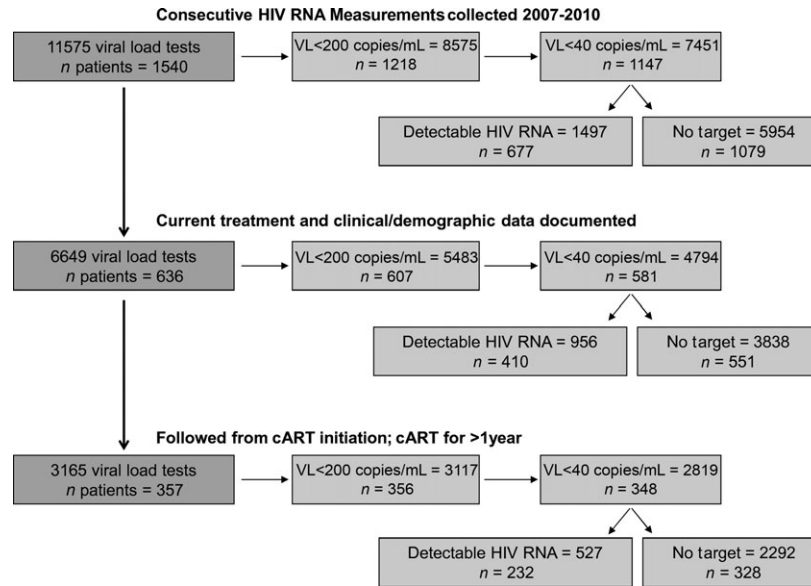


Fig. 1 A schematic representation of the available data from 11 575 consecutive HIV RNA tests performed between 2007 and 2010. cART, combination antiretroviral therapy; VL, viral load.

period, 607 (95.4%) received HIV therapy resulting in viral load suppression to < 200 copies/mL over 5483 measurements in total. From these samples, viral load results < 40 copies/mL were obtained from 87.4% of measurements, of which 19.9% had detectable HIV RNA target. Over half of the 581 patients having results reported as undetectable had measurements both with and without detectable target [$n = 380$ individuals; median (IQR) 11 (8–13) visits per person]. On average, residual viraemia was observed in 24.5% of measurements from these patients.

Further longitudinal analyses were undertaken among 356 patients who had been followed since commencement of combination antiretroviral therapy (cART) for more than 1 year and who had achieved viral suppression to < 200 copies/mL during follow-up. This data set comprised 3117 viral load measurements < 200 copies/mL with an average of 8.76 measurements per person [standard deviation (SD) 4.4]. Within this data set, 90.4% of viral load results were measured at < 40 copies/mL, of which 18.7% demonstrated detectable HIV RNA target. While no HIV RNA target was detected in any measurements for 116 patients [median (IQR) 7 (2–11) visits per person], residual viraemia was observed in approximately 23% of measurements [median (IQR) 11 (8–13) visits per person] among the 212 patients having results both with and without detectable target.

Demographics and patient summaries are provided in Table 1, stratified according to year of cART initiation, 1996–2001 and 2002–2009, to reflect changes in prescribing patterns. Of patients commencing treatment in

the years 1996–2001, 60% (99 of 165) received a PI as their first-line cART; these patients had significantly lower CD4 T-cell numbers at baseline (difference 110 cells/ μ L; $P = 0.003$) and were approximately 5 years older at the time of analysis ($P = 0.05$) compared with patients receiving an NNRTI (predominantly nevirapine). In contrast, over the latter years a PI was chosen as first-line cART for only 28% (53 of 192) of patients. Among patients of the latter cohort, baseline viral load values were higher among the PI recipients ($P = 0.004$) but cART class differences in baseline CD4 count and age were not observed. Plots of the observed proportions across initial class and era of treatment are provided in Fig. 2.

Predictors of virological response to HIV RNA levels < 40 copies/mL

Initial univariate analyses examined the impact of each baseline variable, in turn within each cohort, as a potential predictor of achieving viral load levels reported as undetectable (HIV RNA < 40 copies/mL). The analyses revealed a consistent effect of current NNRTI therapy across both eras of treatment initiation (1996–2001, OR 2.26, $P = 0.004$; 2002–2009, OR 1.98, $P = 0.01$; Table 2). Prior virological failure marked by a rebound of HIV RNA > 400 copies/mL was also associated with a reduced likelihood of achieving subsequent viral load levels < 40 copies/mL (1996–2001, OR 0.35, $P = 0.001$; 2002–2009, OR 0.28, $P < 0.0001$). Higher pretreatment HIV viral load

Table 1 Demographic variables among individuals with virological suppression to < 200 copies/mL and on documented combination antiretroviral therapy (cART) > 1 year (n = 356)

	cART initiated 1996–2001			cART initiated 2002–2009		
	Initial regimen class			Initial regimen class		
	PI (n = 99)	NNRTI (n = 66)	P ^a	Init PI (n = 53)	NNRTI (n = 138)	P ^a
Male	86 (87)	53 (80)	0.3	40 (75)	114 (83)	0.3
Caucasian	81 (82)	49 (74)	0.3	37 (70)	98 (71)	0.9
Number of visits [median (IQR)]	11 (8–13)	9.5 (5.25–12)	0.03	7 (3–11)	8 (5–12)	0.07
At initiation of cART						
Age (years) [median (IQR)]	41.11 (32.8–48.3)	36.78 (30.3–46.0)	0.05	42.97 (34.2–48.1)	41.12 (34.3–49.2)	0.7
Viral load (log ₁₀ copies/mL) [median (IQR)]	5.19 (4.6–5.7)	5.01 (4.7–5.5)	0.3	5.00 (4.8–5.6)	4.98 (4.5–5)	0.004
CD4 count (cells/μL) [median (IQR)]	204 (68–361)	314 (205–490)	0.003	280 (160–372)	294 (192–396)	0.6
Included in initial regimen						
Atazanavir	0 (0)	–	–	26 (49)	–	–
Lopinavir	4 (4)	–	–	26 (49)	–	–
Ritonavir	13 (13)	–	–	52 (98)	–	–
Indinavir/nelfinavir/saquinavir	95 (96)	–	–	1 (2)	–	–
Efavirenz	–	25 (38)	–	–	101 (73)	–
Nevirapine	–	41 (62)	–	–	37 (27)	–
At first visit included in analysis						
Age (years) [median (IQR)]	50.20 (42.8–58.2)	45.26 (37.5–54.4)	0.007	45.27 (35.7–51.1)	43.03 (36.6–51.2)	0.6
Duration of therapy (years) [median (IQR)]	9.89 (8.6–10.4)	8.69 (6.7–9.5)	< 0.0001	1.33 (1.1–3.0)	1.59 (1.2–3.3)	0.5
CD4 count (cells/μL) [median (IQR)]	580 (384–756)	650 (443–856)	0.07	462 (338–754)	512 (381–756)	0.5
Viral load < 40 copies/mL	81 (82)	58 (88)	0.4	44 (83)	124 (90)	0.2
No detectable HIV RNA target	64 (65)	43 (65)	> 0.9	30 (57)	103 (75)	0.02
Prior rebound in viral load to > 400 copies/mL	68 (69)	37 (56)	0.1	13 (25)	25 (18)	0.3
Class change from first cART	37 (37)	23 (35)	0.9	12 (23)	19 (14)	0.2
Included in regimen						
Atazanavir	35 (35)	12 (18)	0.02	28 (53)	15 (11)	< 0.0001
Lopinavir	8 (8)	9 (14)	0.3	12 (23)	3 (2)	< 0.0001
Ritonavir	56 (57)	19 (29)	0.0005	41 (77)	19 (14)	< 0.0001
Indinavir/nelfinavir/saquinavir	12 (12)	6 (9)	0.6	0 (0)	1 (1)	> 0.9
Efavirenz	12 (12)	16 (24)	0.06	6 (11)	79 (57)	< 0.0001
Nevirapine	25 (25)	27 (41)	0.04	6 (11)	40 (29)	0.01
Tenofovir	34 (34)	17 (26)	0.3	27 (51)	69 (50)	> 0.9
Abacavir	46 (46)	28 (42)	0.6	20 (38)	52 (38)	> 0.9
Lamivudine	77 (78)	48 (73)	0.5	32 (60)	79 (57)	0.7

PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; IQR, interquartile range.

Values are n (%), unless otherwise stated.

Significant values are shown in bold.

^aP values for comparisons between treatment classes obtained from either a Fisher test (categorical variables) or a Mann–Whitney U test (continuous measures).

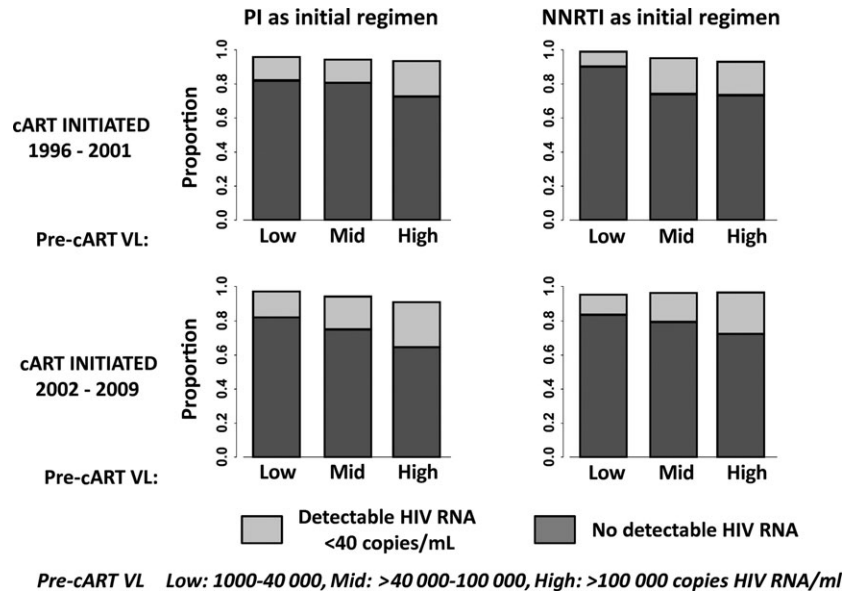


Fig. 2 Observed categories of virological suppression as proportions of the HIV RNA measurements < 200 copies/mL, according to the initial class of combination antiretroviral therapy (cART) [protease inhibitor (PI) or nonnucleoside reverse transcriptase inhibitor (NNRTI)] and era of treatment initiation. Pre-cART viral load (VL): low, 1000–40 000 copies/mL; mid, > 40 000–100 000 copies/mL; high, > 100 000 copies/mL.

levels were associated with poorer treatment response in the later cART cohort (OR 0.52; *P* = 0.02) but no significant effect was evident among the patients who commenced treatment in the earlier era and thus had longer follow-up (OR 0.8; *P* = 0.3).

When these variables were considered together in multivariate analyses, the ability to achieve viral load levels < 40 copies/mL remained strongly associated with current NNRTI therapy across both treatment eras. No other variable retained independent significance among those individuals who initiated treatment prior to 2002, while for

those in the latter treatment era the detrimental influence of elevated pretreatment HIV RNA levels (OR 0.5 per log₁₀ copies/mL increase; *P* = 0.01) and prior history of virological failure (OR 0.18; *P* < 0.0001) remained highly significant. Interestingly, a longer duration of cART was associated with an improved likelihood of achieving viral load levels of < 40 copies/mL in the latter era (OR 1.27; *P* = 0.005) but not among those who initiated treatment prior to 2002 (OR 0.98; *P* = 0.8). Once current regimen and other influencing factors were taken into account, no

Table 2 Predictors of viral suppression to HIV RNA levels < 40 copies/mL among 356 individuals with viral load < 200 copies/mL and on combination antiretroviral therapy (cART) for at least 1 year

	cART initiated 1996–2001 (<i>n</i> = 165 patients; <i>n</i> = 1597 measurements)				cART initiated 2002–2009 (<i>n</i> = 191 patients; <i>n</i> = 1520 measurements)			
	Univariate		Multivariate		Univariate		Multivariate	
	OR (95% CI)	<i>P</i> ^a	OR (95% CI)	<i>P</i> ^b	OR (95% CI)	<i>P</i> ^a	OR (95% CI)	<i>P</i> ^b
Pre-Rx VL (per log ₁₀ copies/mL)	0.8 (0.52–1.25)	0.3	0.91 (0.51–1.63)	0.7	0.52 (0.31–0.88)	0.02	0.5 (0.29–0.87)	0.01
Pre-Rx CD4 count (per 100 cells/μL)	1.05 (0.91–1.21)	0.5	1.1 (0.91–1.33)	0.3	0.98 (0.84–1.15)	0.8	1.03 (0.88–1.22)	0.7
Years on cART (per year)	0.95 (0.85–1.05)	0.3	0.98 (0.82–1.18)	0.8	1.08 (0.96–1.21)	0.2	1.27 (1.07–1.5)	0.005
Current age (per year)	0.92 (0.70–1.19)	0.5	0.83 (0.58–1.18)	0.3	1.17 (0.92–1.49)	0.2	0.96 (0.74–1.26)	0.8
Male	1.52 (0.67–3.45)	0.3	1.17 (0.38–3.62)	0.8	1.17 (0.58–2.34)	0.7	0.87 (0.38–2)	0.7
Caucasian	2.36 (1.16–4.83)	0.02	2.29 (0.87–5.99)	0.09	1.16 (0.63–2.16)	0.6	1.04 (0.53–2.03)	0.9
Current NNRTI	2.26 (1.30–3.94)	0.004	2.06 (0.99–4.28)	0.05	1.98 (1.17–3.37)	0.01	1.99 (1.14–3.48)	0.02
Prior rebound in VL to > 400 copies/mL	0.35 (0.19–0.66)	0.001	0.54 (0.22–1.33)	0.2	0.28 (0.16–0.50)	< 0.0001	0.18 (0.1–0.35)	< 0.0001

OR, odds ratio; CI, confidence interval; Pre-Rx, pretreatment; VL, viral load; NNRTI, nonnucleoside reverse transcriptase inhibitor. Significant values are shown in bold.

^a*P*-value from univariate logistic regression.

^b*P*-value from multivariate logistic regression, with adjustment for all tabled variables.

significant additional effect of cART class at treatment initiation was observed.

Predictors of detectable HIV RNA among patients with viral load results < 40 copies/mL

As shown in Table 3, univariate analyses of the HIV RNA measurements that were below the detection limit found that detectable HIV RNA target was consistently associated with pretreatment viral load levels across both cART eras (1996–2001, OR 1.47 per log₁₀ copies/mL increase, $P = 0.01$; 2002–2009, OR 1.96, $P = 0.003$). Additionally, prior virological failure was associated with an increased likelihood of detectable HIV RNA among patients who had commenced treatment in the earlier cART era (OR 2.08; $P = 0.001$). This was particularly true when prior viral rebound could be attributed to treatment interruption (OR 2.35; $P = 0.0002$) and remained significant when analyses were restricted to only those measurements at least 1 year subsequent to the rebound. Current NNRTI therapy was associated with a reduced likelihood of detectable HIV RNA only among patients of the later cohort (OR 0.53; $P = 0.008$), while no significant effect of demographic factors, time on therapy or pretreatment CD4 levels could be detected in analyses involving either cohort.

Results of the multivariate analysis closely mirror those from the univariate analysis, indicating relative independence of the contributing factors within each cohort. Notably, the association of pretreatment viral load with occurrence of detectable HIV RNA remained significant for both treatment eras (1996–2001, OR 1.51 per log₁₀ copies/mL increase, $P = 0.01$; 2002–2009, OR 1.96, $P = 0.004$). As observed in the analyses determining pre-

dictors of viral suppression to levels < 40 copies/mL, no additional effect of initial cART class was observed among patients of either treatment era once the predictive factors of pretreatment viral load, prior virological failure and current regimen were taken into account.

Combined analyses

In the observed proportions of viral suppression categories (Fig. 2) and the results of combined analyses across treatment eras (Fig. 3), we again found a consistent influence of pretreatment HIV RNA levels on obtaining detectable target in patients with a viral load < 40 copies/mL. The effect was observed in both treatment groups (current PI: univariate OR 1.51 per log₁₀ copies/mL increase, $P = 0.02$; current NNRTI: OR 1.70, $P = 0.002$), with no significant difference between the treatment classes in the magnitude of this observed effect ($P > 0.4$ for the interaction term). The detrimental impact of prior virological failure was only found to be significant among patients currently on an NNRTI ($P = 0.02$), again most particularly for patients with a treatment interruption ($P = 0.008$), but there appeared to be little difference in efficacy between nevirapine and efavirenz. While we observed an increased likelihood of detectable HIV RNA target among patients on atazanavir compared with other drugs of the PI class (OR 1.65; $P = 0.04$), the effect diminished in analyses restricted to patients only on ritonavir-boosted PI regimens (OR 1.46; $P = 0.1$).

The likelihood of achieving virological suppression to < 40 copies/mL was particularly reduced among patients who had experienced prior virological rebound (current PI: OR 0.46 per log₁₀ copies/mL increase, $P = 0.02$; current NNRTI: OR 0.46, $P = 0.002$), while the impact of pre-

Table 3 Predictors of detectable HIV RNA among 348 individuals with HIV RNA levels < 40 copies/mL and on combination antiretroviral therapy (cART) for at least 1 year

	cART initiated 1996–2001 (<i>n</i> = 161 patients; <i>n</i> = 1430 measurements)				cART initiated 2002–2009 (<i>n</i> = 187 patients; <i>n</i> = 1389 measurements)			
	Univariate		Multivariate		Univariate		Multivariate	
	OR (95% CI)	<i>P</i> ^a	OR (95% CI)	<i>P</i> ^b	OR (95% CI)	<i>P</i> ^a	OR (95% CI)	<i>P</i> ^b
Pre-Rx VL (per log ₁₀ copies/mL)	1.47 (1.09–1.96)	0.01	1.51 (1.09–2.08)	0.01	1.96 (1.26–3.03)	0.003	1.96 (1.25–3.03)	0.004
Pre-Rx CD4 count (per 100 cells/μL)	1.00 (0.91–1.10)	1.0	1.05 (0.95–1.17)	0.3	0.96 (0.84–1.10)	0.6	0.95 (0.83–1.08)	0.4
Years on cART (per year)	0.98 (0.90–1.06)	0.6	0.93 (0.85–1.03)	0.2	1.01 (0.92–1.11)	0.9	0.96 (0.86–1.08)	0.5
Current age (per year)	1.03 (0.86–1.23)	0.8	1.10 (0.92–1.33)	0.3	1.06 (0.87–1.30)	0.5	1.02 (0.82–1.27)	0.9
Male	0.77 (0.45–1.37)	0.4	0.93 (0.50–1.70)	0.8	1.59 (0.87–2.86)	0.1	1.56 (0.81–3.03)	0.2
Caucasian	0.63 (0.38–1.05)	0.08	0.70 (0.41–1.20)	0.2	1.10 (0.65–1.85)	0.7	0.91 (0.53–1.56)	0.7
Current NNRTI	0.77 (0.52–1.14)	0.2	0.86 (0.56–1.31)	0.5	0.53 (0.34–0.85)	0.008	0.54 (0.34–0.86)	0.01
Prior rebound in VL to > 400 copies/mL	2.08 (1.35–3.23)	0.001	1.96 (1.19–3.13)	0.008	1.41 (0.81–2.43)	0.2	1.54 (0.88–2.78)	0.1

OR, odds ratio; CI, confidence interval; Pre-Rx, pretreatment; VL, viral load; NNRTI, nonnucleoside reverse transcriptase inhibitor. Significant values are shown in bold.

^a*P*-value from univariate logistic regression.

^b*P*-value from multivariate logistic regression, with adjustment for all tabled variables.

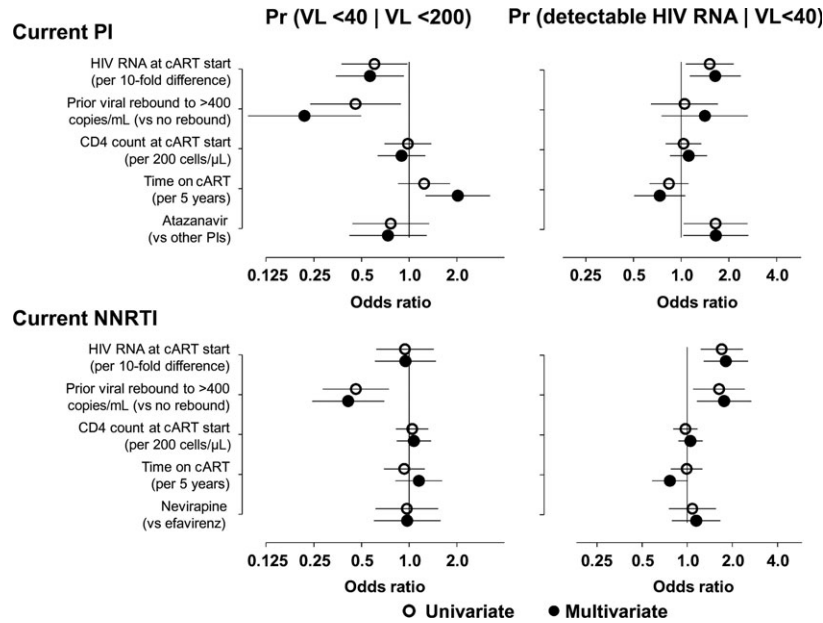


Fig. 3 Odds ratios (\pm 2 standard errors) from longitudinal logistic regression modelling of virological suppression rates according to class of current therapy. cART, combination antiretroviral therapy; PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; VL, viral load.

treatment viral load was evident among those currently receiving a PI (OR 0.60; $P = 0.04$) but not an NNRTI ($P = 0.9$). With regard to the potential impact of detectable HIV RNA on subsequent risk of virological failure, we found that, among viral load measurements < 40 copies/mL, 2.7% of those with detectable residual viraemia were followed by a viral load above 200 copies/mL compared with 1.2% of those where no target was detected (OR 1.77; 95% CI 1.48–2.10; $P = 0.001$ with adjustment for baseline viral load, time on therapy and current class of regimen).

Discussion

In the analysis of viral load data obtained from a large single-centre HIV-infected cohort, we identified disease- and treatment-related factors associated with the likelihood of observing detectable target PCR amplification in a standard HIV RNA assay (Cobas Ampliprep/Cobas TaqMan HIV-1 test, version 1) below the reported detection limit for this assay (40 copies/mL). The study included a large majority of patients receiving effective therapy, with HIV RNA results < 40 copies/mL demonstrated in 85–90% of those with viral load results < 200 copies/mL. It is notable that the proportion of samples with “residual viraemia” remained stable when comparing the overall sample set (20.1% of 7451 results < 40 copies/mL) to the restricted data set with comprehensive treatment history (19.9% of 4794 results < 40 copies/mL) as well as the

longitudinal data set where viral load results could be assessed from the commencement of combination HIV therapy (18.7% of 2819 results < 40 copies/mL).

The principal finding of our analyses is a strong and consistent association of pretreatment HIV viral load with the persistence of detectable HIV RNA among patients who would otherwise be considered to be on suppressive HIV treatment. Strikingly, this association remained stable with adjustment for other demographic and clinical factors, and remained relevant for patients who had been on therapy for many years. As noted in Fig. 2, this effect appears to be evident at viral load ranges < 100 000 copies/mL (10^5 copies/mL), suggesting that consideration of earlier treatment initiation may have long-term beneficial effects on virological response. This result is consistent with a recent substudy analysis of the AntiRetroviral Therapy with TMC114 ExaMined In Naive Subjects (ARTEMIS) trial, which also identified pretreatment viral load as the major determinant of achieving HIV RNA levels < 5 copies/mL among antiretroviral treatment-naïve patients randomized to receive tenofovir/emtricitabine plus either darunavir/ritonavir or lopinavir/ritonavir, irrespective of treatment choice [10].

We note that, while PI therapy was consistently associated with a reduced likelihood of achieving a viral load measurement < 40 copies/mL, as noted in a meta-analysis of 48-week efficacy data from randomized clinical trials [9], this did not translate to a consistent reduction in the ability to achieve a result lacking any detectable HIV

RNA. However, we did find evidence of an increased likelihood of residual viraemia associated with atazanavir therapy, even after adjustment for any potential effects of baseline viral load, CD4 T-cell count and demographic variables. It is notable in this respect that ritonavir-boosted atazanavir alone may have somewhat limited efficacy as a treatment simplification strategy [11,12] compared with lopinavir or darunavir [13], which suggests that there may be possible differences in virological efficacy within the HIV PI class that are not readily explained in terms of drug resistance. In the setting of combined antiretroviral treatment, however, it is noteworthy that a recent clinical trial has not identified atazanavir as a risk factor for achievement of viral load results < 1 copy/mL using an ultrasensitive viral load assay, compared with efavirenz or darunavir [14].

The impact of treatment interruptions on the likelihood of residual viraemia is yet to be fully explored, but our observed association of detectable HIV RNA with prior loss of virological control, particularly when attributable to a prior period off therapy, supports the findings of a smaller scale study that also examined markers of persistent low-level viraemia [15]. We have also noted in this study that residual viraemia was associated with an increased risk of progressing to higher plasma HIV RNA levels (> 200 copies/mL), although any interpretation of these findings is limited by the fact that medication adherence has not been formally assessed as a potential confounding factor. Our findings also support previous studies indicating that residual viraemia is a true virological entity with identifiable risk factors, rather than a product of viral load assay error at the limits of detection. This view is also reinforced through studies of the effects of HIV treatment intensification (measured using ultrasensitive viral load assays), which have shown that residual viraemia is a stable state that appears to be unaffected by the addition of an integrase inhibitor [6] or triple-class cART regimens (i.e. NRTI/PI/NNRTI) [5]. These findings and those of other HIV clinical trials [9,13] also suggest that the inability of standard treatment regimens to fully suppress plasma HIV RNA levels in some patients is likely to reflect latent infection in long-lived cells [3,16,17] that are minimally influenced by treatment-induced reductions in viral replication [18,19].

This concept of a stable, very long-lived reservoir of latently infected cells as a source of residual viraemia despite effective HIV treatment is also strongly supported by our finding that baseline plasma HIV RNA levels continue to predict virological response over extended periods of treatment – in this case 10 years and beyond. This effect does not appear to be determined by the pretreatment (nadir) CD4 T-cell count, nor is it influenced in any

detectable way by age, gender or race in this analysis. This finding is consistent with others' analyses of viral load decay during treatment that have shown an eventually steady-state viral load that reflects the level of pretreatment viraemia and the size of the latently infected CD4 T-cell population [16–20].

This study has several limitations, not least of which is the determination of residual viraemia using the Cobas Ampliprep/Cobas TaqMan HIV-1 test (version 1) assay, rather than a dedicated ultrasensitive viral load assay. As with any observational cohort study, it is important to interpret findings with caution in light of potential sample biases such as variation in treatment prescribing practices. Nevertheless, this retrospective analysis has the distinct advantage that the primary outcome measure (detectable HIV RNA below the assay threshold of 40 copies/mL) was not known to clinicians involved in the care of these patients, and therefore could not influence treatment decisions during the study period. Thus, at least one source of potential confounding has been eliminated.

Acknowledging these caveats, the association of higher pretreatment viral load measurements with persistently detectable HIV RNA during long-term HIV treatment is biologically intuitive and, as noted above, is supported by other studies. Tellingly, this effect has remained consistent across various stratifications of patient groups and over extended periods of follow-up, a result that is also consistent with available evidence. Taken together, these findings have some interesting implications for the timing of HIV treatment and for the development of management strategies aimed at optimizing HIV treatment outcomes and minimizing persistent immune activation in the face of lifelong treatment. In particular, the dominant influence of pretreatment HIV RNA level on long-term virological outcomes – and the lack of any discernible effect of CD4 T-cell count on these outcomes – challenges “early” *vs.* “late” treatment based on CD4 T-cell levels as the dominant topic for debate in contemporary HIV management, instead arguing for a greater appreciation of the influence of plasma HIV RNA levels across the spectrum of HIV-induced immune deficiency. This concept is supported by a recent meta-analysis of HIV treatment efficacy [21], which concluded that initial antiretroviral treatments for HIV-1 are more effective when commenced at plasma viral loads < 100 000 copies/mL and recommended that rising viral load should be considered an indication for starting treatment. At the same time, there is decreasing emphasis on CD4 T-cell levels as the basis for commencing HIV treatment following the recent conclusion and publication of the Strategic Timing of Antiretroviral Treatment (START) trial, which has conclusively demonstrated the clinical benefits of

commencing HIV therapy in patients with normal CD4 T-cell counts [22].

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Representative real-time polymerase chain reaction (PCR) amplification plots demonstrating HIV-1 amplification (lower trendline) relative to control (upper trendline).

Supplementary figure for Chapter 2:

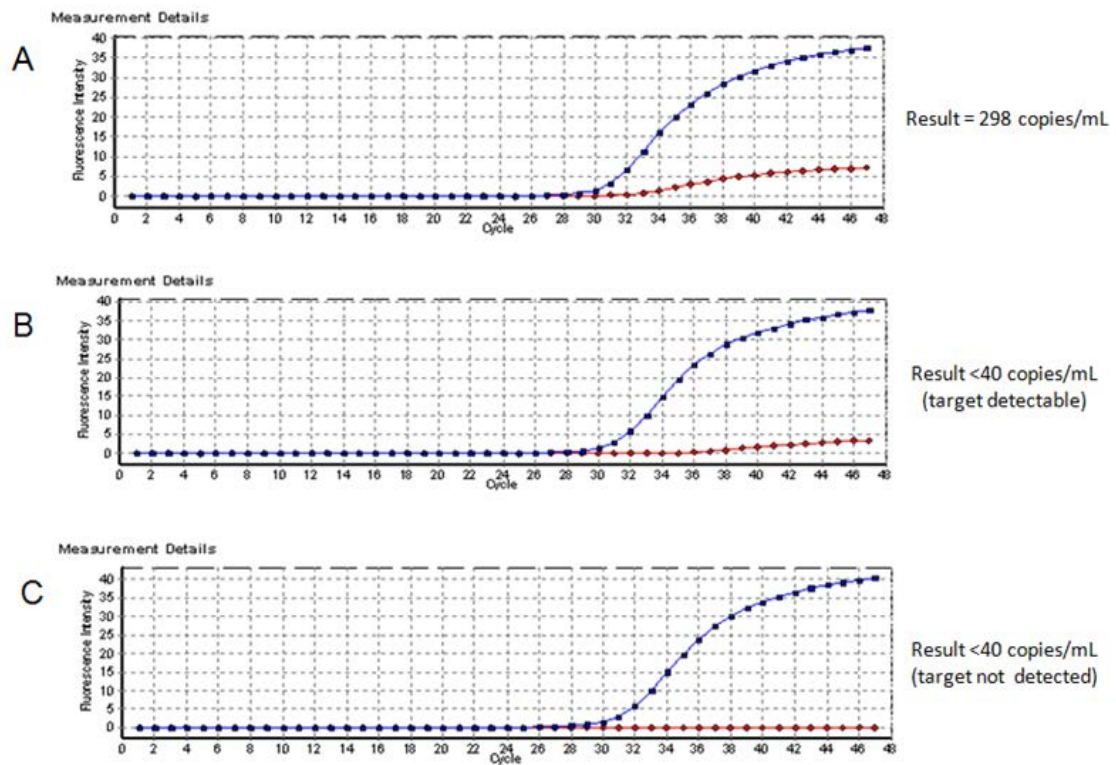


Fig. S1: Representative real-time polymerase chain reaction (PCR) amplification plots demonstrating HIV-1 amplification (lower red trendline) relative to control (upper blue trendline). The results show either the HIV-1 amplification being >40 copies/mL (A); HIV-1 amplification between 0-40 copies/mL (B) and a “target not detected” result (C) which showed no detection of target HIV-1 for > 15 cycles beyond the expected crossing threshold of 32 cycles.

Summary for Chapter 2.

In this chapter the determinants of plasma HIV-1 RNA suppression below the threshold (<40 copies/mL) of the routinely used method (the Cobas Taqman HIV system) were investigated in 11,575 consecutive tests. The data suggests that 20% of patients tested had residual viraemia with low-level viral replication below the detection limit of the standard HIV-1 RNA assay (<40copies/mL) and that the residual viraemia was associated with pre-treatment HIV-1 RNA levels and some antiretroviral therapy. These findings support the concept of a stable long-lived HIV reservoir of latent infected cells as a source of residual viraemia even in the context of successful ART, and in the context of this thesis suggest that the reporting of detectable HIV-1 RNA target when using assays such as the Cobas Taqman HIV system may have an application in routine patient monitoring. While this study has been able to demonstrate the prevalence of detectable HIV-1 RNA among plasma samples from patients receiving HIV therapy, the ultimate utility of this approach depends on the biological and clinical consequences of residual viraemia which is relatively unknown.

To date, there is limited data available that identifies potential consequences of long term residual viraemia in patients on HAART or the long term clinical implications. It is known, however, that duration of HAART, treatment intensification or previous treatment failures do not significantly reduce or alter residual viraemia levels [104, 105]. Detectable residual viraemia may increase the risk of poor virological control [106, 107] and potentially predict viral blips [108] that have been associated with immune activation [109]. Additionally, residual viraemia increased in poor immunological responders, particularly correlating with the activation of CD4⁺ and CD8⁺ T cells potentially leading to persistent immune dysfunction [110]. Monocytes have also been identified as a source of residual viraemia [110] implying there are at least two sources of residual viraemia.

The direct role residual viraemia has on immune activation therefore requires further investigation especially given evidence linking HIV-1 elite controllers, who have residual viraemia, with the presence of increased immune activation levels [111]. It has also been shown that persistent production of low level circulating HIV virions supply antigens at low levels, which in turn, could activate monocytes [112] and pDCs [113], both of which have been implicated in immune activation in the setting

of HAART. This issue will be addressed further in Chapter 4, in a large cohort-based study of plasma biomarkers that reflect interferon-inducible monocyte activation pathways. In this study we demonstrate a specific association between plasma soluble CD163 levels and residual viraemia, thereby providing a plausible link between residual viraemia and systemic inflammation. It is also notable in this study that the specific influence of residual viraemia was only observed in the case of soluble CD163, and was not demonstrated for plasma CXCL10 levels (which correlated closely with plasma viraemia more generally) or soluble CD14 (which was unaffected by virological suppression at any detectable level).

Chapter 3:

Elevated plasma soluble CD14 and skewed CD16+ monocyte distribution persist despite normalisation of soluble CD163 and CXCL10 by effective HIV therapy: A changing paradigm for routine HIV laboratory monitoring?



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Attributions:

AC designed and performed all the assays, the analysis, the interpretations of the findings, the literature review and the manuscript writing. RK and SF supervised the flow cytometry development component and reviewed the manuscript. MF and CB reviewed the manuscript. DN supervised the development of the assays, interpreted the findings and critically reviewed the manuscript. All authors approved the final version.

AC: 75%

RESEARCH ARTICLE

Elevated Plasma Soluble CD14 and Skewed CD16+ Monocyte Distribution Persist despite Normalisation of Soluble CD163 and CXCL10 by Effective HIV Therapy: A Changing Paradigm for Routine HIV Laboratory Monitoring?

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

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Abstract

Objective: We investigated plasma and flow cytometric biomarkers of monocyte status that have been associated with prognostic utility in HIV infection and other chronic inflammatory diseases, comparing 81 HIV⁺ individuals with a range of treatment outcomes to a group of 21 healthy control blood donors. Our aim is to develop and optimise monocyte assays that combine biological relevance, clinical utility, and ease of adoption into routine HIV laboratory practice.

Design: Cross-sectional evaluation of concurrent plasma and whole blood samples.

Methods: A flow cytometry protocol was developed comprising single-tube CD45, CD14, CD16, CD64, CD163, CD143 analysis with appropriately matched isotype controls. Plasma levels of soluble CD14 (sCD14), soluble CD163 (sCD163) and CXCL10 were measured by ELISA.

Results: HIV status was associated with significantly increased expression of CD64, CD143 and CD163 on CD16⁺ monocytes, irrespective of the virological response to HIV therapy. Plasma levels of sCD14, sCD163 and CXCL10 were also significantly elevated in association with viremic HIV infection. Plasma sCD163 and CXCL10 levels were restored to healthy control levels by effective antiretroviral

therapy while sCD14 levels remained elevated despite virological suppression ($p < 0.001$).

Conclusions: Flow cytometric and plasma biomarkers of monocyte activation indicate an ongoing systemic inflammatory response to HIV infection, characterised by persistent alterations of CD16⁺ monocyte expression profiles and elevated sCD14 levels, that are not corrected by antiretroviral therapy and likely to be prognostically significant. In contrast, sCD163 and CXCL10 levels declined on antiretroviral therapy, suggesting multiple activation pathways revealed by these biomarkers. Incorporation of these assays into routine clinical care is feasible and warrants further consideration, particularly in light of emerging therapeutic strategies that specifically target innate immune activation in HIV infection.

Introduction

Monocytes are a heterogeneous cell population arising from the myeloid lineage that provide a link between innate and adaptive immunity. They can be classified according to cell surface expression of CD14 (a lipopolysaccharide receptor) and CD16 (FcγRIII, a low affinity Fc receptor) into three subsets known to have different phenotype and functions. The more numerous “classical” CD14⁺⁺/CD16⁻ monocytes appear to be more granulocyte-like in that they are well-equipped for innate immune responses involving trans-endothelial migration and phagocytosis [1], while the remaining CD16⁺ monocytes have been more recently subclassified into “intermediate” (CD14⁺⁺/CD16⁺) and “non-classical” (CD14⁺/CD16⁺⁺) populations that appear to have more in common with dendritic cells and macrophages [1], in that they exhibit greater potential for HLA-restricted antigen presentation and pro-inflammatory cytokine production [2], migrating in response to distinct subset-specific chemokine/ligand gradients [3]. With regard to HIV infection, there has been a great deal of interest in the potential role of CD16⁺ monocytes, particularly CD14⁺⁺/CD16⁺ intermediate monocytes, in disease pathogenesis given that these populations (which co-express CCR5) [3] are permissive to HIV infection [4] and are capable of transferring HIV infection across the genital mucosal barrier [5] as well as into the central nervous system [6]. Expansion of this intermediate monocyte population also appears to be associated with cardiovascular events in subjects referred for elective coronary angiography [7] and has been noted in acute coronary syndromes as well as in chronic HIV infection [8].

Additional cell surface markers may further characterise CD16⁺ monocyte function, particularly in the context of HIV infection and/or inflammation. These include the angiotensin converting enzyme (CD143), expressed predominantly on CD14⁺⁺/CD16⁺ intermediate monocytes, which has been linked to mortality and cardiovascular disease in haemodialysis patients [9]; as well as the scavenger receptor CD163 which has been shown to be significantly elevated in the context

of HIV infection [10, 11]. In contrast, the high affinity Fc γ receptor 1 (CD64) is principally expressed on CD14⁺⁺/CD16⁻ classical monocytes, where it may serve as a biomarker of type I interferon activation in autoimmune diseases [12]. Monocyte CD64 expression may restrict productive HIV-1 infection by facilitating viral phagocytosis and degradation [13].

Several plasma biomarkers of monocyte activity have also been linked to HIV disease progression, including soluble CD14 (sCD14) which has been shown to predict all-cause mortality in HIV patients [14], even in the setting of undetectable plasma HIV RNA levels that would generally define effective HIV therapy [15, 16]. Elevated levels of soluble CD163 (sCD163) have also been shown to be associated with arterial inflammation and cardiovascular disease in HIV-infected patients [16], although in this case sCD163 levels appear responsive to HIV therapy [17]. Similarly, plasma levels of CXCL10 (also known as interferon gamma-induced protein 10 [IP-10]) are induced by HIV infection [18] but are also readily reduced by effective therapy [19].

Taken together, these data suggest that laboratory evaluation of monocyte populations may provide important prognostic information during HIV management that is currently 'invisible' through standard assessments of plasma HIV RNA levels and CD4 T cell counts. In this study we have therefore sought to develop and optimise methods that reveal monocyte status in a manner that combines biological relevance, clinical utility, and ease of adoption into routine HIV laboratory practice.

Methods

Patients attending the Royal Perth Hospital (RPH) Immunology clinic and healthy control blood donors attending the Australian Red Cross Blood Service (ARCBS) were recruited for this study. Informed written consent was obtained from the patients participating in this investigation. This consent form was reviewed and accepted by the ethics committees. Written ethics committee approvals for this investigation were also received from Royal Perth Hospital (EC2012/170), Murdoch University (2012/216) and the Australian Red Cross Blood Service (11-08WA-15). After site approvals and patient consent, whole blood was collected into EDTA anti-coagulated tubes from 81 HIV-1 positive patients and 21 healthy controls.

Sample Collection

Plasma was collected from EDTA whole blood samples within 6 hours of collection by centrifugal force of 2000 rpm for 20 mins. Plasma was removed and stored at -80°C until required for the enzyme-linked immunosorbent assay (ELISA). For flow cytometry analysis, whole blood (WB) samples were kept at room temperature and processed within 24 hours after collection.

Whole blood flow cytometric analysis

The eight monoclonal antibodies (mAbs) utilised in this study were designed using the BD FACSelect multicolour panel designer then purchased from BD Biosciences, unless otherwise indicated, and stored at 4°C until required.

After assessing the effect of storage time on whole blood samples, mAb titrations and the effect of washing cell preparations (data not shown) we assessed the above cohorts using an 8 channel multicolour flow cytometer (FACSCanto II) for measuring monocytes and monocyte activation status using the following conjugated antibodies; 0.5 µl APC-H7 mouse anti-human CD14 (clone MφP9), 1 µl V450 mouse anti-human CD16 (clone 3G8), 1 µl V500 mouse anti-human CD45 (clone HI30), 1 µl PerCP-Cy5.5 mouse anti-human CD56 (clone B159), 2.5 µl PE-Cy7 mouse anti-human CD64 (clone 10.1-this was sub-aliquoted and stored to prevent degradation from repeated exposure to light) and 5 µl APC mouse anti-human hematopoietic progenitor cell (clone BB9), 1 µl anti-human CD163-Alexa Fluor488 (clone 215927) and 2 µl anti-human NKG2C-phycoerythrin (clone 134591) from R&D systems. The appropriately matched isotype controls were utilised for each mAb, as negative controls, and fluorescence minus one (FMO) staining was also utilised as a control procedure.

The mAbs were pipetted into a 3DT tube (BD Falcon) followed by the addition of 50 µl of well-mixed, reverse pipette WB. After gentle mixing the sample tubes were incubated for 15 min at room temperature in the dark. Red blood cells were lysed by adding 500 µl of 1 × FACSlyse red cell lysis buffer (BD Biosciences), gently mixed then incubated for a further 15 min at room temperature in the dark. The stained cell preparation was acquired on the flow cytometer within 30 mins after incubation.

Sample acquisition was performed using 3-laser, 8-color configuration on the FACSCanto II flow cytometer with FACSDiva 6.1.1 software (BD Biosciences). The performance and quality control of the instrument was assessed and recorded daily utilising CST beads (BD Biosciences).

Fluorescence compensation was required and assessed as per kit instructions with anti-mouse Igk CompBeads (BD Biosciences) as well as the above mentioned mAbs. Compensation values were calculated automatically and accepted when all fluorochrome-paired values were <30%. Voltages for forward scatter (FSC) and side scatter (SSC) were adjusted giving a FSC voltage of 385 and a SSC voltage of 450. This enabled distinct and clear separation of the monocyte cell population from lymphocyte and granulocyte cell populations. Sample preparation tubes were acquired by collecting 100,000 events using the acquisition gate on the lymphocyte cell population, ensuring at least 1000–5000 events were collected for the monocyte population.

Analysis of monocyte subsets and activation markers

After successful acquisition the data files were saved to the BD FACS database then exported. Data was imported into Kaluza Flow Cytometric analysis software (version 1.1) where monocyte analysis was performed.

Duplets were removed from the analysis by gating on the singleton cells from a FSC-Area and FSC-Height plot. Lymphocytes and monocytes were visualised and gated on by using forward scatter/side scatter (FSC/SSC) dot plot. Monocyte subsets were identified by plotting CD45/FSC-A from the monocyte gate, then plotting the CD14/CD16 fluorescence from the CD45⁺ gate. The three monocyte subsets were defined by using isotype controls and FMO as cutoffs (classical monocytes (CD14⁺⁺/CD16⁻), the intermediate monocytes (CD14⁺⁺/CD16⁺) and the non-classical monocytes (CD14⁺/CD16⁺⁺). To determine the presence of monocyte activation markers, CD64, CD163 or CD143 were plotted against SSC-A from each monocyte subset (i.e. classical, intermediate and non classical). The percentage of cells positive was determined from the number present in the parent group.

Measurement of plasma sCD14, sCD163 and CXCL10 levels by ELISA

For the quantitative determination of plasma biomarker levels, ELISAs were utilised as per manufacturer's recommendations unless otherwise stated. R&D Systems, Quantikine ELISAs were used to determine sCD14, sCD163 and CXCL10 levels. Each sample was assayed in duplicate then the optical densities (OD) were measured at an absorbance of 450 nm and 570 nm. The latter wavelength corrects for any optical imperfections and is therefore subtracted from the 450 nm. Standards and controls were included as per kit instructions. Log/log standard curves were generated by averaging optical density readings from the standard results. To determine biomarker concentrations, plasma sample OD readings were also averaged and concentrations were determined from the log/log standard curve.

Statistical analysis

Statistical data analysis was performed using SPSS version 21. Normality of data distribution was assessed using the Shapiro-Wilk test. Based on these results, one way ANOVA and Mann-Whitney tests were utilised to compare HIV+ and healthy control groups, with appropriate correction for multiple comparisons when >2 groups were compared. Bivariate correlation and multivariate linear regression analyses (with logarithmic data transformation to normalise distribution where appropriate) were utilised to estimate associations between plasma biomarkers and monocyte subsets with HIV status, HIV viral load, absolute CD4 counts, CD4:8 ratio, gender and age.

Results

Influence of HIV status on monocyte populations by flow cytometry analysis

The study population included 81 individuals with HIV infection and 21 healthy controls, covering a wide range of CD4 T cell counts and plasma HIV RNA levels and an age range from 20-70 years ([Table 1](#)). Within the HIV group there were 48 participants with viral load results <40 copies/mL (31 male, 17 female), 12 with viral load levels between 40 and 400 copies/mL (11 male, 1 female), and 21 with viral load levels >400 copies/mL (15 male, 6 female).

The identification of monocyte subsets based on CD14 and CD16 expression was investigated in a whole blood flow cytometry assay as shown in [Fig. 1](#). Upon identification of the monocyte subsets, the expression of CD64, CD143 and CD163 proteins were then assessed. For example, [Fig. 1C, 1D and 1E](#) show CD163 expression on classical, intermediate and non-classical monocytes respectively.

We initially investigated whether there was a difference in the proportion of monocyte subsets between HIV-infected and healthy control subjects. We found no influence of HIV status on either classical monocytes (HIV- median 78% (SE 1.4), HIV+ median 80% (SE 1.9), $p=0.75$), intermediate monocytes (HIV median 4.2% (SE 0.5), HIV+ median 3.8% (SE 0.3), $p=0.18$), or non classical monocytes (HIV median 5.3% (SE 0.3), HIV+ median 4.8% (SE 0.3), $p=0.65$) using Mann-Whitney tests to compare groups. There was no detectable influence of gender ($p>0.05$, Mann-Whitney) or correlation with age ($p>0.05$, Spearman's rho) for any monocyte subset.

Comparing participants according to HIV viral load status (21 controls, 60 with 'suppressed' HIV RNA levels <400, and 21 with 'viremic' HIV RNA levels >400 copies/mL), we found significantly higher levels of intermediate CD14⁺⁺/CD16⁺ monocytes in viremic HIV⁺ cases compared with suppressed HIV ($p_{\text{adjusted}}=0.01$), although no significant differences between viremic HIV⁺ and control groups ($p_{\text{adjusted}}=0.95$). This was associated with a corresponding trend on classical CD14⁺⁺/CD16⁻ monocytes, in which levels were lower among viremic HIV⁺ patients compared with those with suppressed viral loads ($p=0.08$). The proportion of non-classical CD14⁺/CD16⁺⁺ monocytes was similar between all groups ($p>0.45$). There was no detectable association of CD4 T cell count or T cell CD4:8 ratio with any monocyte subset (all $p\geq 0.7$).

Influence of HIV status on monocyte CD64, CD163 and CD143 cell surface expression

The expression of CD64, CD163 and CD143 proteins on all monocyte subsets was explored using whole blood immunophenotyping as described above. As expected and shown in [Fig. 2](#), classical CD14⁺⁺/CD16⁻ monocytes showed the highest overall levels of CD64 [[1](#)] and CD163 [[10](#), [11](#)] expression (98% and 79% respectively), while higher overall levels of CD143 expression were observed in CD16⁺ monocyte populations [[9](#)].

Table 1. Demographic and clinical characteristics of HIV-infected and healthy control subjects.

Category	Subcategory	HIV ⁺ group	Controls
Gender	Males (n)	57	10
	Females (n)	24	11
	Total (n)	81	21
Age (years)	Males mean (range)	45 (20–65)	56 (45–70)
	Females mean (range)	37 (25–59)	41 (23–66)
Percentage CD4 (%)	Males mean (range)	27 (5–53)	NT
	Females mean (range)	28 (4–48)	NT
Absolute CD4 (cells/ μ L)	Males mean (SD, range)	603 (301, 140–1369)	NT
	Females mean (SD, range)	611 (324, 104–1188)	NT
CD4:CD8 ratio	Males mean (SD, range)	0.63 (0.41, 0.07–2.36)	NT
	Females mean (SD, range)	0.71 (0.38, 0.07–1.48)	NT
HIV viral load (copies/ml)	Males mean (SD, range)	30,905 (84,837, <40–446684)	NT
	Females mean (SD, range)	16,662 (43240, <40–173780)	NT
Duration of HIV (years)	Males mean (SD, range)	8.6 (7.0, 1 mth–24 yrs)	NT
	Females mean (SD, range)	8.9 (6.9, 1 mth–24 yrs)	NT
Duration of ART at time of MAM (months)	Males n=45, mean (SD, range)	96.3 (77, 1–240)	NT
	Females n=22, mean (SD, range)	67.9 (57.3, 1–192)	NT
Months suppressed (<400cpm) at time MAM	Males n=42, mean (SD, range)	49.2 (46.7, 1–184)	NT
	Females n=18, mean (SD, range)	49.8 (43.9, 1–149)	NT
Months suppressed (<40cpm) at time MAM	Males n=31, mean (SD, range)	30 (37.8, 2–183)	NT
	Females n=17, mean (SD, range)	30.1 (22.3, 1–66)	NT
ART naïve	Males (n)	12	NT
	Females (n)	2	NT

NT=Not tested.

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Comparing HIV infected and control groups (Mann-Whitney), we found that HIV status was associated with significantly higher CD64 expression on all three monocyte subpopulations (Fig. 2A) with a difference of approximately 14% in both intermediate CD14⁺/CD16⁺ monocytes (median (SE): 88% (1.2) vs 74% (3.3), $p < 0.001$) and non-classical CD14⁺/CD16⁺ monocytes (27% (2.3) vs 14.5% (2.1), $p < 0.001$).

HIV infection was also associated with higher monocyte CD163 expression for both intermediate CD14⁺/CD16⁺ monocytes (79% (2.2) vs 65% (3.5), $p = 0.048$) and non-classical CD14⁺/CD16⁺ monocytes (26% (1.9) vs 19% (2.1), $p = 0.017$) (Fig. 2B). Despite low overall CD143 expression, HIV status was associated with higher expression on CD14⁺/CD16⁺ intermediate monocytes (4.0% (0.6) vs 2.2% (0.3), $p = 0.01$) and CD14⁺/CD16⁺ monocytes (5.5% (0.9) vs 2.0% (0.4), $p = 0.01$) among HIV⁺ participants (Fig. 2C).

We then sought to examine the association of plasma HIV RNA levels with these cell surface markers within the two CD16⁺ monocyte populations, comparing the normal healthy control group, HIV⁺ participants with viral suppression (viral load <400 copies/ml) and those with higher levels of viremia

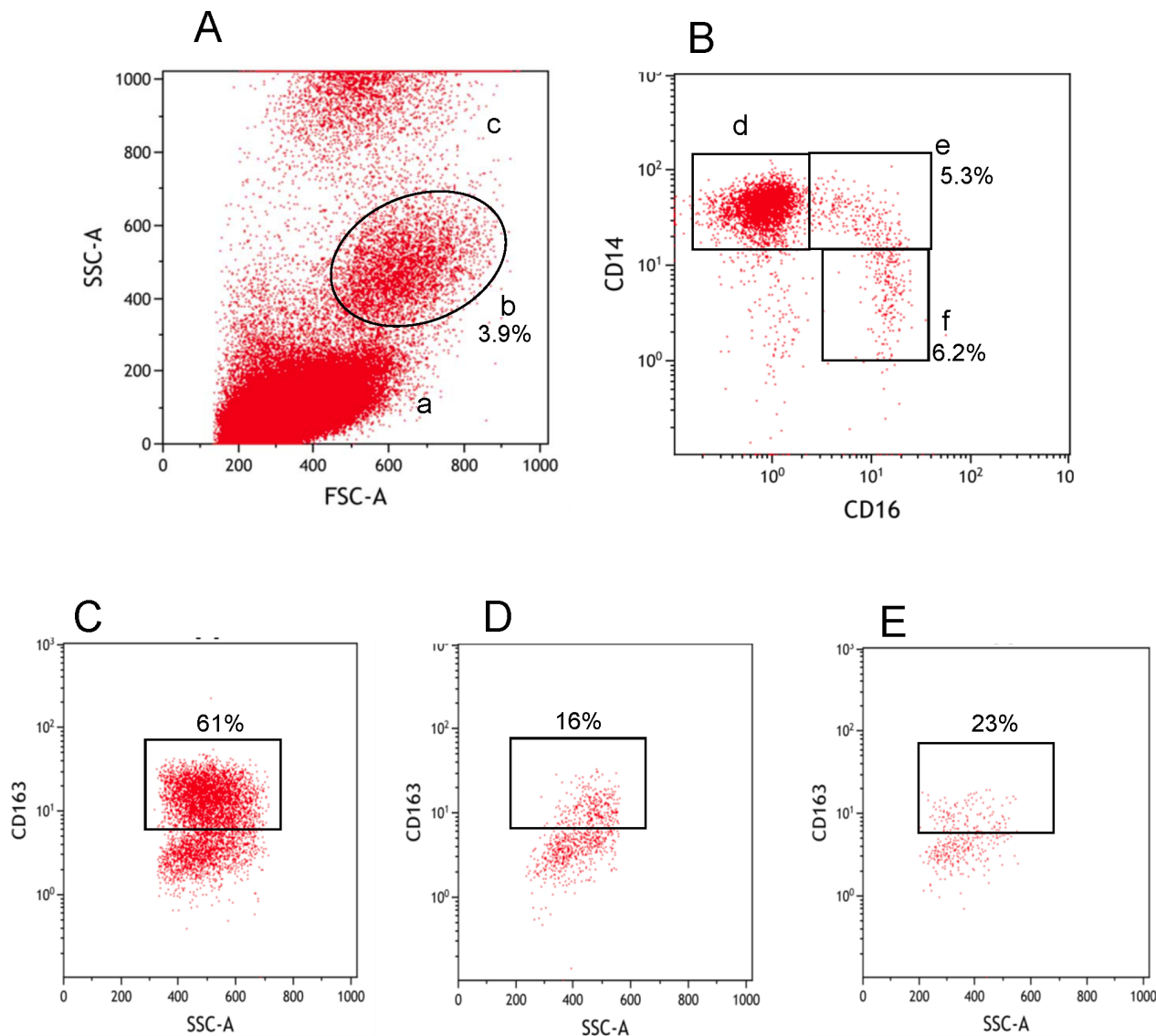


Fig. 1. Phenotypic identification of monocytes and monocyte subsets. Whole blood monocytes were identified by a forward scatter and side scatter plot (A). Lymphocytes (a), monocytes (b) and the granulocytes (c) are shown. The three monocyte subsets were identified from the monocyte population (b) by CD14 and CD16 expression (B). Classical monocytes (d-CD14⁺/CD16⁻), intermediate monocytes (e-CD14⁺/CD16⁺) and non-classical monocytes (f-CD14⁺/CD16⁺) were gated for further analysis of activation markers. For example figures C, D and E show CD163 expression from d, e and f respectively.

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(HIV-1 viral load >400 copies/ml), with additional stratification by gender as shown in Fig. 3. This analysis revealed significant between-group differences in CD64 expression involving both intermediate monocytes (Fig. 3A, $p < 0.005$) and non-classical monocytes (Fig. 3B, $p < 0.03$), indicating that although effective ART was associated with reduced CD64 expression compared with viremic infection, levels were not normalised to those seen in the healthy control group. No additional influence of age or gender was detected in either of the CD16⁺ monocyte populations, in multivariate regression analyses ($p > 0.5$). Similar

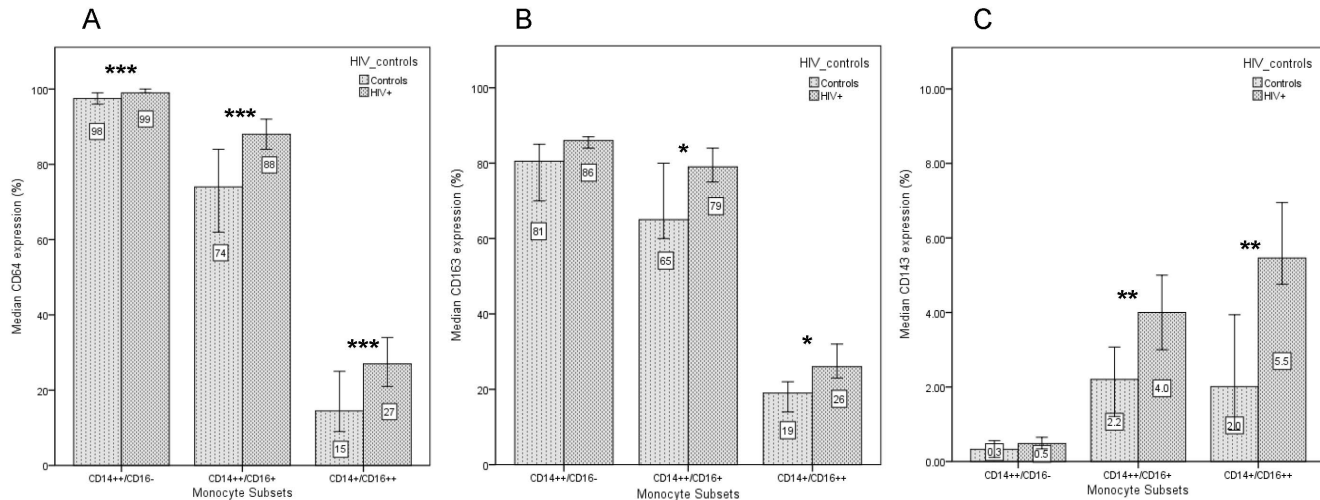


Fig. 2. The median cell surface expression and 95% confidence intervals are shown for CD64 (A), CD163 (B) and CD143 (C) on monocyte subsets in HIV and control subjects. Expression on classical monocytes is significantly different between HIV and control groups for CD64 ($p < 0.001$) but not for CD163 or CD143. Expression of all cell surface markers on CD16⁺ monocytes, however, are significantly different between the HIV infected and control groups (* $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$).

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multivariate analyses within the HIV⁺ group identified a marginal association of lower CD4 T cell counts as well as higher HIV RNA levels on CD64 expression in both intermediate monocytes (CD4 count, $p = 0.08$; HIV RNA, $p = 0.004$) and non-classical monocytes ($p = 0.06$, $p = 0.001$ respectively).

In contrast, we found that HIV⁺ status was associated with elevated CD143 expression levels on both CD16⁺ monocyte populations, regardless of viral load suppression ($p < 0.01$ for all HIV⁺ vs. control comparisons, $p > 0.05$ for comparisons of suppressed vs. viremic HIV⁺ groups), although overall effect sizes were small as shown in Fig. 3C and 3D. Similarly, higher levels of CD163 expression on intermediate CD14⁺/CD16⁺ monocytes were observed in the suppressed HIV⁺ group compared to controls (Fig. 3E; $p = 0.02$) whilst in the non-classical CD14⁺/CD16⁺⁺ monocytes higher levels were observed in the viremic HIV⁺ group compared to controls (Fig. 3F; $p = 0.049$), with no discernible difference between the suppressed and viremic groups ($p = 0.98$). No significant influence of age, gender or CD4 T cell count on CD143 or CD163 monocyte expression in any monocyte population could be detected in regression analyses, including within analyses restricted to the HIV⁺ study group (all $p > 0.2$).

Influence of HIV status on plasma levels of sCD14, sCD163 and CXCL10

Comparing normalised (log-transformed) levels of plasma biomarkers in the HIV-infected and healthy control groups (ANOVA with Bonferroni correction as appropriate for multiple comparisons), we found a highly significant difference in plasma sCD14 levels in the HIV⁺ group compared to controls (Fig. 4A; $p < 0.001$),

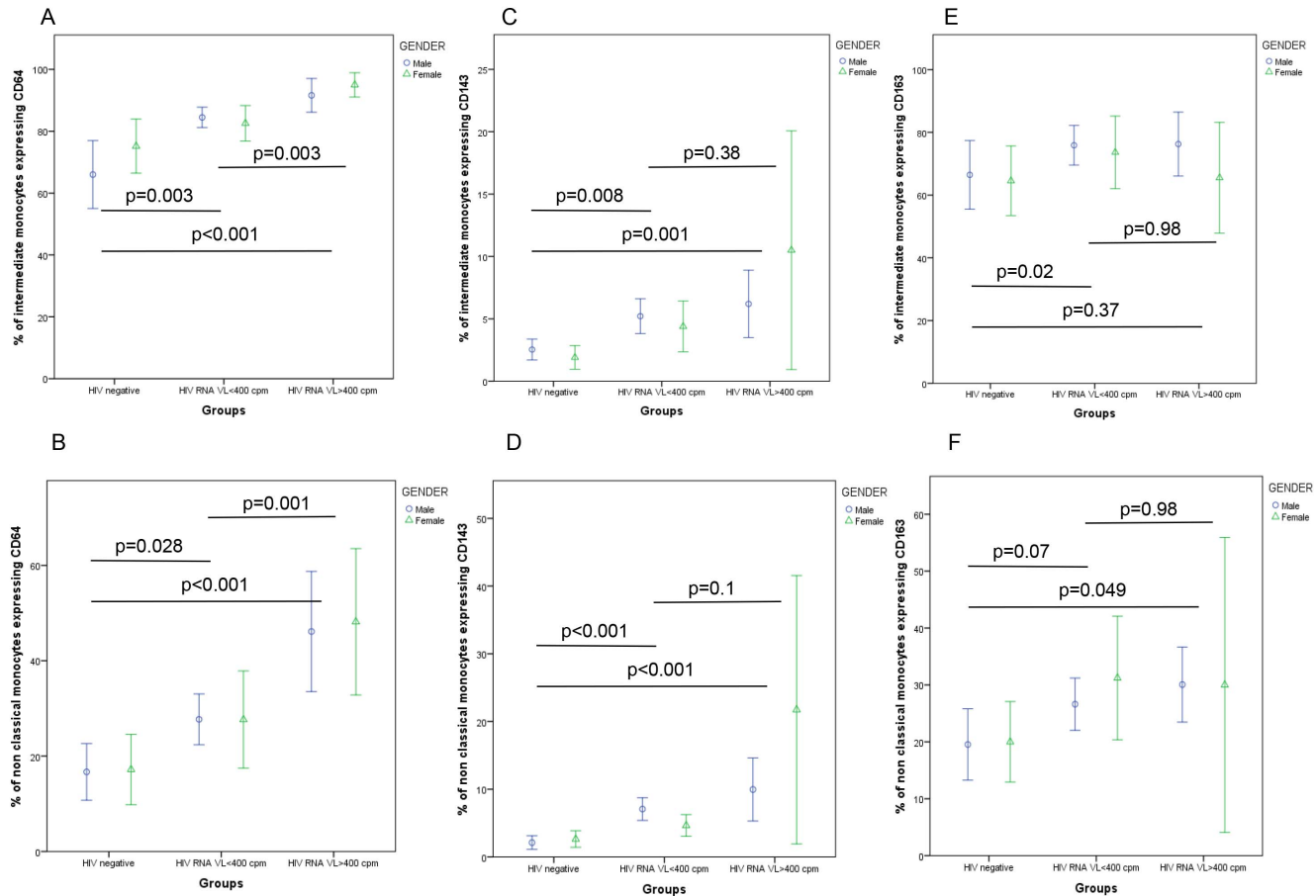


Fig. 3. HIV viral load status has different influences on monocyte cell surface expression. Expression of CD64 on intermediate (A) and non-classical (B) monocytes, CD143 on intermediate (C) and non-classical (D) monocytes, and CD163 on intermediate (E) and non-classical (F) monocytes are shown below. (Data presented as mean values and 95% confidence intervals; p-values derived from Kruskal-Wallis tests).

doi:10.1371/journal.pone.0115226.g003

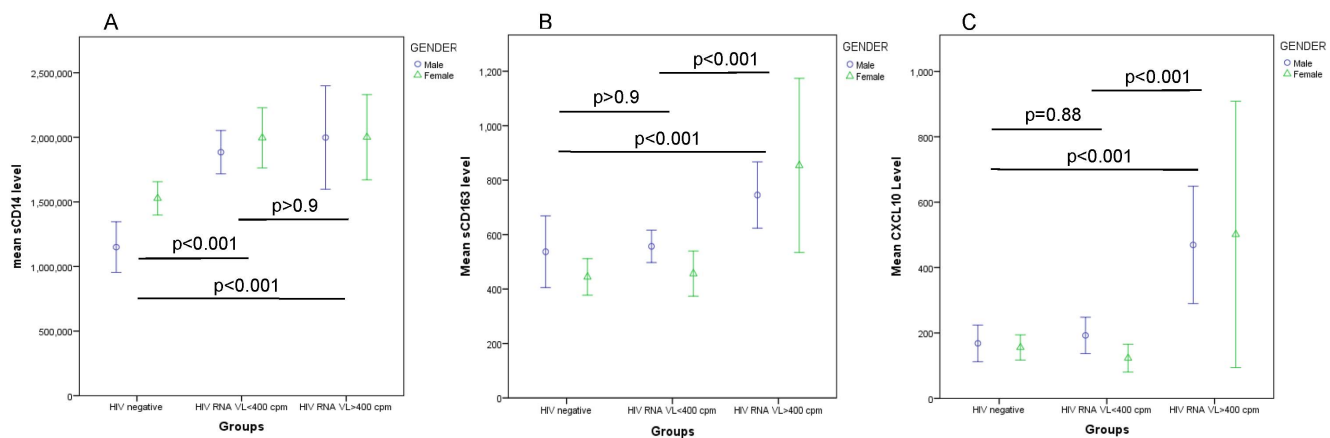


Fig. 4. sCD14 levels are significantly elevated in HIV patients irrespective of HIV viral load status (A). In contrast sCD163 levels are only elevated in viremic HIV patients (B) and a similar relationship is seen with CXCL10 levels (C). (Data presented as mean values and 95% confidence intervals; p-values are derived from log transformed data and ANOVA tests were adjusted for multiple comparisons).

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with no detectable influence of HIV suppression on sCD14 levels compared with the viremic HIV group ($p > 0.9$). Age and gender also had no influence in adjusted analyses ($p = 0.72$ and $p = 0.21$ respectively). Restricting analyses to the HIV⁺ group, there was no evidence of an association between sCD14 and plasma HIV RNA levels ($p = 0.7$) or CD4 T cell count or T cell CD4:8 ratio (both $p > 0.6$).

Plasma levels of sCD163 were also elevated in the HIV group overall ($p < 0.05$), but in striking contrast to sCD14, we found that sCD163 was significantly increased in viremic compared to both virally-suppressed patients (Fig. 4B; $p < 0.001$) and healthy controls ($p < 0.001$) whilst sCD163 levels in suppressed patients were similar to healthy controls ($p > 0.9$). Within the HIV⁺ group, sCD163 levels were independently associated with plasma HIV RNA levels ($p < 0.01$) as well as T cell CD4:8 ratio ($p = 0.003$) in multivariate analysis, with no additional effect of age ($p = 0.3$) or gender ($p = 0.7$).

Elevated CXCL10 levels were also specifically associated with viremic HIV infection compared to both controls (Fig. 4C; $p < 0.001$) and suppressed HIV groups ($p < 0.001$), with no additional effect of gender ($p = 0.3$) or age ($p = 0.8$). CXCL10 levels were associated with plasma HIV RNA levels ($p < 0.001$) but not absolute CD4 T cell count ($p = 0.1$) or CD4:8 ratio ($p = 0.5$) within the HIV⁺ group.

We also investigated if the duration of effective HIV treatment had an influence on these plasma biomarker levels, considering 48 patients who had achieved virological suppression < 40 copies/mL for at least one month prior to assessment (31 males and 17 females, with mean duration of viral suppression 30 months (SD 6.8) and 30 months (SD 5.4) respectively). Here, the duration of virological suppression had no detectable influence on plasma levels of CXCL10 ($p = 0.47$), sCD163 ($p = 0.17$) or sCD14 ($p = 0.49$). Similar results were obtained when considering viral load levels < 400 copies/mL as evidence of virological suppression (42 males and 18 females, mean duration of viral suppression < 400 copies/mL 49 months (SD 7.2) and 50 months (SD 10.3), with no evidence of an association with CXCL10 ($p = 0.29$) or sCD14 ($p = 0.65$). There was a trend observed for sCD163 ($p = 0.08$), although this lost significance ($p = 0.34$) after adjustment for the influence of CD4:8 ratio ($p = 0.008$) noted earlier. No significant effect of HIV treatment duration, or time since HIV diagnosis, was observed for plasma biomarker levels (all $p > 0.3$).

Correlations between flow cytometry and plasma biomarkers

Comparing plasma biomarkers in the first instance, we found that CXCL10 levels strongly correlated with sCD163 levels in the overall dataset ($r = 0.50$, $p < 0.001$) and within the HIV⁺ group ($r = 0.58$, $p < 0.001$), while there was no correlation between plasma levels of sCD14 and CXCL10 ($r = 0.08$, $p = 0.5$) or sCD163 ($r = 0.06$, $p = 0.5$).

We next sought to examine correlations between plasma biomarkers and flow cytometry expression profiles within this study population, with investigation of significant bivariate correlations in subsequent multivariate regression analysis

with log-transformed plasma biomarkers as the dependent variable. Here we found that sCD14 correlated independently with non-classical monocyte CD143 expression ($r=0.3$, $p=0.006$), as well as with intermediate monocyte expression levels of CD64 ($r=0.3$, $p=0.001$) providing a modest overall R^2 value of 0.07 ($p=0.03$). The significance of these variables was lost after inclusion of HIV status as a covariate (β 0.34, $p=0.003$), suggesting that HIV infection *per se* was the dominant determinant of sCD14 levels. For CXCL10, we found that a combination of sCD163 (β 0.43, $p<0.001$) and non-classical monocyte CD64 expression (β 0.30, $p=0.001$) provided the best model (R^2 0.37, $p<0.001$), with no evidence of an additional effect of HIV status ($p=0.22$). Soluble CD163 variability was best explained by a model including CXCL10 (β 0.47, $p<0.001$), although in this case combined with non-classical monocyte CD143 expression (β 0.27, $p=0.002$). This final model (R^2 0.36, $p<0.001$) was also unaffected by the addition of HIV status ($p=0.54$). No effect of age, gender or CD4 count was detected.

Discussion

This study was primarily concerned with the evaluation of methods relevant to monocyte activation during HIV infection and treatment, which could be readily adopted in routine laboratory practice. This may be particularly relevant in the current treatment era, when the availability of effective HIV therapy (defined by suppression of plasma HIV RNA levels and recovery of normal CD4⁺ T cell counts) has brought into question the prognostic value of regular CD4 T cell monitoring once these treatment goals have been achieved [20]. In this context, there is an opportunity to explore novel aspects of chronic HIV infection that currently remain ‘invisible’ in routine care despite being prognostically important [14, 15], and which may not be influenced by what would otherwise be considered effective therapy.

The results of our flow cytometric analyses strongly support the concept that monocytes are phenotypically altered in the presence of HIV infection, with a skewing of the distribution towards CD16-expressing monocytes that have been previously associated with a range of adverse outcomes in both HIV-infected and non-infected populations [7–9]. We also observed increased cell surface expression of CD64, CD143 and CD163 on these CD16⁺ monocytes, which were not reduced to healthy control levels despite virologically-suppressive ART. These results suggest that chronic HIV infection promotes CD16⁺ monocyte activation, overriding the ‘default’ apoptotic program that generally limits monocyte maturation and monocyte survival (with a half-life of approximately three days) [21]. Within this highly dynamic system, the persistence of pro-inflammatory cell surface markers on CD16⁺ monocytes in the face of suppressive HIV treatment is noteworthy.

Measurement of the plasma biomarkers sCD14, sCD163 and CXCL10 in this cohort also revealed distinct associations with HIV disease and treatment. In these

analyses, sCD14 levels were significantly elevated in the HIV⁺ group, irrespective of viral load level, CD4 T cell counts, or demographic variables. This stability of sCD14 levels has been previously noted over extended periods of follow-up among patients on HIV therapy [14, 19, 22], with further evidence that sCD14 levels are established at a 'set point' level very early in the course of HIV infection [23]. The fact that sCD14 levels are chronically increased with HIV infection compared to controls in this and other studies [22, 24], and that sCD14 levels are also strongly associated with mortality risk in the general population [25] as well among those with HIV infection [14], suggest that variability is prognostically important and is determined by HIV-specific factors as well as those shared among the general population. In this respect, a recent genome wide association study has identified heritable factors accounting for ~30% of this variation, while sCD14 levels were also significantly associated with cardiovascular risk factors (smoking, diabetes mellitus, fasting glucose and hypertension; all $p < 0.001$) as well as with C-reactive protein and IL-6 [25]. Similar associations between sCD14 and cardiovascular risk factors have also been discovered in a study of HIV-infected patients [26].

In contrast, we found that both CXCL10 and sCD163 levels were elevated in association with viremic HIV infection – with significant linear correlations between plasma HIV RNA levels and these variables – but that suppressive ART restored plasma levels to healthy control values. In the case of CXCL10 this is in keeping with previous studies [18, 19], as well as observations in acute HIV-1 infection that plasma CXCL10 levels provide a very early predictive measure of set-point viral load, CD4 T cell activation and disease progression [27] most likely through direct stimulation of monocyte and plasmacytoid dendritic cell CXCL10 production via toll-like receptor ligation [18]. Similar evidence has been provided for a correlation between plasma HIV RNA levels and sCD163 [17], and the interrelationship between sCD163 and CXCL10 identified in this study has also been noted in rheumatoid arthritis [28] – suggesting common activation pathways for these molecules.

In terms of the utility of these assays in a routine HIV laboratory setting, our data indicate that virological suppression (a known variable in HIV care) would be anticipated to have a favourable influence on plasma CXCL10 and sCD163 levels – potentially limiting the independent value of these tests in routine management. In contrast, many of the observed effects of HIV infection on CD16⁺ monocyte surface expression profiles (CD64, CD143 and CD163) and plasma sCD14 levels were not corrected by HIV treatment, nor were they associated with other variables considered in these analyses (gender, age, CD4 T cell count). Given available evidence that these monocyte biomarkers have prognostic significance in the general population as well as among those with HIV infection – including when standard CD4 T cell monitoring approaches carry limited prognostic value in the setting of 'successful' therapy [20, 29] – we would suggest that consideration of their inclusion in routine care is warranted. This is particularly relevant in light of emerging HIV treatment strategies that seek to reduce systemic immune activation (including sCD14 levels), either through modifying antiretroviral

therapy [30,31], or introducing adjunctive therapies such as statins [32], low-dose corticosteroids [33], or IL-7 treatment [34]. In these circumstances the ability to assess who will benefit from these treatment strategies, and then to monitor treatment response, will be critically important.

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Author Contributions

Conceived and designed the experiments: AC SF DN. Performed the experiments: AC. Analyzed the data: AC DN. Contributed reagents/materials/analysis tools: AC CB MF SF RK DN. Wrote the paper: AC DN CB MF SF RK. Served as the chief investigators: AC CB DN.

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Supplementary information for Chapter 3: The method development and validation of the flow cytometry monocyte activation marker panel.

3.1 Background

This supplementary component of Chapter 3 presents the development and optimisation of the eight colour flow cytometry method used for assessing monocyte cell populations in HIV infected individuals and a healthy control population. The process of assay development features: 1) instrumental setup and validation controls including compensation and brightness assessments, 2) staining and gating controls including isotype control (IC) determination and fluorescence minus one (FMO) staining controls, and 3) biological/experimental controls including normal or untreated samples. Through this process, the FMO controls help identify gating boundaries when using multi-channel panels, isotype controls help identify staining issues, and unstained controls show the background or auto-fluorescence of the system.

3.2 Fluorochrome selection for the eight colour monocyte activation marker (MAM) assay

Designing an eight colour flow cytometry assay has its challenges, although this process has been simplified by the development of commercial product websites. In this case, the design of the eight colour MAM panel, shown throughout Chapter 3, was selected utilising the BD multicolour panel designer (<https://www.bdbiosciences.com/paneldesigner/>). This simplified the process of matching antigen density to fluorochrome brightness, by selecting the antigen with the highest density to be matched to the fluorochrome with the lowest intensity (Figure S1). The panel of fluorochromes available for consideration for the work up of the MAM panel are highlighted in Table S1 (blue) along with the final MAM panel selection (shown in light blue and turquoise). Primary, secondary and tertiary antigens are classified according to how well they have been characterised and their level of expression (i.e. CD45; well characterised and easily classified positive or negative while CD143 poorly classified and expressed at low levels).

Additionally, the fluorochrome present on tandem dye conjugates (i.e. PE-Cy7) degrade with exposure to light and temperature therefore to avoid this occurring, the

tandem conjugates used throughout the MAM assays were stored in small volumes at 4°C until required.

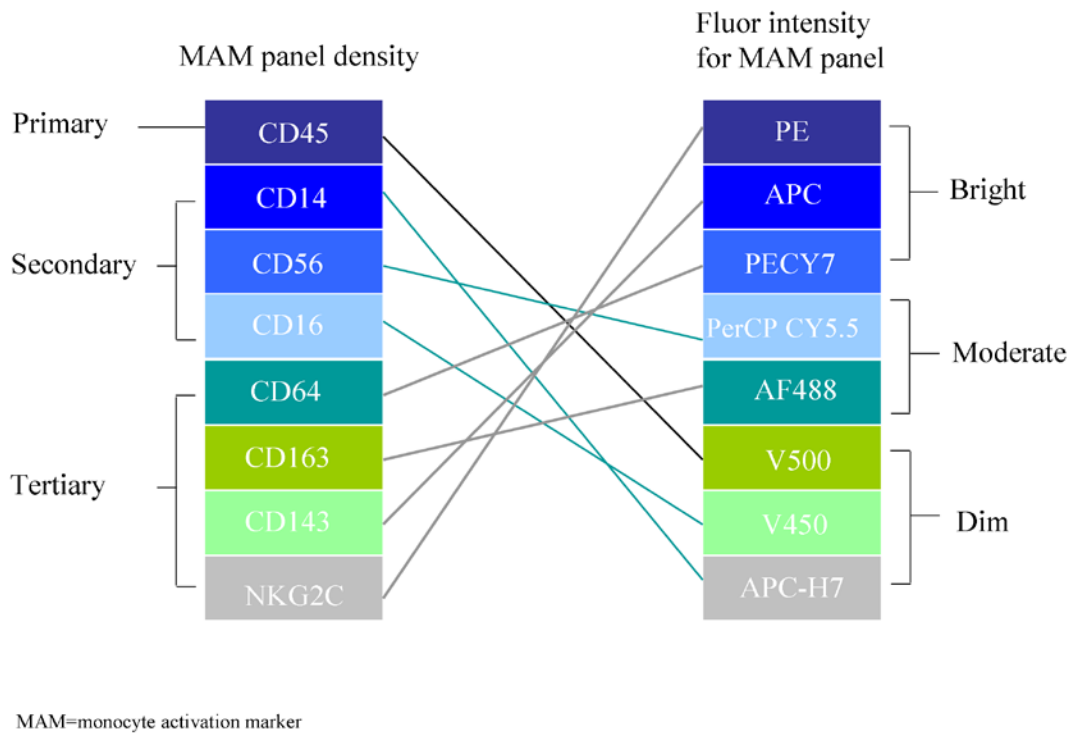


Figure S1: Monocyte activation marker panel matching procedure. Antigen density (left panel; primary, secondary and tertiary antigens) are matched to fluor intensity (right panel; bright, moderate or dim).

3.3 Instrument set up

The FACSCanto II instrument used for the eight colour monocyte expression assay was monitored daily within the Royal Perth Hospital Haematology department. Daily maintenance of the instrument involved running the BD Cytometer Setup Beads (CST) to monitor the 3 laser/eight colour settings. Optimisation of photomultiplier tube (PMT) voltages occurred monthly to obtain maximum resolution between negative and positive populations of a fluorescence channel.

3.4 Compensation evaluation

Colour compensation analysis was performed at the beginning of the assay optimisation then repeated every six months or when the instrument was calibrated. Compensation was used to reduce the fluorescence spill-over (optical background) from laser channels as the more colours used resulted in more spill-over which in turn

resulted in higher background. The compensation set up was performed using the BD™ CompBeads Set as per manufacturer’s instructions. Voltages were adjusted specifically for V450 with V500; PE with PerCP-CY5.5; and PerCP-CY5.5 with PECY7 to ensure precise compensation with minimal spill-over. Once established the compensation profile was stored for future use.

Table S1: The antibody-fluorochrome pairs were selected by using the BD multicolour panel designer (<https://www.bdbiosciences.com/paneldesigner/>). The final selection of the monocyte activation marker panel, used in the evaluation, are shown in light blue and turquoise (grey = not available, royal blue = available but not considered).

Wavelength (nm)	Violet Laser 405nm			Blue Laser 488 nm					Red Laser 640nm				
	488	491	500	519	519	578	695	785	600	668	719	785	785
Antibody	V450	AM Cyan	V500	FITC	AF488	PE	PerCP-Cy5,5	PECy7	APC	AF647	AF700	APC-Cy7	APC-H7
CD163													
CD143 (BB9)													
CD14													
CD16													
CD45													
CD64													
CD56													
NKG2C													

■ Becton Dickinson fluorochromes used
■ R+D Systems fluorochromes used
■ Other available fluorochromes
■ Not available (at Sept 2012)

3.5 Establishing monocyte activation marker parameters

Once the assay compensation was established, each antibody was tested against a donor HIV negative control, using the manufacturer’s instructions, to identify whether all antibodies worked efficiently. The corresponding ICs were also tested. Initially tests were carried out using two protocols. One protocol (wash protocol - Figure S2-A) required a centrifugation wash step (after cell staining) followed by a red cell lysis step while a second protocol (no wash protocol - Figure S2-B) was used without a centrifugation wash step after the cells were stained. The results from this experiment demonstrated that a centrifugation wash step changed the size of some monocytes. Figure S2 below clearly shows two monocyte populations, one larger forward scatter

than the other (Figure S2A: T vs F) with “T” expressing more inflammatory monocytes (intermediate; 10.7% vs 3.9% and non classical monocytes; 13% vs 1.5%), while the no wash protocol only identified one monocyte population (Figure S2B: F) and expressed monocytes at levels previously described. Since it is highly important to reflect the *in vivo* biological activity of monocytes without artificially activating the monocytes with washing *in vitro*, the “no wash protocol” was used throughout evaluation as described below.

- I. Add the appropriate volume of antibody to a tube (as identified from titration experiments)
- II. Add 50ul of well mixed whole blood and gently vortex
- III. Incubate for 15 minutes (at room temperature, in the dark)
- IV. Add 500ul of FACSLyse red cell lysis buffer and gently vortex
- V. Incubate for 15 minutes (in the dark, at room temperature)
- VI. Process the samples immediately on the Canto instrument.

Assessing the correct number of events is important because monocytes comprise only 2-12% of the lymphocyte population therefore approximately 5000 monocytes (events) were collected.

After establishing the optimal conditions for each antibody, the next step of the assay workup assessed how well the “pooled” antibody mix worked. The probability of spill-over occurring increases when “pooling” antibodies, therefore it is important to define the best concentration of antibody to use throughout testing without compromising brightness and sensitivity. A titration optimisation step was therefore performed on 50ul of whole blood from healthy control samples. The antibodies were evaluated neat (N), and diluted (N/2, N/5, N/10, N/25, N/50) using flow buffer. To ensure that the intensity of the antibody was not compromised, the most appropriate dilution was determined by selecting the dilution preceding the limiting dilution factor. Once the most appropriate concentration of antibody was determined, all antibodies were pooled and reassessed as per the method above.

The next step was to perform a titration of the whole blood and to test the samples over 76hrs to ensure there was adequate number of cells collected without compromising cellular integrity and intensity of the fluorochromes. Whole blood samples stored at room temperature or at 4°C were used to evaluate the effect of storage temperature on monocyte subsets at times 4hrs, 28hrs, 52hrs and 76hrs after

collection. The final volume of whole blood used in the experiments was 50ul and samples were deemed satisfactory for use for up to 52hrs at room temperature or 76hrs of storage at 4°C (Figure S3).

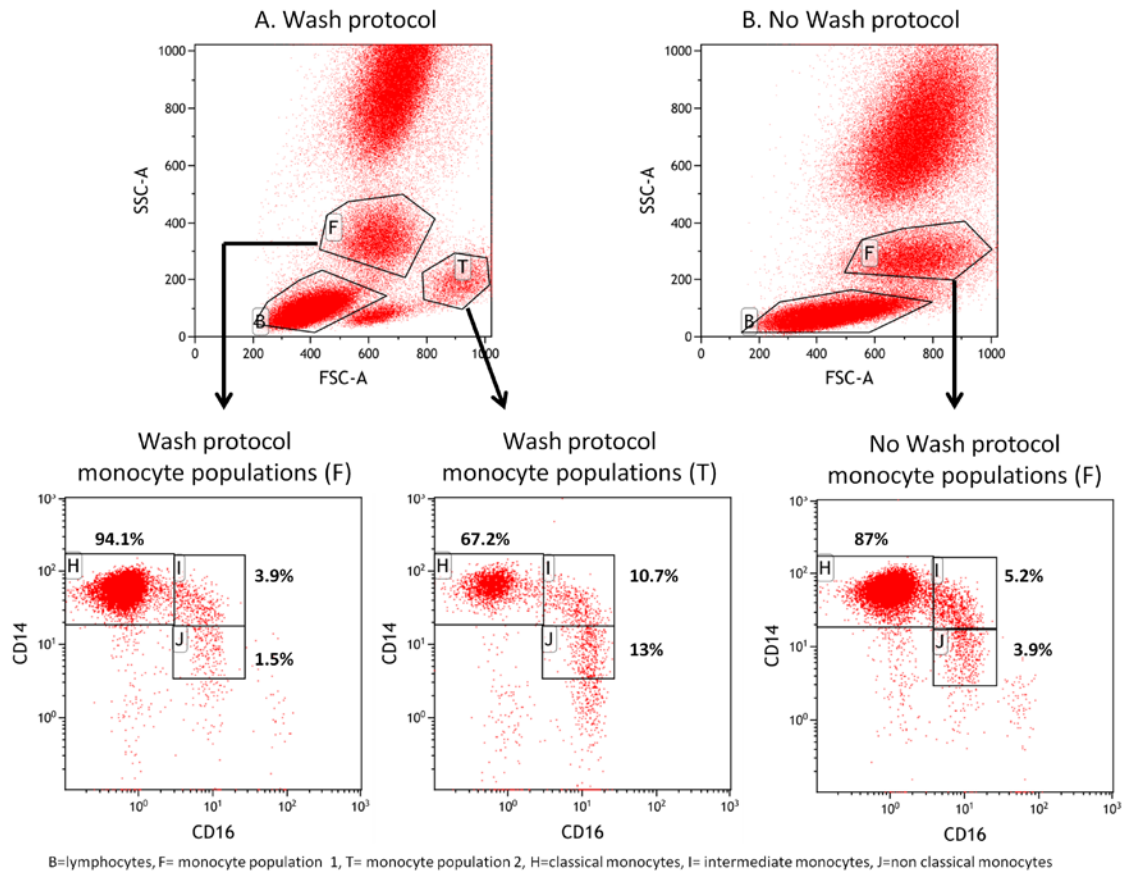
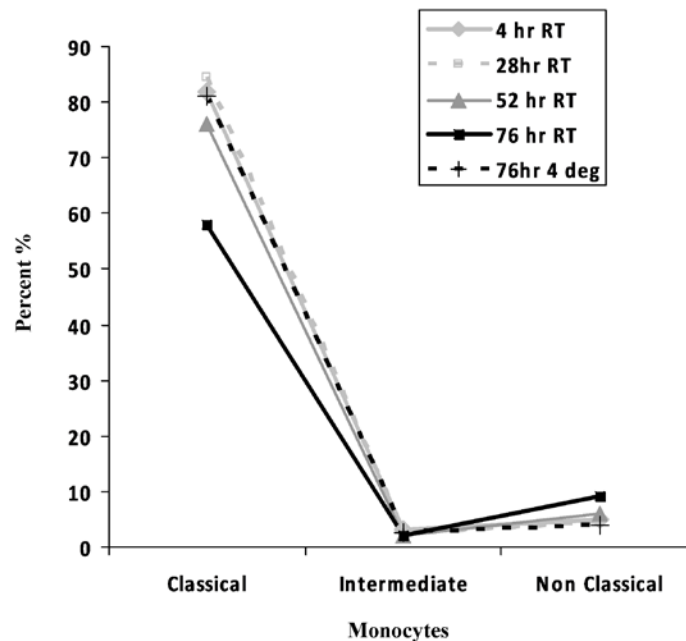


Figure S2: The effect of “washing” monocyte cell populations. Two monocyte cell populations were identified when employing the “wash protocol” (A: F and T) while only one monocyte cell population (B: F) was identified with the “no wash protocol”.

3.6 Gating strategy to identify MAM expression levels

An important part of the assay optimisation was to ensure that the gating strategy was robust. Isotype antibody controls were used to identify staining problems for single antibody use, while FMO staining was used to identify gating boundaries for the 8 colour multi-channel MAM panel. As shown in Figure 1 in Chapter 3, forward-light scatter (FSC) and side-light scatter (SSC) area plots were used to set the gate around the monocyte population. The monocyte population was further identified based on CD45 fluorescence. From here CD14 vs CD16 expression was plotted to determine

the classical, intermediate and non classical monocyte populations. Furthermore, within each monocyte subpopulation, CD64, CD163 and CD143 expression was identified. Of importance, the gating strategy for identifying positive cell populations from FMO and isotype controls should be determined for each individual sample. An example displaying the gating strategy for CD14 and CD16 is shown below (Figure S4).



RT=room temperature, hr=hour, deg=degree

Figure S3: The effect of temperature and time on monocyte subsets. The average percentage of monocyte subsets (classical, intermediate and non classical) taken from 2 healthy donors after storing whole blood at room temperature or at 4°C.

3.7 Safety guidelines utilised for the MAM whole blood phenotyping assay

Another important aspect to consider when developing, optimising and utilising infectious whole blood in the eight colour flow cytometry MAM method, is safety procedures. Throughout this study universal safety precautions and the Royal Perth Hospital health and safety principles and practices were followed to reduce the risk of exposure to hazardous materials and eliminate sample contamination. These practices follow the accreditation standards maintained by the Department of Immunology to ensure safe practices to the samples and the researcher.

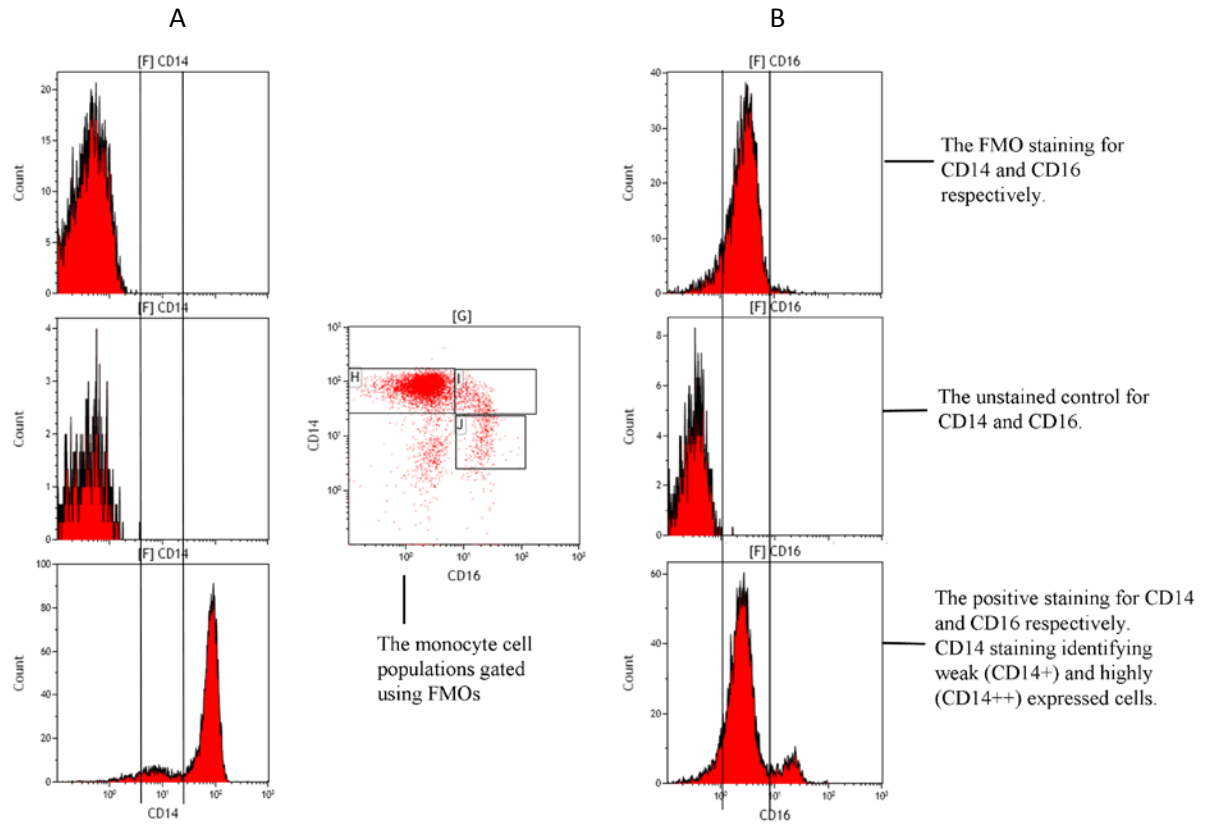


Figure S4: The gating strategy for CD14 (A) and CD16 (B) monocytes. Fluorescent minus one (FMO) controls, unstained controls and samples stained with 8 fluorochromes were used to determine an appropriate gating strategy.

Summary for Chapter 3.

In Chapter 3 “Elevated plasma soluble CD14 and skewed CD16⁺ monocyte distribution persist despite normalisation of soluble CD163 and CXCL10 by effective HIV therapy: a changing paradigm for routine HIV laboratory monitoring?”, the influence of HIV infection and its treatment on monocyte activation was explored. Cell surface expression markers on monocytes were evaluated by flow cytometry and circulating soluble plasma biomarkers were analysed using commercial ELISA assays, among patients with HIV infection as well as healthy control samples. The results showed ongoing systemic immune activation in response to HIV infection, characterised by persistent alterations of CD16⁺ monocyte expression profiles and elevated soluble CD14 levels that were not corrected by antiretroviral therapy and thus likely to be of prognostic significance. The elevated soluble CD14 result is an interesting one given that this marker has been associated with increased mortality risk in patients with HIV infection; however, its role in HIV infection especially involving co-morbidities is still poorly understood and warrants further investigation. For this reason, Chapter 4 will explore potential roles of the three soluble markers of monocyte activation evaluated in Chapter 3 (sCD14, sCD163 and CXCL10) during chronic HIV infection in 475 persons where cardiovascular risk assessments, lipid profiles and HIV treatment information were collected in 2010. The major aim being to identify therapies (HAART, statins and lipid lowering treatments) that may reduce monocyte activation in HIV patients to normal healthy levels, while adding evidence that each of these ‘biomarkers’ reflects distinct biological pathways that influence monocyte activation. Taken together these research findings may be useful in reducing immune dysfunction during chronic HIV infection and limiting age related illnesses during HIV infection.

Chapter 4:
Plasma CXCL10, sCD163 and sCD14 levels have distinct associations with antiretroviral treatment and cardiovascular disease risk factors.



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Attributions:

AC designed and performed all assays along with the analysing and the interpretation of the findings, literature review and manuscript writing. LW helped perform the CVD risk assessment and compile the data. IJ assisted with the statistical analysis and reviewed the manuscript. GG assisted with collating the BMI results and reviewed the manuscript. CB provided supervision and reviewed the manuscript. DN supervised the development of the assays, interpreted the findings and critically reviewed the manuscript. All authors approved the final version.

AC: 70%

RESEARCH ARTICLE

Plasma CXCL10, sCD163 and sCD14 Levels Have Distinct Associations with Antiretroviral Treatment and Cardiovascular Disease Risk Factors

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

We investigate the associations of three established plasma biomarkers in the context of HIV and treatment-related variables including a comprehensive cardiovascular disease risk assessment, within a large ambulatory HIV cohort. Patients were recruited in 2010 to form the Royal Perth Hospital HIV/CVD risk cohort. Plasma sCD14, sCD163 and CXCL10 levels were measured in 475 consecutive patients with documented CVD risk (age, ethnicity, gender, smoking, blood pressure, BMI, fasting metabolic profile) and HIV treatment history including immunological/virological outcomes. The biomarkers assessed showed distinct associations with virological response: CXCL10 strongly correlated with HIV-1 RNA ($p < 0.001$), sCD163 was significantly reduced among 'aviraemic' patients only ($p = 0.02$), while sCD14 was unaffected by virological status under 10,000 copies/mL ($p > 0.2$). Associations between higher sCD163 and protease inhibitor therapy ($p = 0.05$) and lower sCD14 with integrase inhibitor therapy ($p = 0.02$) were observed. Levels of sCD163 were also associated with CVD risk factors (age, ethnicity, HDL, BMI), with a favourable influence of Framingham score $< 10\%$ ($p = 0.04$). Soluble CD14 levels were higher among smokers ($p = 0.002$), with no effect of other CVD risk factors, except age ($p = 0.045$). Our findings confirm CXCL10, sCD163 and sCD14 have distinct associations with different aspects of HIV infection and treatment. Levels of CXCL10 correlated with routinely monitored variables, sCD163 levels reflect a deeper level of virological suppression and influence of CVD risk factors, while sCD14 levels were not associated with routinely monitored variables, with evidence of specific effects of smoking and integrase inhibitor therapy warranting further investigation.

Introduction

In spite of the evident success of highly active antiretroviral therapy (HAART) in suppressing plasma levels of HIV-1 RNA, preventing progressive immune deficiency and ultimately improving patient survival [1], there is increasing evidence that immune activation persists in the face of effective HIV treatment [2,3]. This immune phenotype, which is characterized by prominent monocyte activation [2–4], heightens age related changes [5] and has been associated with increased prevalence and earlier onset of a range of non-infectious co-morbidities among HIV-infected individuals including cardiovascular and liver disease, type II diabetes mellitus and cognitive decline [2–4,6]. The prognostic significance of several innate immune ‘biomarkers’ such as interleukin-6, C-reactive protein and particularly soluble CD14 (sCD14) [7] as strong predictors of mortality in the setting of treated HIV infection, has also now been established [2–4].

We have previously described an ongoing systemic inflammatory response to HIV infection among 81 HIV+ individuals with a range of treatment outcomes compared to 21 healthy control blood donors [8]. Here, untreated HIV infection was characterised by elevated levels of pro-inflammatory CD16⁺ monocytes as well as elevated plasma levels of monocyte-derived, interferon-inducible proteins (sCD14, soluble CD163 and CXCL10). Treatment-associated suppression of plasma HIV-1 RNA levels (i.e. <40 copies/mL) was associated with levels of sCD163 and CXCL10 that were similar to healthy controls, while sCD14 levels remained significantly elevated despite what would otherwise be considered successful HIV treatment [8]. This observation of stable elevated sCD14 levels has also been made by others [9,10], highlighting that plasma ‘biomarkers’ of systemic immune activation have distinct relationships with HIV infection and its treatment.

In this study we have sought to investigate these plasma ‘biomarkers’ further, utilising a larger study population and incorporating analysis of cardiovascular disease risk factors, noting that sCD14 has been positively correlated with smoking, diabetes, fasting glucose and hypertension (all $p < 0.001$), as well as all-cause mortality, in a large US cohort study of older adults [11]. We were also interested to explore the influence of detectable plasma HIV-1 RNA below the standard viral load assay threshold of 40 copies/mL on these biomarkers, having previously demonstrated in a study of >11,000 viral load results that ‘residual viraemia’ could be identified in 20% of samples measured at <40 copies/mL and was strongly predicted by the level of plasma viraemia prior to HIV treatment—even after 10–15 years of suppressive HIV therapy [12].

Our principal aim was to investigate the potential utility of incorporating one or more of these plasma biomarkers into routine HIV management, through an improved understanding of their relationships to known laboratory and clinical variables. This is informed by a growing awareness that monitoring CD4⁺ T cell counts has limited ongoing utility once normal levels have been achieved [13], while other markers of immune function may have more prognostic value [7] as well as providing insights into disease pathogenesis [14] and informing new therapeutic strategies beyond the current antiretroviral treatment paradigm [15].

Materials and Methods

Patient cohort

Patients residing in Western Australia and attending the Royal Perth Hospital (RPH) Immunology clinic in 2010 were recruited for this study. Informed written consent was obtained from the patients participating in this investigation. This consent form was reviewed and

accepted by the ethics committees. Written ethics committee approvals for this investigation were received from Royal Perth Hospital (EC2012/170) and Murdoch University (2012/216).

Plasma collection process

Plasma samples were collected on the day of CVD risk assessment. Plasma was collected from EDTA whole blood samples within 6 hours of collection by centrifugal force of 1000g for 20 mins. Plasma was removed and stored at -80°C until required for the plasma HIV-1 RNA viral load and enzyme-linked immunosorbent assays (ELISA).

Cardiovascular disease risk assessments and HIV laboratory testing

Data on cardiovascular disease risk factors were obtained by physical examination and blood tests assessed at the time of visit. Serum levels of total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglycerides were measured and the total:HDL ratio was subsequently calculated.

HIV-1 RNA levels were measured using the Roche Cobas ultrasensitive ampliprep assay V1 (Roche). CD4⁺ T cell counts, CD4⁺ T cell percentage and CD4:8 ratios were measured from sample acquisitions performed on the FACSCanto II flow cytometer with FACSDiva 6.1.1 software (BD Biosciences).

Physical examinations included records for age (at sample collection), gender, ethnicity, height (cm), weight (kg) and blood pressure monitoring (FsysBP, FDiaBP). Smoking was self-reported and recorded as either being a smoker, a non-smoker or an ex-smoker (within 1 year). Clinical notes were accessed to record whether patients were using ART (protease inhibitor, NRTI, NNRTI and Integrase inhibitor), ACE inhibitors or statin therapy at the time of assessment. Body mass index (BMI) was calculated using height and weight measurements overseen by a dietitian, whilst the Framingham score (mean 5-year CVD risk score) was determined using the National Heart Foundation absolute CVD risk algorithm.

Measurement of plasma sCD14, sCD163 and CXCL10 levels

For the quantitative determination of plasma biomarker levels, ELISAs were utilised, without modification, as previously described [7]. In-house control samples (sCD14 = 400,000 pg/μl; sCD163 = 250 ng/μl; CXCL10 = 125 pg/μl) were included in each assay. The mean concentration values (SD) for the assay controls over all runs were 444,080 pg/μl (17310), 251 ng/μl (16.7) and 120.8 pg/μl (5.76) for sCD14, sCD163 and CXCL10 respectively. The mean R value from all ELISAs was 0.993, 0.997 and 0.998 for sCD14, sCD163 and CXCL10 respectively.

Statistical analysis

Statistical data analysis was performed using SPSS version 21. Data distribution was assessed for normality, with transformation of variables as required. Plasma biomarker data required logarithmic transformation while absolute CD4⁺ cell count, CD4:8 ratio and total cholesterol levels were square rooted. One way ANOVA analysis was utilised to compare variants within the HIV cohort with appropriate correction for multiple comparisons when >2 groups were compared. Univariate correlation and multivariate linear regression analyses were utilised to estimate associations between plasma biomarkers and HIV clinical parameters (viral load status, HIV-1 RNA viral load level, absolute CD4⁺ T cell count, CD4:8 ratio), treatment choice, CVD risk factors (blood pressure and cholesterol variables, smoking, BMI and Framingham score), gender, age and ethnicity. Statistical significance required a p-value of <0.05.

Results

The study population included 474 consecutive patients who attended the Royal Perth Hospital (RPH) Immunology clinic in 2010 who consented to a cardiovascular risk assessment including smoking history, standardised measurements of blood pressure and weight as well as collection of a fasting metabolic profile. Patient characteristics, demographics and clinical details for this study are shown in [Table 1](#).

The overall study population comprised 78.5% males, 68.8% Caucasians, with a mean age of 45 years (range 21–81 years, SD 12.3 years). The overall mean CD4⁺ T cell count was 567 cells/ μ L (range 3–2205 cells/ μ L, SD 319 cells/ μ L) and mean plasma HIV-1 RNA level was 2.4 log₁₀-copies/mL (lcpm) or 251 copies/mL (cpm). Three hundred and sixty-five patients were on

Table 1. Demographics and patient characteristics from 474 HIV positive patients who underwent CVD risk assessments in 2010.

<i>Characteristic</i>	<i>Value</i>
Age at time of assessment, mean years (range)	45 (21–81)
Male sex (n, %)	372 (78.5)
Ethnicity	
- Caucasian (n, %)	326 (68.8)
- Indigenous Australian (n, %)	24 (5.1)
- African (n, %)	57 (12.1)
- Asian (n, %)	66 (14.0)
Current Smoker (n, %)	168 (35.4)
HIV Clinical Parameters at time of assessment	
- Plasma HIV RNA viral load (lcpm)	2.4 (1.6–6.0)
- Aviraemia (n, %)	211 (44.5)
- Residual viraemia <1.6 lcpm (n, %)	60 (12.7)
- HIV RNA viral load 1.6–≤3 lcpm (n, %)	75 (15.8)
- HIV RNA viral load 3–4 lcpm (n, %)	49 (10.3)
- HIV RNA viral load ≥4 lcpm (n, %)	79 (16.7)
- CD4%, mean % (range, SD)	26.1 (1–62, 11)
- Absolute CD4 T cell count, mean (range, SD)	567 (3–2205, 319)
- CD4:8 ratio, mean (range, SD)	0.65 (0.01–3.1, 0.4)
HIV therapy at the time of assessment (n)	365
- NNRTI (n, %)	215 (45.4)
- NRTI (n, %)	348 (73.4)
- HIV Protease Inhibitor (n, %)	161 (34)
- Integrase (n, %)	17 (3.6)
Framingham score (mean, range)	6.67 (0–42)
BMI, kg/m ² (mean, SD)	25 (4.8)
Statin therapy, n (%)	72 (15.2)
Diabetic, n (%)	25 (5.3)
ACE inhibitor, n (%)	54 (11.4)
sCD14 (log pg/ μ l), mean (SD error, range)	6.24 (0.007, 5.65–6.62)
sCD163 (log ng/ μ l), mean (SD error, range)	2.89 (0.009, 2.22–3.42)
CXCL10 (log pg/ μ l), mean (SD error, range)	2.05 (0.018, 1.13–3.22)

lcpm = log copies per mL, SD error = standard error; n = number, BMI; body mass index; ACE = angiotensin converting-enzyme inhibitor; NRTI = Nucleoside Reverse Transcriptase; NNRTI = Non Nucleoside/ Nucleotide Reverse Transcriptase.

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antiretroviral therapy (77%), with undetectable HIV-1 RNA viral load (<40 cpm) noted for 271 patients; 57.2% of the overall cohort, and 74.2% of those on HIV therapy. Within this subset, target HIV-1 amplification could be detected on the Cobas HIV-1 ampliprep system below the assay threshold of 40 cpm, reflecting ‘residual viraemia’ in 60 cases (22.1% of results reported as <40 cpm).

With regard to cardiovascular risk factors, the mean 5-year CVD risk score (Framingham score) in the study population was 6.6% (SD 6.7; range 0–42), including 325 patients (71.0%) in the low risk category (estimated 5-yr CVD risk <10%), 90 patients (19.7%) deemed at moderate risk (5-yr CVD risk 10–15%), and 43 patients within the high risk group (n = 23 (5.0%) with CVD risk 15–20%, and n = 20 (4.4%) with CVD risk >20%). The study group included 168 current smokers (35.4%) and 25 diabetics (5.3%). The average BMI of the study group was 25.0 kg/m² (SD 4.8). Statin therapy was used in 15.2% while ACE inhibitors were used in 11.4% of participants.

Plasma biomarker levels were approximately normally distributed following logarithmic transformation, with mean log values and corresponding plasma concentrations for sCD14, sCD163 and CXCL10 of 6.24 [1,737,800 pg/μl], 2.89 [776 ng/μl] and 2.05 [112.2 pg/μl] respectively (Table 1).

Correlations between HIV-1 clinical parameters, CVD risk and circulating plasma biomarkers

Spearman rank correlations were used to explore associations between circulating soluble plasma biomarkers and HIV clinical and CVD risk parameters. Investigating the influence of HIV clinical parameters (HIV-1 RNA, CD4⁺ T cell count and CD4:8 ratio), we observed strong positive correlations between plasma HIV-1 RNA levels and both CXCL10 and sCD163 (p<0.001, r = 0.5; Fig 1A and 1B) and strong negative correlations for CD4⁺ T cell (p<0.001, r = -0.36) and CD4 ratio (p<0.001, r = -0.37) while we could not find any significant correlations between HIV clinical parameters and sCD14 (p>0.1, Fig 1C).

With respect to CVD risk, we demonstrated a strong and significant negative correlation for sCD163 and CXCL10 and total cholesterol levels (data not shown; p<0.001, r = -0.23), LDL-c (p<0.01, r = -0.18) and HDL-c (p≤0.001, r = -0.18), however these parameters did not correlate with sCD14 levels (p>0.6). There were no significant correlations between the plasma biomarkers and total:HDL cholesterol ratio (p>0.1), blood pressure parameters (p>0.2),

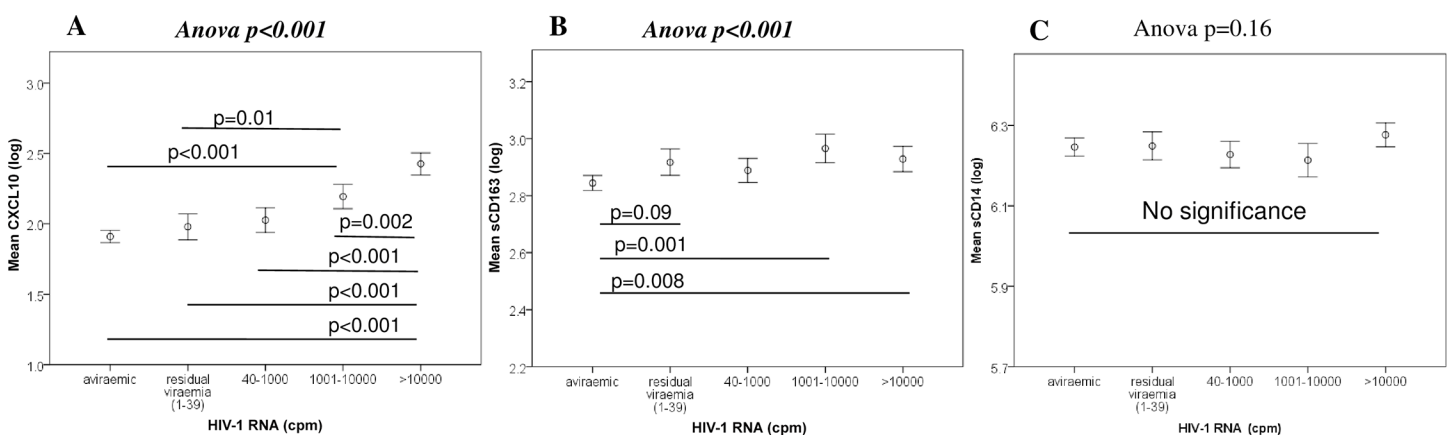


Fig 1. Differing correlation outcomes between the three plasma biomarkers and HIV-1 RNA levels. A significant correlation was recognised between HIV-1 RNA levels with CXCL10 (A) and sCD163 (B) while there was no significance with sCD14 (C).

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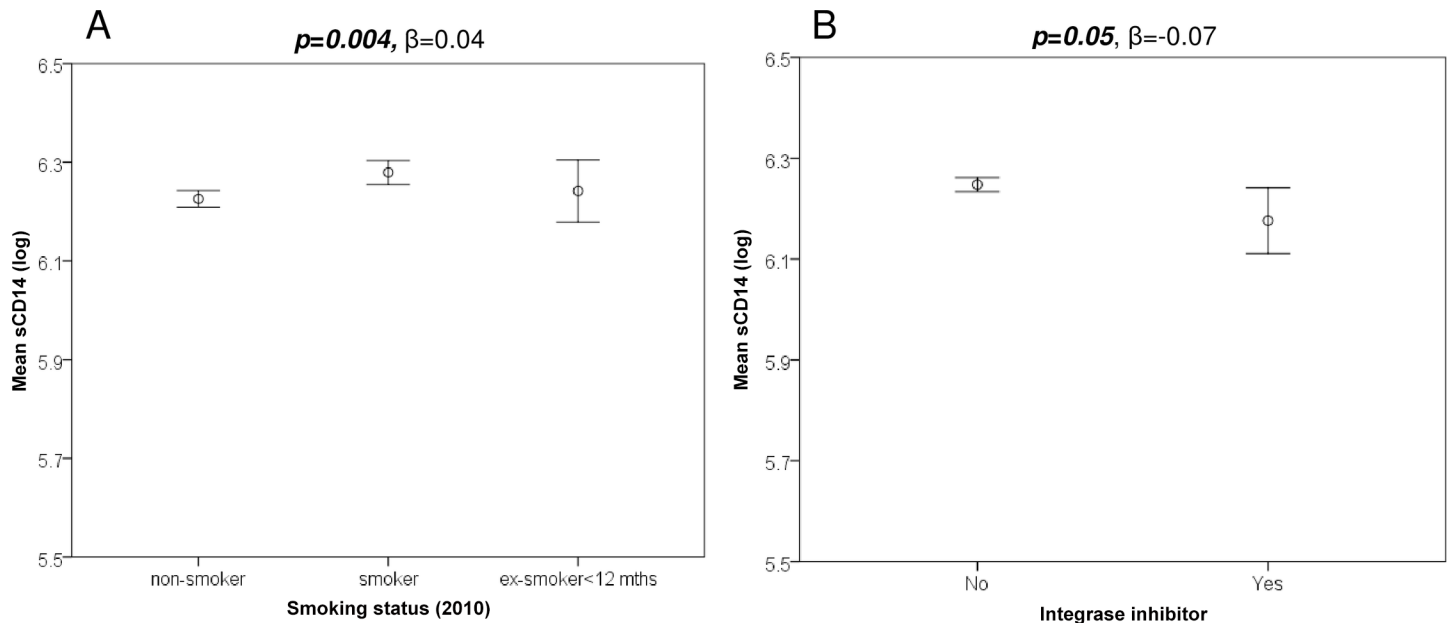


Fig 2. There was a significantly strong correlation between sCD14 and smoking where HIV-1 smokers have higher sCD14 levels than HIV-1 non-smokers while patients on an integrase inhibitor had significantly lower sCD14 levels than patients on an alternative treatment.

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Framingham score ($p>0.15$) or BMI ($p>0.07$). Analysis with the Tukey post-hoc test revealed lower sCD14 levels in non-smokers compared to smokers (Fig 2A: $p = 0.004$). Furthermore, there was a significant positive correlation for sCD14 and sCD163 levels with smoking (data not shown; $p = 0.002, r = 0.14$; $p = 0.03, r = 0.1$ respectively) but not CXCL10 ($p = 0.07$).

We also demonstrated correlations between the three plasma biomarkers assessed. Circulating sCD163 had a strong positive correlation with CXCL10 levels ($p<0.001, r = 0.41$, S1A Fig) and sCD14 levels ($p<0.001, r = 0.17$, S1B Fig) however sCD14 did not significantly correlate with CXCL10 ($p = 0.07, r = 0.07$, S1C Fig). We did not identify any correlations for age ($p>0.09$) or gender ($p>0.4$) with circulating plasma biomarkers. Univariate analysis of the plasma biomarkers with ethnicity suggests higher levels of CXCL10 and sCD163 in Indigenous Australians (S2A and S2B Fig) and lower levels of sCD14 in Africans (S2C Fig).

Multivariate regression analysis reveals distinct biomarker associations

Multivariate regression analysis was then undertaken with each plasma biomarker in isolation (Model 1) as well as considering the influence of all three biomarkers in adjusted analyses (Model 2).

CXCL10. In unadjusted analyses for Model 1 (S1 Table), CXCL10 levels were strongly associated with higher HIV-1 RNA viral load ($p<0.0001$) and lower CD4⁺ T cell counts ($p = 0.0001$) as well as lower CD4:8 ratio ($p = 0.008$). Participants on NRTI therapy had lower CXCL10 ($p = 0.0002$) whilst being an Asian ($p = 0.05$) or African male had favourable effect on CXCL10 levels ($p = 0.0001, \beta>0.2$). Framingham score and BMI were not associated with CXCL10 levels, however patients on an ACE inhibitor had significantly higher levels of CXCL10 than patients not on an ACE inhibitor ($p = 0.045, \beta = 0.1$). Lower total cholesterol levels were also associated with elevated CXCL10 levels ($p = 0.02, \beta = -0.37$).

As shown in Table 2 (Model 2), the inclusion of all plasma biomarkers did not abrogate the significant associations of CXCL10 with HIV clinical parameters (CD4⁺ T cell counts

Table 2. Model 2—Multivariate regression results showing significant associations of plasma biomarker with HIV clinical parameters, CVD risk age, gender, ethnicity and smoking after adjusting for CXCL10, sCD163 and sCD14.

Biomarker Variable	CXCL10			sCD163			sCD14		
	β	std error	p	β	std error	p	β	std error	p
Age at 2010	-	-	-	0.002	0.001	0.03	0.001	0.0006	0.08
Gender	-	-	-	-	-	-	0.031	0.018	0.08
Ethnicity	-0.15	0.046	0.001	0.107	0.042	0.012	-0.058	0.022	0.009
Gender:Ethnicity	-0.224	0.063	0.0004	-	-	-	-	-	-
Smoking	-	-	-	-	-	-	0.043	0.015	0.004
Residual viraemia	-	-	-	0.058	0.026	0.025	-	-	-
VL5	0.28	0.058	<0.0001	-	-	-	0.07	0.023	0.002
SQR CD4:8	-0.202	0.080	0.012	-	-	-	-	-	-
SQR CD4	-0.01	0.003	0.0005	-	-	-	-0.002	0.001	0.07
NRTI	-0.156	0.046	0.0007	-	-	-	-	-	-
NNRTI	-	-	-	-	-	-	0.052	0.018	0.004
PI	-	-	-	-	-	-	0.041	0.018	0.026
Integrase	-	-	-	-	-	-	-0.078	0.037	0.037
HDL	-	-	-	-0.04	0.023	0.09	-	-	-
BMI	-	-	-	0.004	0.002	0.017	-	-	-
Framingham score	-	-	-	0.05	0.025	0.049	-	-	-
CXCL10	-	-	-	0.18	0.026	<0.0001	-0.023	0.022	0.3
sCD163	0.51	0.079	<0.0001	-	-	-	0.13	0.039	0.0009
sCD14	-0.06	0.096	0.53	0.16	0.056	0.003	-	-	-

“-“p = >0.01; std = standard error.

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(p = 0.0005) and CD4:8 ratio (p = 0.012) and HIV-1 RNA viral load (p<0.0001), ethnicity (p = 0.001), NRTI therapy (p<0.001) or with sCD163 (p<0.0001). It did however nullify the significant association with ACE inhibitor treatment and cholesterol levels. Interestingly, the adjusted multivariate regression analysis confirmed an interaction between gender and ethnicity (Table 2) which attributed to significantly lower CXCL10 levels in Asian (p = 0.001, β = -0.15) and African males (p<0.001, β = -0.22).

sCD163. In unadjusted analysis plasma levels of sCD163 were significantly reduced among ‘aviraemic’ patients only (p = 0.02, β = 0.06) and otherwise remained stably elevated across all levels of virological suppression (Model 1; S1 Table). Higher levels were associated with lower CD4⁺ T cell counts (p = 0.01, β = -0.004), as previously noted for CXCL10. Several CVD risk factors were associated with sCD163, namely age (p = 0.001, β = 0.003), ethnicity (p = 0.01, β = 0.1), HDL (p = 0.048, β = -0.05) and BMI (p = 0.009, β = 0.005), with a favourable influence of Framingham score <10% (p = 0.04, β = 0.06). Additionally, the level of circulating sCD163 was increased when the choice of HIV treatment was a PI (p = 0.05, β = 0.04) but decreased if participants were on NRTIs (p = 0.04, β = -0.06).

Including all the biomarkers in the regression analysis for sCD163 (Model 2; Table 2) showed a positive association with both CXCL10 (p<0.0001) and sCD14 (p = 0.003). Interestingly sCD163 remained significantly associated with ‘aviraemic’ status only (p = 0.02) and with several CVD risk factors including age (p = 0.03), ethnicity (p = 0.01), BMI (p = 0.02), Framingham score <10% (p<0.05). Univariate associations with CD4⁺ T cell counts and choice of therapy were abrogated in adjusted analyses. Of interest, the adjusted multivariate regression analysis confirmed that sCD163 levels were significantly higher among indigenous cases (p = 0.012, β = 0.11).

sCD14. Analysing the determinants of sCD14 levels in Model 1 (S1 Table) revealed no significant influence of virological status under 10,000 cpm ($p = 0.8$; data not shown) although sCD14 levels were incrementally higher when HIV-1 RNA levels were $>10,000$ cpm ($p = 0.003$, $\beta = 0.06$). Soluble CD14 levels were higher among smokers ($p = 0.002$, $\beta = 0.05$), lower in Africans ($p = 0.009$, $\beta = -0.06$) with no effect of other CVD risk factors or overall Framingham score, apart from age ($p = 0.04$, $\beta = 0.001$). Interestingly, lower sCD14 levels were associated with use of integrase inhibitor therapy ($p = 0.02$, $\beta = -0.09$; also Fig 2B).

After adjusting for soluble biomarkers in Model 2 (Table 2), sCD14 levels remained strongly associated with sCD163 ($p < 0.001$), HIV-1 RNA level $>10,000$ copies/mL ($p = 0.002$), smoking ($p = 0.004$), ethnicity ($p = 0.009$) and choice of HIV treatment—with a beneficial effect of integrase inhibitor therapy ($p = 0.037$). In this analysis, the univariate association with age was abrogated ($p = 0.08$). Interestingly, the adjusted multivariate regression analysis confirmed that sCD14 levels were significantly lower for African cases ($p = 0.009$, $\beta = -0.06$).

Discussion

This study confirms our previous finding that CXCL10, sCD163 and sCD14 have distinct although overlapping associations with different aspects of HIV infection and treatment [8], as well as cardiovascular disease risk factors and demographic variables (Fig 3). These relationships are in keeping with an increasingly refined understanding of these plasma biomarkers and their place within the immune environment. For example, CXCL10 was initially identified as an interferon-gamma-induced protein (also named interferon inducible protein-10) and a ligand for CXCR3 [16,17], although in the context of HIV infection there is evidence that the strong relationship between plasma viraemia and CXCL10 [5, 8–10,18] is likely to be mediated via IFN- α -induced toll-like receptors 7 and 8 [18]. Circulating plasma virions therefore provide the major stimulus for CXCL10 secretion from monocytes and monocyte-derived dendritic cells [18], which in turn inhibits IFN- γ signalling and adaptive immune responses [19]. In this respect, CXCL10 appears to have its most important prognostic role in early HIV infection, where elevated levels independently predict disease progression rate [20] even among HIV-controllers with low levels of viraemia [21], and are also associated with risk of transmitting or acquiring HIV infection [22]. In this setting, the potential utility of CXCL10 measurement in clinical practice is likely to diminish in light of recent evidence that early treatment of HIV infection is beneficial irrespective of baseline CD4⁺ T cell count or plasma viral load [1].

Soluble CD163 provides an interesting contrast in that elevated plasma levels are associated with a range of cardiovascular risk factors (age, HDL cholesterol, body mass index), and perhaps most importantly with the overall Framingham cardiovascular risk score. This is in keeping with a number of studies that have identified associations between sCD163 and cardiovascular inflammation and atherosclerotic plaque formation in the setting of HIV infection [14,23–25] as well as in the general population [23,26,27]. This association appears to be underpinned by the role of activated monocyte-derived macrophages within atherosclerotic vessels [28] as well as in adipose tissue [29,30] in producing sCD163. This pathway involves tumor necrosis factor-alpha (TNF- α) and ADAM-17 [30,31], thus providing a link between inflammation and atherosclerotic risk factors including oxidised lipids that may be particularly relevant in HIV infection [32,33] but are rarely measured in routine care.

We previously found that sCD163 levels were elevated in the setting of untreated HIV infection, while levels among those on suppressive antiretroviral therapy were comparable to healthy controls [8]. Here we extend this observation, noting that sCD163 levels were significantly reduced only among those patients with no detectable plasma HIV-1 RNA, and remained relatively elevated in the 60 cases with residual viraemia (22.1% of results reported as

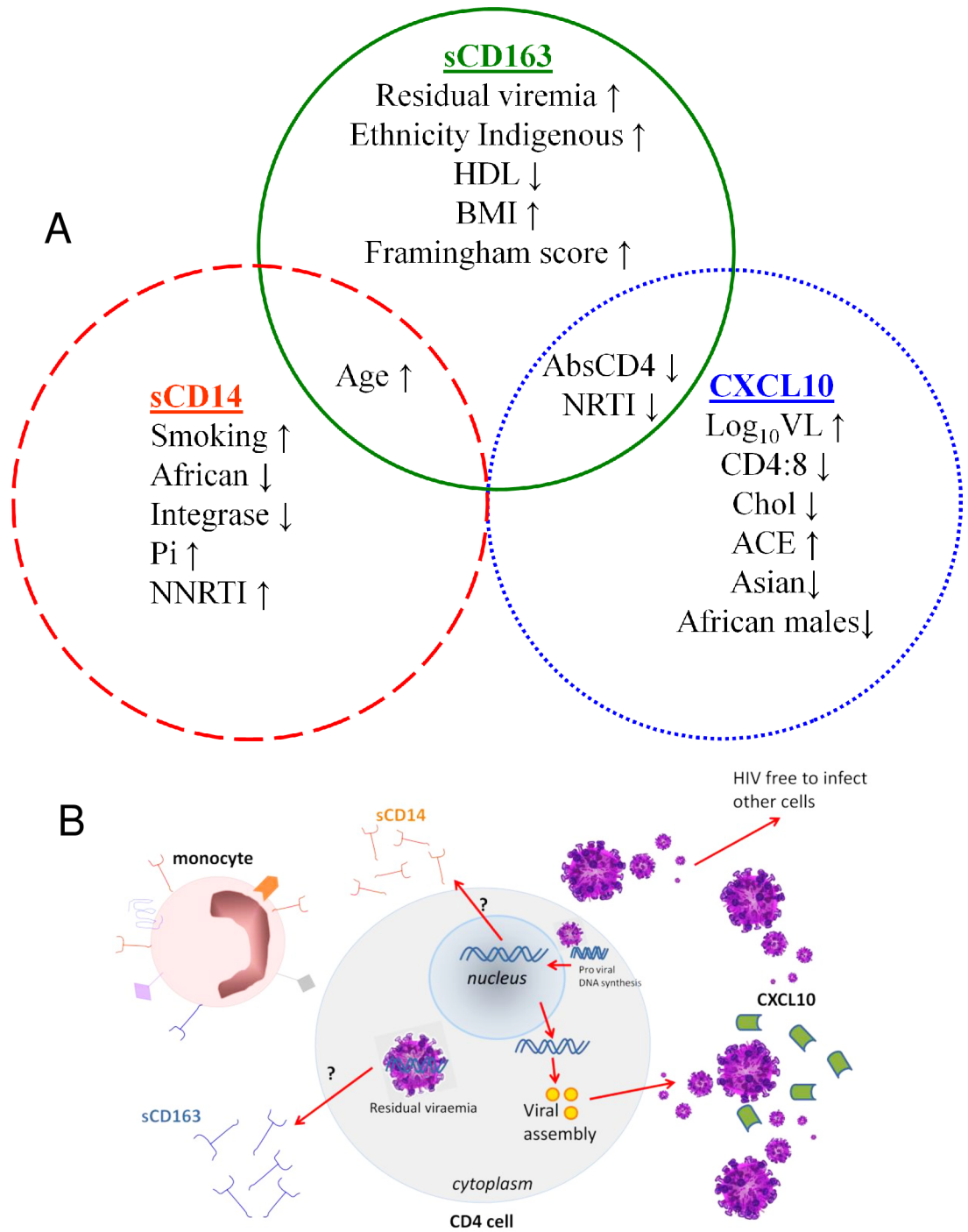


Fig 3. CXCL10, soluble CD14 and soluble CD163 have distinct although overlapping associations with cardiovascular disease risk factors, HIV treatment, ethnicity and age (A) along with different aspects of HIV infection (B).

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<40 cpm). This is consistent with previous studies that have identified the TNF- α pathway as a sensor of low-level viraemia [34,35], although to our knowledge sCD163 has not been previously studied in this context. Given previous evidence that persistent low-level viraemia

originates largely from a reservoir of long-lived, latently-infected CD4⁺ T cells [36,37], it is interesting to note that ADAM-17 and TNF- α (the major stimuli for sCD163 shedding [31]) are implicated in the replication of quiescent CD4⁺ T lymphocytes initiated by exosomes from HIV Nef-expressing cells [38].

These findings suggest that measuring soluble CD163 in routine HIV care has the potential to capture important prognostic information regarding cardiovascular risk [14,23–25] and other co-morbid conditions [39] as well as HIV treatment responses beyond the detection threshold of routine viral load measurements.

The measurement of soluble CD14 in this study population confirmed previous observations by ourselves [8] and others [9,10,40,41] that levels remain elevated irrespective of the level of plasma HIV suppression. In contrast to the broad influence of cardiovascular risk factors on sCD14 levels in the general population [11], we did not observe any influence of individual risk factors or the overall Framingham score—suggesting that HIV infection itself provided an overriding stimulus. We did however observe a significant influence of cigarette smoking, which was not examined specifically in the population-based study but has been identified previously in the setting of HIV infection [42]. Moreover, we identified a favourable effect of integrase inhibitor-based HIV treatment on sCD14 levels in this study, despite the small number of patients receiving this regimen in 2010 ($n = 17$). This effect has also been observed by others [43,44] although not universally [40,45], and at this stage an underlying mechanism has not been identified although an association between sCD14 and integrated HIV DNA has been observed in one study [46]. This warrants further study, along with the impact of smoking, particularly given the established mortality risk associated with elevated sCD14 levels [7] as well as the ongoing strong influence of smoking on mortality among HIV⁺ individuals [47,48].

The major strength of this analysis is the statistical power associated with a large sample pool with complete HIV treatment history and full CVD risk assessments. Limitations include the lack of subclinical measurements of vascular disease, so that we were unable to confirm previous findings of associations between sCD14 or sCD163 and cardiovascular disease or mortality. This study lacked an HIV negative control group, however, we have previously provided evidence of significantly lower sCD14 in an HIV negative population compared to treated and untreated HIV groups, while sCD163 and CXCL10 were reduced to levels seen in HIV uninfected controls when treated with antiretroviral therapy [8].

In summary, this study supports growing evidence that monitoring plasma immune activation markers has prognostic significance throughout the course of HIV management. We provide further evidence that these biomarkers, although linked through common associations with monocyte activation and interferon signalling, reveal distinct aspects of the inflammatory response and indeed of the HIV replication cycle. We found that levels of CXCL10 correlated with routinely monitored variables particularly with plasma viraemia levels, while cell-associated virus appears to be the major stimulus for sCD163 levels, reflecting a deeper level of virological suppression. Levels of sCD163 also appear to capture the influence of a broad range of CVD risk factors, potentially revealing insights into the inflammatory component of cardiovascular disease that may be particularly relevant in the setting of HIV infection. Lastly, while levels of sCD14 are not associated with routinely monitored variables, this study suggests integrated virus is associated with sCD14, and that cessation of smoking along with the use of integrase inhibitor therapy could significantly reduce levels of this monocyte activation marker, in turn potentially decreasing mortality risk during HIV infection. With the ongoing identification of pathways leading towards monocyte activation, targeting these sources (i.e. ADAM17, IFN- α , TNF- α) could lead to useful therapeutic strategies to reduce immune activation, which has been shown to be associated with the development of age-related disease and mortality in a

treated HIV setting. Further research into these mechanisms is warranted, underpinned by the evolution of new HIV monitoring strategies that reflect the underlying chronic inflammatory disease burden and provide insights into pathogenesis and response to treatment.

Supporting Information

S1 Fig. Correlations between plasma biomarkers show strong correlation between sCD163 and CXCL10 (A) and sCD163 and sCD14 (B) while there was no correlation between sCD14 and CXCL10 (C).

(TIF)

S2 Fig. The levels of the different plasma biomarkers correlate with ethnicity. CXCL10 was significantly lower in Asian but higher in Indigenous Australians (A), sCD163 was significantly higher in Indigenous Australians (B) while sCD14 was significantly lower in Africans (C).

(TIF)

S1 Table. Model 1- Multivariate regression results for CXCL10, sCD163 and sCD14 plasma biomarkers showing significant associations with HIV clinical parameters, CVD risk age, gender, ethnicity and smoking.

(DOCX)

Author Contributions

Conceived and designed the experiments: AC LW IJ GG CB DN. Performed the experiments: AC. Analyzed the data: AC IJ DN. Contributed reagents/materials/analysis tools: AC LW IJ GG CB DN. Wrote the paper: AC LW IJ GG CB DN.

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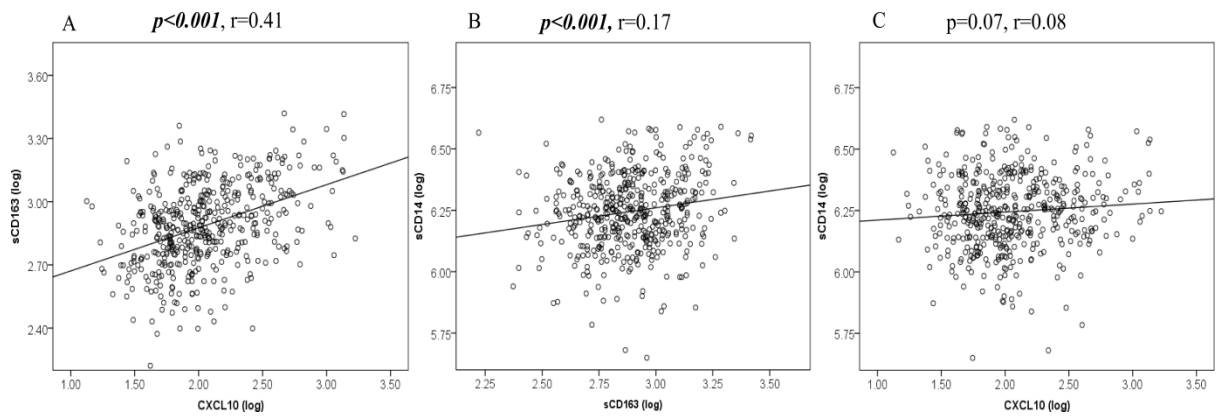
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Chapter 4 supplementary Table and Figures.

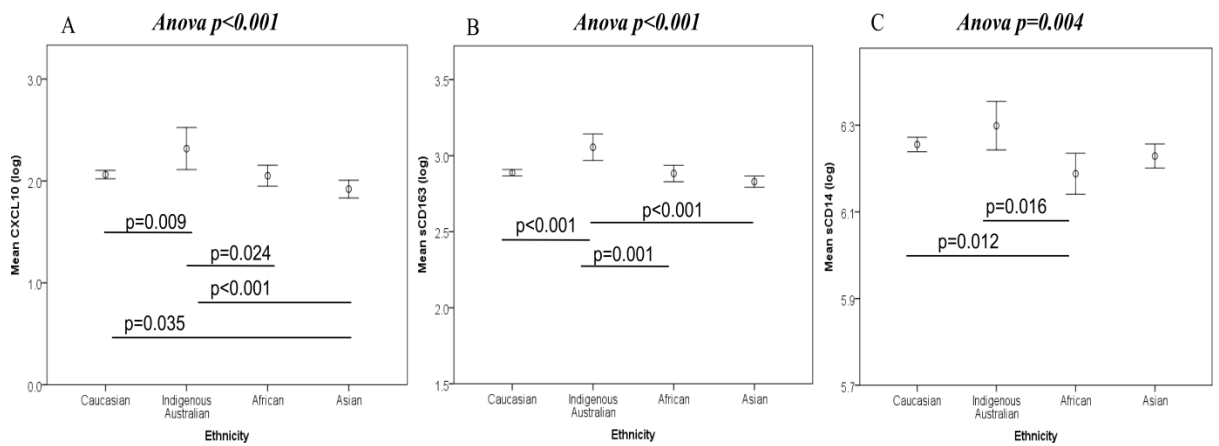
Supplementary Table S1: Model 1- Multivariate regression analyses results. CXCL10, sCD163 and sCD14 plasma biomarkers showed significant associations with HIV clinical parameters, CVD risk age, gender, ethnicity and smoking.

<i>Biomarker</i>	<i>CXCL10</i>			<i>sCD163</i>			<i>sCD14</i>		
<i>Variable</i>	β	std	p	β	std	p	β	std	p
Age at 2010	-	-	-	0.003	0.001	0.001	0.001	0.0006	0.044
Gender	-	-	-	-	-	-	0.035	0.018	0.057
Ethnicity	-0.18	0.047	0.0002	0.116	0.045	0.011	-0.058	0.022	0.009
Gender:Ethnicity	-0.26	0.065	0.0001	-	-	-	-	-	-
Smoking	-	-	-	-	-	-	0.046	0.015	0.002
Residual viraemia	-	-	-	0.062	0.027	0.022	-	-	-
VL3	0.08	0.043	0.056	-	-	-	-	-	-
VL4	0.12	0.063	0.054	0.082	0.037	0.028	-	-	-
VL5	0.29	0.060	<0.0001	-	-	-	0.064	0.022	0.003
SQR CD4:8	-0.22	0.083	0.0085	-	-	-	-	-	-
SQR CD4	-0.01	0.003	0.0001	-0.004	0.001	0.011	-0.002	0.001	0.051
NRTI	-0.18	0.048	0.0002	-0.059	0.028	0.039	-	-	-
NNRTI	-	-	-	-	-	-	0.047	0.018	0.008
PI	-	-	-	0.038	0.019	0.052	0.042	0.018	0.021
Integrase	-	-	-	-	-	-	-0.088	0.038	0.019
Cholesterol (log)	-0.37	0.158	0.02	-	-	-	-	-	-
HDL	-	-	-	-0.049	0.025	0.048	-	-	-
BMI	0.006	0.003	0.078	0.005	0.002	0.009	-	-	-
Framingham score	-	-	-	0.056	0.027	0.038	-	-	-
ACE Inhibitor	0.099	0.049	0.045	-	-	-	-	-	-

CVD= cardiovascular disease; VL= Viral load; residual viraemia= $0 < VL \leq 1.6$; VL3= $1.6 < VL \leq 3$; VL4= $3 < VL \leq 4$; VL5= $4 < VL \leq 6$; SQR= squared; BMI= body mass index; NRTI= nucleoside reverse transcriptase inhibitor; NNRTI= non nucleoside reverse transcriptase inhibitor; PI= protease inhibitor; HDL= high density lipoprotein; ACE= angiotensin converting-enzyme inhibitor.



Supplementary Figure S1: Correlations between plasma biomarkers. Strong correlation between sCD163 and CXCL10 (A) and sCD163 and sCD14 (B) were identified while there was no correlation between sCD14 and CXCL10 (C).



Supplementary Figure S2: Plasma biomarker marker levels correlate with ethnicity. CXCL10 was significantly lower in Asian but higher in Indigenous Australians (A), sCD163 was significantly higher in Indigenous Australians (B) while sCD14 was significantly lower in Africans (C).

Summary for Chapter 4.

Throughout Chapter 4 the aim was to explore potential roles and associations between the plasma biomarkers representing monocyte activation, CXCL10, sCD163 and sCD14 with the choice of HIV-1 treatment regimen, HIV-1 treatment response and cardiovascular disease risk factors during chronic HIV infection in 475 persons within the Royal Perth Hospital HIV cohort. The results reveal distinct and sometimes overlapping associations with different aspects of HIV infection and CVD risk factors to provide an increased understanding on the role these biomarkers have with respect to immune activation.

We have shown that CXCL10, sCD163 and sCD14 have distinct associations with different aspects of HIV infection and treatment, with several novel findings that have potential implications for clinical management and disease monitoring. For example, results show CXCL10 levels correlate strongly with the level of plasma viraemia, while elevated levels of sCD163 levels persist even among those with residual viraemia (i.e. detectable HIV-1 RNA with a viral load <40 copies/mL) but are reduced when there is no detectable HIV-1 RNA. Soluble CD163 levels are also influenced by a range of CVD risk factors, potentially reflecting the intersection of traditional and HIV-specific risk factors for atherosclerosis.

We have also found that sCD14 levels remain elevated despite HIV therapy, with no evidence that virological suppression at any level has a beneficial effect. This highlights a potentially important gap in the current approach to monitoring HIV infection over time, noting that sCD14 levels have been shown to predict overall mortality in the general population as well as those with HIV infection. In this regard, we have demonstrated specific effects of smoking and integrase inhibitor therapy that warrant further study.

The results presented in Chapter 4 builds on a growing level of interest in the role of innate immune activation during long-term HIV management, with strong evidence that deleterious systemic inflammation persists despite successful virological suppression and CD4⁺ T cell recovery. Looking into the future, incorporating biomarkers into routine care can now be seen as a powerful tool to optimise clinical practice and improve individualised treatment and patient care, without the need for extra patient bleeds as the biomarkers can be tested from the same plasma sample as the HIV-1 RNA test or HIV-1 genotyping assay.

A collective summary for Chapters 2, 3 and 4.

The main conclusions derived from the observations presented in Chapters 2, 3 and 4 include:

1) The routine method used at RPH for detecting plasma HIV-1 RNA (Cobas Ampliprep/Cobas TaqMan HIV-1 test) performed well at amplifying and identifying residual viraemia in previously reported “undetectable” (<40cpm) patients. Remarkably, 80% of HIV patients in the RPH cohort had plasma HIV-1 RNA levels <40 copies/mL and in 20% of these, residual viraemia was detectable below this threshold. This residual viraemia was strongly associated with pre-treatment HIV-1 RNA levels, suggesting earlier treatment based primarily on the level of viraemia may be beneficial.

2) The distribution of monocyte subsets is altered with HIV infection with an increased proportion of CD16⁺ monocytes (inflammatory and patrolling monocytes) that are not normalised with HAART.

3) The large HIV/CVD cohort studied in Chapter 4 showed high levels of CXCL10 in untreated HIV infection, with strong correlations between plasma HIV-1 RNA levels and CXCL10 levels suggesting it is the circulating HIV-1 virus that stimulates the production of CXCL10 potentially through pDC and monocytes by directly stimulating TLR pathways (TLR7/8). In contrast, we noted that sCD163 levels were only influenced by HIV treatment when there was no detectable HIV-1 RNA (ie. beyond the reportable assay threshold of 40 cpm). The level of sCD163 detected was also associated with CVD risk factors including an overall Framingham risk score, suggesting that this biomarker may reflect the inflammatory component of atherosclerosis risk in this population. Finally, we found that sCD14 levels remain significantly elevated in the setting of HIV infection, irrespective of HIV-1 RNA levels, although our results and others suggest that integrase inhibitor therapy may be associated with beneficial effects on sCD14 (though not restoring them to normal levels). Smoking was also found to have a negative impact on circulating sCD14 levels, which is a noteworthy observation given the high prevalence of smoking among patients with HIV infection [114] and its impact on mortality in this setting [115].

These results can inform further research aimed at understanding mechanisms driving immune activation, inflammation and residual viraemia, along with elucidating the

biological roles of these biomarkers. These issues will be explored briefly below, to highlight possible future pathways to study.

4.1. Impact plasma biomarkers have on HIV replication, associations with HIV treatment choice and response to HAART

An overall representation of the effect HIV-1 replication has on plasma biomarker levels, pre and post HAART, is represented in Figure 1.

The interferon inducible protein-10 (CXCL10) was shown to strongly correlate with HIV-1 RNA levels, as treatment with HAART reduced circulating levels of CXCL10 to levels comparable with healthy controls. Plasma HIV-1 particles are therefore a major stimulus for CXCL10 secretion from monocytes and pDC [95], which in turn inhibits IFN- γ signalling, thus regulating overall immune responses including the inhibition of the adaptive immune responses [116]. The pathways involved in HIV-1 induced CXCL10 production include signalling through the TLR7/8 pathway, as it has been previously shown that blocking TLR7/9 effectively blocks CXCL10 production [95]. These toll-like receptors are expressed intracellularly on endosomes in response to CpGDNA (TLR9) or ssRNA (TLR7). Both TLR7/8 and TLR9 trigger MyD88 dependant immune activation of monocytes and pDCs [117] by promoting IFN- α production.

Once HAART is administered and maintained, HIV viral replication is diminished along with CXCL10 levels, CXCL10 functions by binding to CXCR3 (receptor 3) to activate the GPCR and MAPK pathways via adenylated cyclases. This leads to a number of effects including chemokine activity, actin reorganisation which enables effective antiviral response, cell migration and immunity and inflammation. Both immune cells and non immune cells produce CXCL10 in response to inflammation [118, 119] helping recruit effector cells (monocytes, T lymphocytes and NK cells) to the site of inflammation to promote antiviral responses [120]. During early HIV infection CXCL10 levels were shown to be associated with rapid disease progression [121] and it has been shown that HIV-1 Tat can synergise with IFN- γ to increase the production of CXCL10 in monocytes via the MAPK pathway [122]. This could be important when exploring the relationship between immune activation and risk of HIV-1 transmission among serodiscordant couples. Mechanisms identified during early HIV-1 infection may explain why HIV-1 transmission was significantly associated with elevated CXCL10 levels in HIV-1 seroconverting partners [123],

suggesting it may have a role in viral entry [124]. Additionally, a study on CXCL10 levels in the genital mucosa of high risk seronegative women showed remarkably low levels of CXCL10, potentially promoting protection [125]. For these reasons, exploring pathways and mechanisms relating to the HIV-1 induced CXCL10 requires further investigation.

In chapter 3 we identified increased sCD163 levels in untreated HIV patients and decreased levels, similar to those seen in healthy controls, in a treated HIV setting. In Chapter 4 we have added to this outcome by comparing patients with an “aviraemic” status with patients who had detectable HIV-1 RNA below the reported assay threshold of 40 copies/mL (residual viraemia), and to those with HIV-1 RNA >40 copies/mL. Our results showed sCD163 was significantly reduced among aviraemic patients only and remained elevated in patients with residual viraemia. Surprisingly, sCD14 levels, which are associated with increased risk of mortality, remained elevated regardless of the level of HIV suppression, but were significantly reduced among patients receiving integrase inhibitor treatment. The mechanism for this has yet to be determined although, as suggested in Chapter 4 and in Figure 1, an association between sCD14 and integrated HIV DNA has been reported [126] along with increased levels of 2-LTR episomes as discussed below, all of which warrant future investigations.

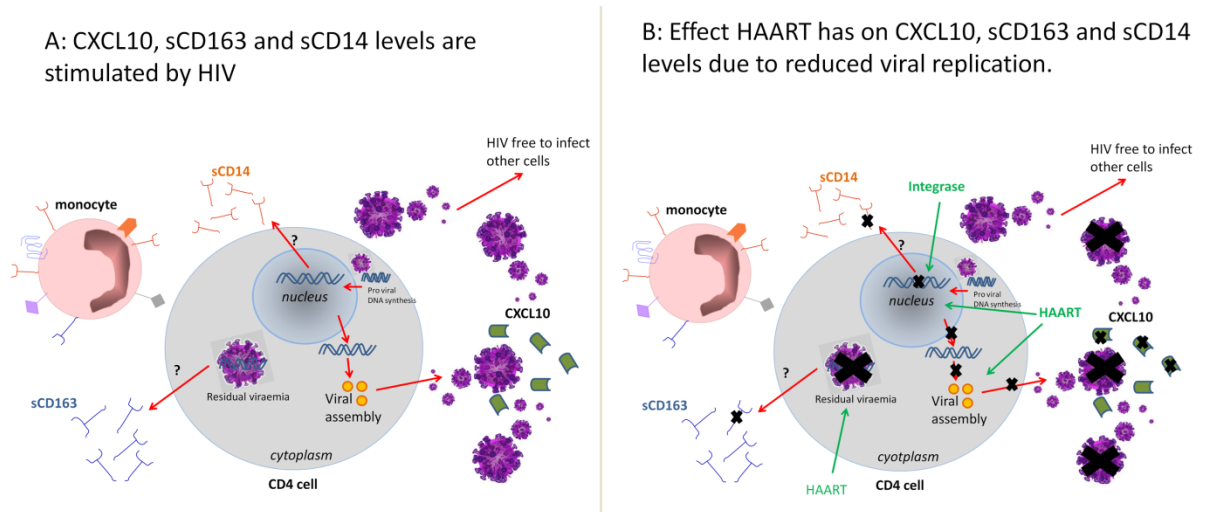


Figure 1: The effect HIV-1 and HAART has on plasma biomarkers. CXCL10, sCD163 and sCD14 were elevated in the presence of untreated HIV infection (A) however under HAART, 3 levels of effects were observed at different stages of HIV-1 replication. CXCL10 and sCD163 are reduced while only integrase inhibitors reduced sCD14 but not to normal levels.

4.2 Implications of persistent residual viraemia

With the introduction of HAART many HIV patients have successfully controlled HIV-1 viraemia to below the detection level of routine diagnostic methods. Despite this success some patients have persistent HIV-1 residual or low level viraemia and the relationship between residual viraemia and immune activation requires ongoing investigation. It is known that the duration of HAART or some treatment intensification drugs do not significantly reduce or alter residual viraemia levels [104, 105]. Detectable residual viraemia has been linked to increased risk of poor virological control [106, 107, 127] and potentially predicts viral blips [108] that have been associated with immune activation [109]. Residual viraemia is increased in poor immunological responders, correlating with the activation of CD4⁺ and CD8⁺ T cells potentially reflecting persistent immune dysfunction [110]. The relationship between residual viraemia and latently infected cells has been investigated with conflicting results. Genetic similarities between reactivated latent HIV-1 compared to residual viraemia during ART have been observed [128] while other studies show genetic differences between residual viraemia sequences and the heterogeneous forms of reactivated virus [129,130] leading to suggestions that residual viraemia does not commonly originate from CD4⁺ T cells. To support this, monocytes have also been identified as a source of residual viraemia [110] confirming there are at least two cellular sources of residual viraemia. The results presented in this dissertation indicate that residual viraemia was associated with increased levels of sCD163 (monocyte activation), although at this stage it is unknown if latently-infected monocytes and/or T lymphocytes provide the stimulus for sCD163 secretion. As discussed below and shown in Figure 2, mechanisms have been described that provide plausible links between cell-associated HIV proteins (Nef in particular) and the release of sCD163 from its membrane-bound precursor.

4.3 Cardiovascular disease risk factors and associations with plasma biomarkers

The biomarker assessments outlined in Chapter 4 resulted in very interesting associations with CVD risk factors, including the most striking association between sCD163 and Framingham score as well as age, high density lipoprotein, cholesterol and body mass index. Together the results suggest a role for inflammatory pathways

associated with atherosclerotic plaque formation seen both in the HIV [68, 92, 131] and healthy populations [92,132,133]. The associations appear to be related to the role that activated monocyte-derived macrophages play within the atherosclerotic vessels in producing sCD163 [134]. The link between inflammation and atherosclerotic risk factors may lie with pathways involving TNF alpha and a disintegrin and metalloproteinase (ADAM17). A possible scenario is presented in Figure 2 showing the potential role Nef particles play in stimulating and activating ADAM17 to cleave membrane bound CD163 to free soluble CD163 [135]. This occurs through the co-expression of the Nef signaling block (A) that activates Erk1/2. Erk1/2 is the pivotal kinase activating ADAM17 (B), and it also requires the interaction of ADAM17 with Eed and paxillin (C). Together the complete “Nef signalling complex” of Nef/Eed/paxillin leads to the activation of ADAM17 (D). Activated ADAM17 cleaves membrane bound CD163 at the “ARG-SER-SER-SER-ARG” site to release it as soluble CD163 (E) [136]. The “Nef signaling complex” can also induce the secretion of ADAM17 via extracellular vesicles (F) and also activates to release TNF. This process requires activated ADAM17 to cleave pro-TNF to release mature TNF (G). The mature TNF binds to the TNF receptors to promote the reactivation of HIV-1 and also induces CXCL10 production via the NFkB activation pathway (H).

Monocyte ADAM17 facilitates endothelial migration by accelerating the rate of diapedesis [137] by cleaving Mac-1 therefore regulatory mechanisms of diapedesis, may be important for the development of therapy to decrease CVD risk and for atherosclerosis; all of which warrants further investigation.

Another potential key driver of immune activation in HIV, particularly with respect to CVD risk factors, is coinfection with CMV. In an HIV setting CMV infection has been shown to be associated with higher CD8 T-cell counts, lower CD4/CD8 ratio and increased inflammation [138] while a recent study indicated CMV/HIV coinfection (based on CMV IgG positivity at baseline) was associated with increased risk of SNAEs particularly cardiovascular events [139]. These results are interesting though the exact mechanisms were not identified and the plasma biomarkers sCD14 and sCD163 were not assessed.

4.4 Clinical relevance of plasma biomarkers and monocyte subsets during HIV infection

While the primary concern in Chapter 3 was to evaluate methods relevant to monocyte activation during HIV infection and treatment by focussing on flow cytometry and ELISA-based methods, the focus for Chapter 4 involved identifying the role plasma biomarkers play in CVD disease risk in an HIV setting. The results from both chapters have helped further define the potential clinical relevance and prognostic role of each biomarker in routine laboratory practice, and the potential value of these assays in identifying those who may benefit from alternative treatment strategies and monitoring the treatment response. These analytes are currently "invisible" in existing routine laboratory practices and HIV management, but may assume increasing importance in the current era of HIV management where patients are likely to experience age related illnesses, even in the presence of successful antiretroviral treatment.

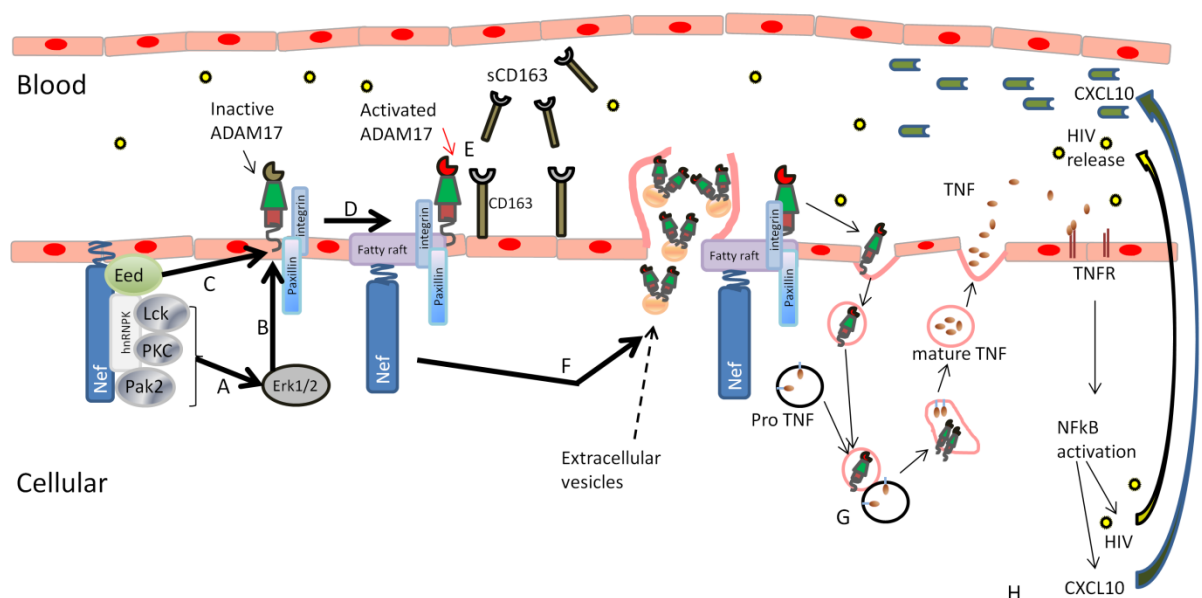


Figure 2: The potential role Nef particles play in stimulating and activating ADAM17. Nef particles activates ADAM17 to 1) cleave membrane bound CD163 to its free soluble CD163 form; and 2) release TNF, which promotes the reactivation of HIV and the release of CXCL10. Adapted from Lee and Etzerodt [135, 136].

The results presented here indicate that CXCL10 is useful in predicting HIV disease progression rate (due to strong positive correlations with HIV-1 RNA levels [140]) however implementing CXCL10 assays into clinical practice may provide little additional clinical value particularly in light of recent studies indicating early treatment of HIV is beneficial regardless of HIV-1 RNA levels or CD4⁺ T cell counts [141]. In a non-HIV setting CXCL10 levels have been shown to be increased in patients with coronary artery disease (CAD) when compared to control cases, although in keeping with the results presented in Chapter 4, CXCL10 did not however have predictive power for CAD risk [142, 143].

There is, however, the potential to include sCD163 into routine monitoring as it captures important information pertaining to 1) treatment responses beyond those that are currently reported in laboratory practices, 2) cardiovascular disease risk within the HIV setting and 3) the potential to capture prognostic information relating to other comorbidities [144].

The inflammatory marker, sCD14, can help predict mortality even after accounting for HIV-1 RNA levels [28] therefore monitoring systemic immune activation, particularly sCD14 levels, during HIV management could offer important prognostic information that could ultimately improve standard of care and improve treatment strategies beyond the current paradigm of achieving undetectable plasma HIV-1 RNA levels and normal CD4⁺ T cell counts – noting that both of these outcomes have little influence on sCD14 levels. Finally, the elevated level of CD16⁺ monocytes (intermediate and non-classical monocyte) regardless of HIV-1 RNA levels is worth monitoring routinely especially as effective HIV therapy has brought in to question the prognostic value of regular (quarterly) CD4⁺ T cell count monitoring once HIV-1 RNA is successfully suppressed to <40cpm [101]. Activation of CD16⁺ monocytes may be important mediators and markers of CVD risk [86], neurocognitive disorders [145] and breast cancers [146]. Monitoring monocyte subsets during clinical practice could therefore be important in identifying patients with high inflammatory responses and immune activation and further clarify the role monocytes play in disease progression though more work is required in this area particularly in larger cohorts.

Additional laboratory tests could be employed to assist in patient care by monitoring ongoing HIV viral replication. In HAART patients, linear HIV-1 cDNA integrates into host DNA which promotes HIV replication. This coincides with increased levels of immune activation. With raltegravir intensification however, particularly for those

on a protease inhibitor, linear HIV-1 cDNA integration reduces, unintegrated episomal cDNA levels (2-LTR circles) increase, and markers of immune activation are normalised [147]. These results suggest active replication exists and this could reflect the overall burden of cell associated HIV during HAART which drives immune activation. Quantitative PCR methods could be utilised in a laboratory setting to measure 2-LTR circles to monitor HAART efficacy and to detect the level of ongoing replication (Figure 3).

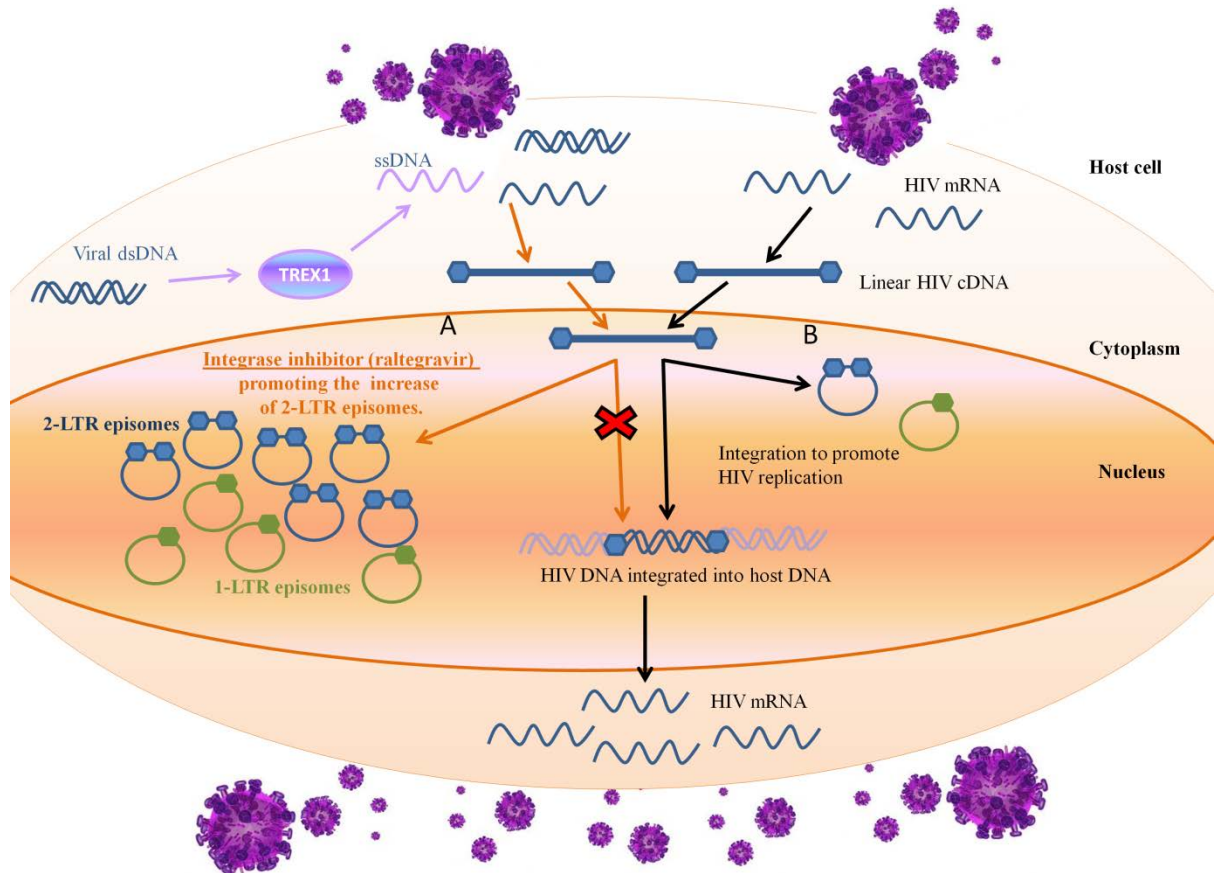


Figure 3: The impact of raltegravir intensification on HIV-1 2-LTR circles in HAART-suppressed subjects. Raltegravir blocks integration to promote episome formation by blocking the integration of linear viral cDNA (A), and subsequently, the DNA is circularized by host DNA repair enzymes to form episomes containing two copies of the viral long terminal repeat (2-LTR circles) or undergoes recombination to form a 1-LTR circle. Therefore, an increase in episomal cDNA occurs when active replication is inhibited by raltegravir. A lower level of 2LTR is present when HIV DNA integrated into the hosts DNA (B). *Adapted from Buzon M [147].*

An overall representation of HIV pathogenesis and its relationship with plasma biomarker levels while on HAART is shown in Figure 4. As described in the introduction to this dissertation, monocyte activation is established in the early phase of HIV infection (innate immune response) and is depicted by the increase in plasma biomarkers including sCD163, CXCL10 and sCD14 and an increase in CD16⁺ monocytes. We have shown that by reducing plasma HIV-1 RNA levels with HAART we can reliably reduce CXCL10 levels, and in the case of sCD163 levels this beneficial effect requires a deeper level of virological suppression without detectable residual viraemia. In contrast, sCD14 levels remain unaffected by virological suppression, although preliminary evidence of the beneficial effects of integrase inhibitor treatment suggest that HIV integration may be an important factor. Taken together these research findings indicate that reducing immune dysfunction during chronic HIV infection may have a role in limiting age-related illnesses during HIV infection, and that monitoring monocyte-derived inflammatory markers is feasible and should be considered in routine management.

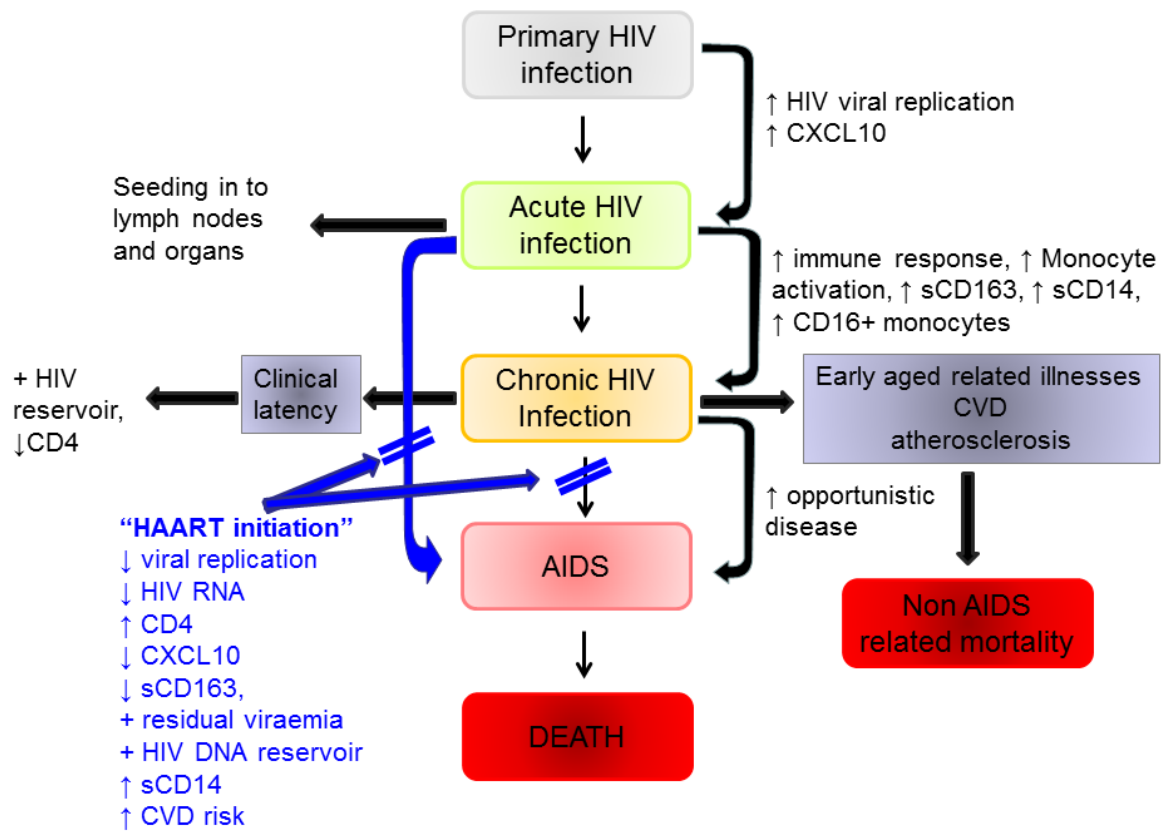


Figure 4: HIV pathogenesis is altered by antiretroviral therapy, as are plasma biomarkers and monocyte activation associated with immune activation.

4.5 Type 1, 2 and 3 Interferon responses during HIV infection

Interferons (IFN) are regulators of the innate immune responses induced to provide protection against early HIV infection as well as offering additional immune responses during acute and chronic immune activation. There are three types of IFNs including human Type I IFNs (IFN- α has 13 known subgroups, IFN- β , IFN- ω , IFN- κ , IFN- ϵ) Type II (IFN- γ) and Type III (IFN- λ). Most cells within the human body can produce IFN however pDCs are powerful producers of Type I and III IFNs in response to DNA or RNA viruses [148]. Type III IFNs primarily act on epithelial cells and prevent viral invasion through mucosal areas [149]. The primary producers of IFN- γ are CD4⁺ and CD8⁺ T cells along with NK cells which provide antiviral responses to HIV and to other pathogens.

The induction of IFNs in combating HIV infection involves many pathways as shown in Figure 5. Adaptors (TRIF, MyD88, MAVS and STING), kinases (TBK1, IKKs) and transcription factors (IRF3, IRF7), along with NF κ B, bind to IFN promoters to facilitate the transcription of IFN genes and the process is dependant on the level of HIV-1 proviral DNA synthesis present. Once produced, IFNs act on cells by binding to cell surface receptors (Type 1-IFNAR1 and IFNAR2; Type II- IFNGR1 and IFNGR2 and Type III (IFN- λ 3- IFNLR1 and IL10R2). The binding of IFNs to the receptors triggers intracellular signalling pathways leading to the transcription of IFN stimulated genes (ISGs; Figure 6). There are over 2,000 ISGs, many of which are responsible for the antiviral control and immunomodulation during infection. Some of the antiviral restriction factors induced by IFNs include APOBEC3G/3F, SAMHD1, tetherin and TRIM5 (Table 1) however HIV can counteract many aspects of the IFN response including inhibiting IRF and NF κ B functions and inhibiting antiviral restriction factors (i.e. APOBEC3G, SAMHD1 and tetherin) through the action of HIV accessory proteins Vif [150], Vpx [151] and Vpu [152] respectively. HIV can also block IFN induction in DC and macrophages by the dysregulation of TANK-binding kinase 1 (TBK1). Here, HIV-1 accessory proteins, Vpr and Vif, blocks the IFN induced signalling pathway by inhibiting phosphorylation of TBK1 [153].

It is interesting to note that some high risk cohorts, including sex workers, MSM and discordant couples, remain HIV free potentially due to the significantly higher level of APOBEC3G shown in PBMCs and cervical tissue of HIV-exposed seronegative groups [154, 155] suggesting potent restriction factors could offer a barrier against

HIV infection during early exposure. Another potential barrier against HIV infection is the inhibition of the hosts' cytoplasmic exonuclease TREX1. When TREX1 is inhibited, HIV infection of cells triggers Type I IFN expression and secretion via the STING/TBK1/IRF3 dependent pathway [156] (Figure 5). Avoiding this process promotes HIV integration into host DNA and ultimately HIV replication. For this reason the inhibition of TREX1 during early HIV infection may offer an important immune based therapy, however improved understanding of how TREX1 pathways function is required, especially since the accumulation of DNA due to TREX1 mutations has shown to be associated with systemic lupus erythematosus (SLE) [157].

Table 1: The antiviral capability of important host restriction factors during HIV infection.

Antiviral restriction factor	Antiviral capability
APOBEC3G	Restricts HIV replication by editing C to U nucleotides in HIV DNA, introducing stop codons, inhibiting reverse transcriptase and therefore integration
TRIM5	Blocks the un-coating of invading HIV virions
Tetherin	Blocks the release of HIV enveloped virus
SAMHD1	Inhibits replication in myloid cells

It is reasonable to envisage there may be competition between recognising and masking HIV-1 cDNA by cytoplasmic receptors and cellular factors respectively and TREX1 function to clear HIV [158]. An interesting line of future studies would be the analysis of TREX1 gene sequences between high risk-uninfected subjects and HIV-infected subjects, looking for sequence differences associated with HIV infection, DNA integration and residual viraemia.

During acute HIV infection a cytokine storm erupts, including the rapid release of IFN- α from pDCs and a slow release of IFN- γ from T cells and NK cells. This early induction of IFNs is thought to aid in HIV control however, it could potentially be detrimental as the increased immune activation may lead to the influx of monocytes/macrophages and T cells providing an environment to promote HIV

replication which coincides with the increase in HIV-1 RNA levels [159, 160] thus highlighting both protective and infection-promoting mechanisms that involve IFN signalling. High levels of IFNs have been noted once HIV replication has been established and throughout disease progression, which may lead to damaging effects during ongoing HIV infection, therefore striking a balance between the role IFNs play during early and late HIV infection requires ongoing research.

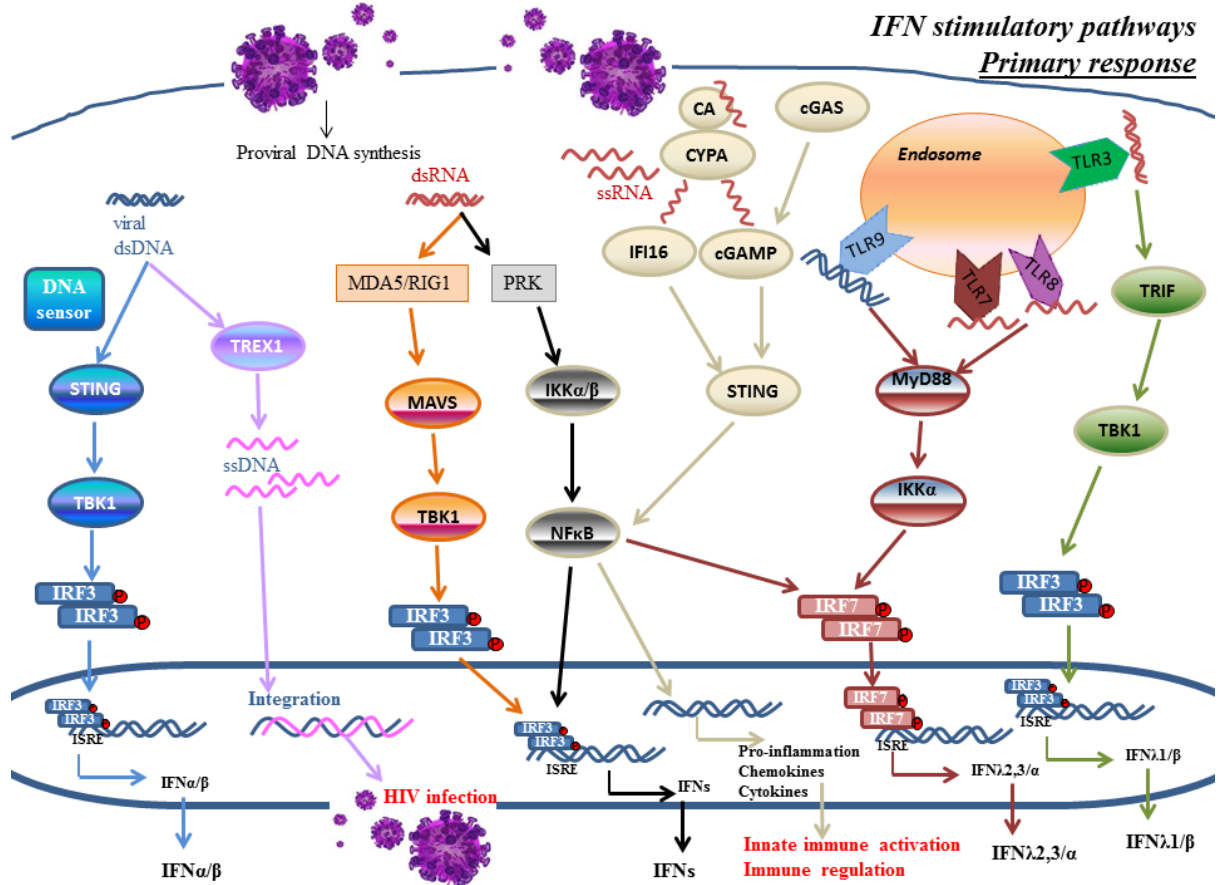


Figure 5: HIV infection can induce Type I, II and III IFNs. These processes are facilitated through alternative pathways and depend on the level of proviral DNA synthesis.

In summary, the complex relationship between HIV sensing by the innate immune system, through the induction of antiviral response factors and immune surveillance factors, that provide an antiviral environment and the rapidly mutating capabilities of HIV to promote viral invasion [158] requires further understanding to help guide therapeutic strategies.

4.6 Therapeutic strategies to reduce systemic immune activation

A significant barrier faced when searching for ways to improve the long term outcome for people living with HIV is the persistence of immune dysfunction that drives cellular activation while on effective HAART. It is well established that inflammation and immune activation exist during HIV infection, whether it is early in the acute phase of HIV infection, in untreated or treated settings when HIV-1 RNA levels are below the detectable limits of commercial assays, in long term non-progressors or in elite controllers [161]. In all cases inflammation and immune activation does not always normalise with HAART and studies have shown a direct link with mortality and morbidity during HIV infection [26, 28].

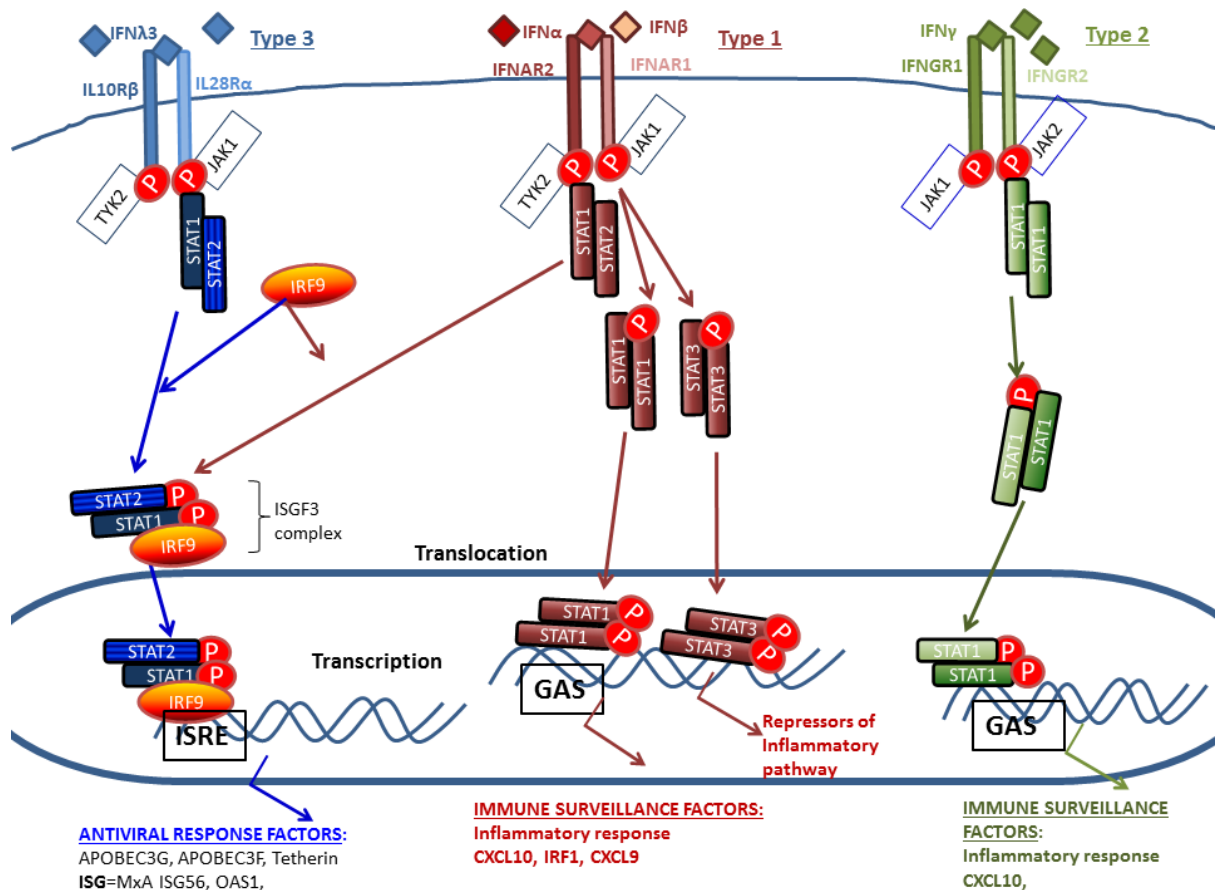


Figure 6: The secondary response pathways induced from Type I, II and III IFNs. The antiviral responses and immune surveillance factors are dependant on the type of IFN stimulation.

Although challenging, treatment with HAART in the first 10-20 days after HIV-1 transmission tends to reverse mucosal and systemic inflammation [162, 163]. As this is challenging, targeting the mediators of immune activation could be pursued as an alternative strategy to reduce immune activation and mortality within the HIV cohort. As described in the introduction, microbial translocation plays a role in establishing HIV infection during early exposure. A recent study focused on the effect of a polyherbal formulation had on immune activation [164]. Although LPS levels were not altered, the sCD14 levels were significantly reduced indicating sCD14 as a marker of monocyte activation that is not restricted to activation by LPS. A number of alternative studies investigated the supplementation of HAART with probiotics. The results showed some reduction in immune activation and inflammation [165, 166]. The “PROOV IT” trial is ongoing however it investigates the use of probiotics to reduce inflammation and enhance gut health in an HIV setting [167]. Although immune activation has shown to be reduced in these circumstances the exact mechanisms are yet to be identified and therefore require further investigation.

Several other studies have delved into defining mechanisms and drugs to target and reduce immune activation. Determining outcomes for patients already on HAART and who undergo treatment intensification with the integrase inhibitor raltegravir or with maraviroc have been studied [168, 169]. Intensification with raltegravir showed a decrease in T cell activation and HIV viraemia however in this study immune activation persisted [168]. This suggests low level viraemia may be the key player in promoting immune dysfunction (immune activation) in T cells potentially triggering the activation of monocytes and pDCs. Residual viraemia has also been highlighted as a possible barrier to producing an effective HIV cure [170]. The intensification of HIV treatment with maraviroc (a CCR5 antagonist) in a small group of patients experiencing HIV associated neurocognitive disorders (HAND) showed promising results particularly with a significant decrease in the proportion of circulating intermediate monocyte (inflammatory) and non-classical monocyte (patrolling) subsets, a reduction in monocyte HIV DNA level and significant reduction in sCD163 levels while improving neuropsychological performance [169]. The results presented in Chapters 3 and 4, showed a decrease in sCD14 (a monocyte activation marker) when an integrase inhibitor was used, indicating immune activation pathways may be blocked when inhibiting integration of HIV viral ssDNA into the host genome, while sCD163 was significantly associated with residual viraemia and CVD risk factors

therefore providing a potential basis for examining the effects of maraviroc intensification therapy.

The use of aspirin has been studied to determine its effect on immune activation and inflammation [171]. This study found low dose aspirin may be a potential intervention for the HIV-infected population as it reduces platelet/endothelial activation (by decreasing p-Selectin, in-turn minimising activation of integrins - which is required for ADAM17 activation), immune activation and inflammation. One week of aspirin treatment successfully enhanced TNF- α levels released from monocytes while decreasing but not normalising activated CD4⁺ and CD8⁺ T cells along with sCD14, IL-6, d-dimer and hsCRP levels; however only CD8 T cells reached levels observed in uninfected controls. Interestingly activated platelets secreting p-Selectin also activate monocytes and DCs to produce inflammatory cytokines and chemokines [172]. Overall, aspirin may have a place in effectively reducing immune activation and inflammation, although further studies are needed on a large scale to expand on potential mechanisms and pathways.

The role played by IFNs during early HIV infection also offers an interesting insight into HIV protection particularly given high risk groups exposed to HIV produce high levels of IFNs to avoid infection [148, 149]. IFN treatment (IFN- α 2b) has been used in acutely infected HIV patients to stimulate a primary anti-HIV antibody response [173] while IFN inhibitors have been tried in chronic HIV patients. An asymptomatic HIV study group was vaccinated against IFN- α and showed reduced levels of circulating IFN- α and HIV events. Another study looked at ways to reduce immune activation and IFN- α level in HIV chronically infected patients by using chloroquine, an endosomal TLR inhibitor [174, 175]. In vitro studies with chloroquine demonstrated reduced T cell activation in HIV untreated patients [176] while in ART treated patients with CD4⁺ T cells <200 copies/ml chloroquine decreased immune activation by reducing TLR4 expression on CD14 monocytes, reducing activated CD4⁺ T cells and inflammatory responses along with a reduction in IFN- α producing pDCs [177]. Interestingly the effect of chloroquine in an alternative study, in HAART naive patients with CD4⁺ T cell >400 copies/ml, showed T cell activation did not reduce, T cell numbers depleted while HIV viral replication increased [178] indicating that during early HIV infection, inhibiting IFN responses provides a platform for HIV establishment. Another strategy looked to suppress HIV replication through the use of pegylated (peg) interferon alfa-2a [179]. Despite the small number of virologically

suppressed subjects on HAART with CD4⁺ T cells counts >450 copies/ml, results suggest, after the discontinuation of HAART, a reduction in viral replication and levels of integrated DNA was observed, although residual viraemia levels increased. Further work is therefore required on larger cohorts especially given the side effects observed in some patients. Finally, the use of Type III IFN- λ 3 before and during HIV infection has revealed strong antiviral effects by inducing the TLR3 pathway along with adaptors MyD88 and TRIF resulting in increased antiviral factors decreasing viral replication [180] (Figure 7). Although IFN- λ 3 is broadly expressed on many cell types, the expression pattern of IFN- λ 3 receptors is more cell specific [181] therefore, when used as a therapeutic option, it may have fewer side effects over other IFNs hence making it more attractive to use.

A recent study has investigated the effects of combined IL-21 treatment (which promotes maintenance and functionality of Th17 cells critical for mucosal immunity) with ART compared with ART only, looking at both inflammatory markers and viral persistence in SIV infected macaques. Those treated with IL-21 and ART showed improved restoration of intestinal Th17 and Th22 cells, reduction in immune activation in the blood and mucosa along with reduced SIV RNA in plasma and CD4⁺ T cells harbouring replication-competent virus [182]. This study highlights the link between immune activation and residual viraemia and shows an effective way of reducing both and maintaining levels after ART is halted, however it did not examine the effect on monocyte activation. Future exploration in humans could provide a key in utilising IL-21 as an immune based therapy.

Overall, the challenges faced in future immune based therapy to treat HIV infection is to tip the balance in favour of optimising the beneficial antiviral IFN effect while minimising HIV-inducing effects of IFN that promote replication, which may involve different treatment approaches for different stages of HIV disease. High risk patients may benefit from slow release pre exposure IFN therapy while inhibiting IFN during chronic infection may be beneficial, however both scenarios require ongoing research efforts, particularly to avoid side effects associated with IFN therapy. Including therapy to reduce platelet and endothelial activation could also hold benefit in reducing immune activation and inflammation, along with consideration of ART intensification.

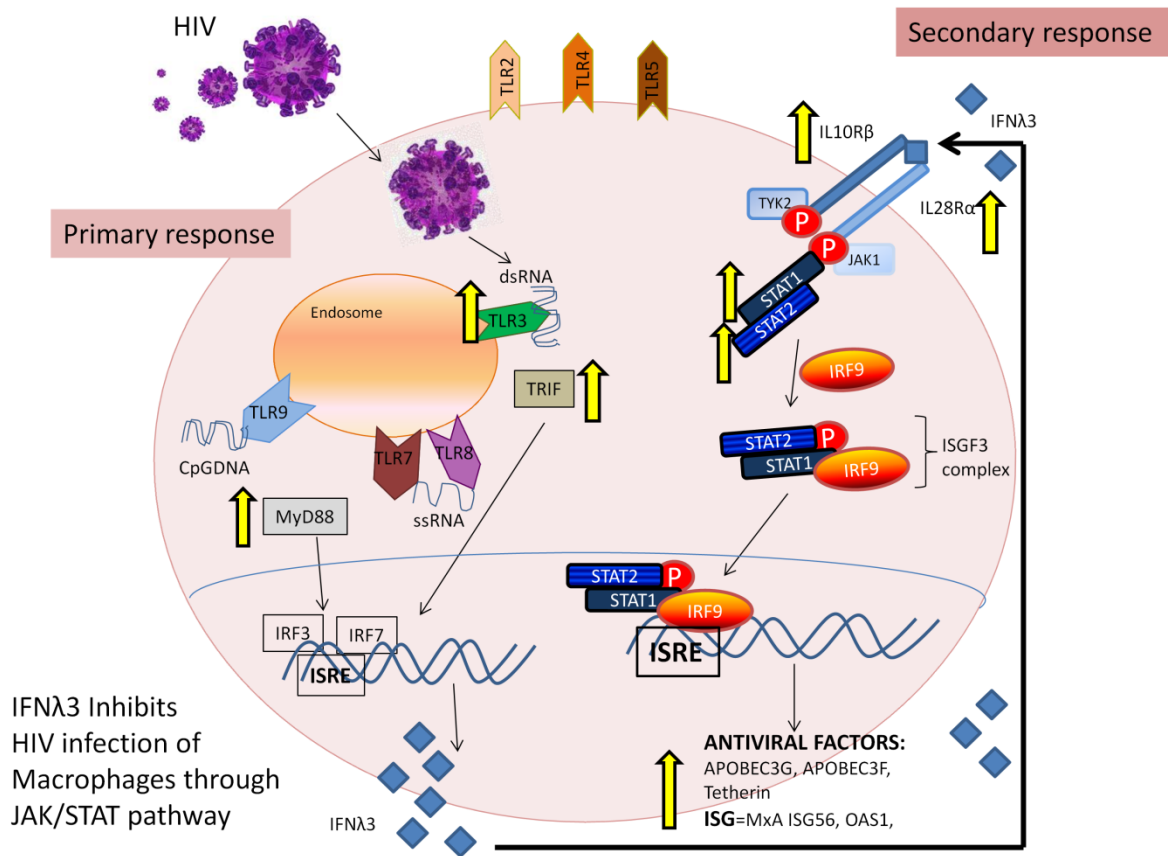


Figure 7: IFN- λ 3 inhibits HIV-1 infection of macrophages through JAK/STAT pathway. Primary and secondary response pathways show: upregulation of TLR3, TRIF and MyD88 adaptors, IRF7 along with STAT1 and STAT2 resulting in the upregulation of antiviral factors (indicated by yellow arrows). IFN- λ 3 receptors are expressed on only a few cell types making IFN- λ 3 a good candidate for therapy with less side effects and inhibiting HIV replication.

4.7 Importance of early treatment to reduce immune activation

It is now well established that HIV infection (treated and untreated) is associated with chronic inflammation and immune activation. Activated monocyte/macrophages (soluble CD14) and high levels of a number of pro-inflammatory cytokines (IL-6, CRP) are hallmarks associated with chronic immune activation. Effective HAART decreases levels of most of these biomarkers, however levels in many patients on HAART remain higher than those observed in age-matched uninfected adults [26, 28]. Chronic inflammation during both untreated and treated disease is strongly associated with risk of non-AIDS defining morbidity and all-cause mortality [26, 28].

HIV replication contributes to this inflammatory state, therefore earlier use of ART to lessen this process may be beneficial.

The results reported from a two armed randomised study involving untreated HIV adults with a CD4⁺ count of >500 cells/ μ l highlight the importance of immediate initiation of ART. A higher proportion of patients achieved HIV-1 RNA levels <200 copies/ml and quicker recovery of CD4⁺ T cell counts when immediate ART was administered upon diagnosis when compared to deferred ART arm, as would be expected, although the clinical benefits of HIV treatment in terms of morbidity and mortality were not associated with these parameters [141]. In the SMART study, the risk of cardiovascular events was greater in participants randomized to the treatment interruption arm (based on CD4⁺ T cell counts) than for patients who received continuous ART [65]. Treatment initiation also resulted in improvements in biomarkers associated with CVD, including markers of immune dysfunction (monocyte activation [sCD14 and sCD163]), T cell activation, T cell senescence), inflammation (IL-6, CRP), hyper-coagulation (D-dimers) and endothelial dysfunction. Supporting the recommendation for patients to immediately start ART regardless of CD4 T cell count also comes from studies showing ART reduces the risk of HIV transmission [182] including perinatal transmissions [184]. Early treatment, however, relies on the common barrier of early diagnosis. Early diagnosis within transmission networks will be addressed in Chapter 5.

To conclude, the implementation of two major advances in the combination ART era has improved HIV outcomes, one being the use of combination therapy and the other being the use of HIV-1 RNA detection assays to monitor HIV replication. Twenty years on from these achievements, there is now strong evidence supporting the need for alternative monitoring strategies that can identify ongoing immune activation and guide the development of novel therapeutic strategies capable of improving long-term health outcomes for those living with HIV infection. We have shown throughout the chapters and in a large HIV/CVD cohort that immune activation remains present and can be reliably measured in a routine laboratory setting. Moreover, distinct differences between the plasma biomarkers that we have assessed in terms of their associations with HIV treatment response as well as other clinical and demographic variables, highlights gaps in our current understanding of HIV pathogenesis

mechanisms, as well as opportunities for improved approaches to treatment and monitoring in the near future.

Preamble to Chapters 5 and 6: HIV-1 epidemiology in Western Australia and Australia: An overview into HIV-1 diversity and HIV-1 phylogenetic analysis techniques

At the end of 2013 an estimated 35,300 people had been diagnosed with HIV in Australia, including 26,800 people currently living with HIV and 90.7% of whom were males [3]. It is also estimated that 14% of HIV infections remain undiagnosed [3]. Although the numbers are low when compared to the overall world-wide HIV-1 epidemic, the number of new diagnoses in Australia has steadily increased since 1999 against the current world trends [2] (specifically in the last 5 years). New HIV infections diagnosed in Australia since 1984 are depicted in Figure 1. In the early years of the HIV epidemic in Australia, HIV diagnoses were high, peaking in 1987 then followed by a decline to a nadir in 1999 which follows the introduction of antiretroviral therapy (1991) and of durably effective combination treatment regimens in 1996. The majority of HIV infections have been diagnosed in New South Wales (51.7%) followed by Victoria (22.7), Queensland (13%), Western Australia (6.1%), South Australia (4.1%) and other states (2.4%) [3]. The spread of HIV-1 within Australia has, historically, been driven by sexual contact within the MSM population (74.7%) and dominated by HIV-1 B-subtype [6].

HIV transmission through heterosexual contact has been historically low in Australia with only 13.9% of infections reported to be from heterosexual contact. Between 2004 and 2013, there were 372 children born to women with HIV in Australia. This has increased from 9.7 per 100,000 births in 2004-05 to 17.5 in 2012-2013. Among these births, 13 children (3.4%) have been diagnosed with HIV infection [3]. This rate is remarkably low when compared to the world-wide mother to child transmission rate (between 30-45% when not on ART and depends on the duration of breastfeeding; and high (30% in central African countries) [2].

The historical association between HIV-1 subtype and geographical location (C-subtype in sub-Saharan Africa and India, CRF01_AE in south east Asia) is well established, however in recent times there has been a shift in global diversity patterns. Many countries have changed from a predominantly B subtype cohort to one with increasing non-B subtypes [5, 12, 185-188]. Although the number of HIV infections diagnosed in Western Australia is low compared to other states, this state's demographic profile has been characterized by a high percentage of migrants settling

into Western Australia from sub-Saharan Africa, with short term visitors coming into Australia from countries where HIV notifications are high and with the increased travel of Australian residents to countries where HIV notifications are also high (as discussed in Chapter 1). In this setting, an improved understanding of the HIV epidemic at an epidemiological level is a valuable tool for the development of rational prevention and diagnostic strategies.

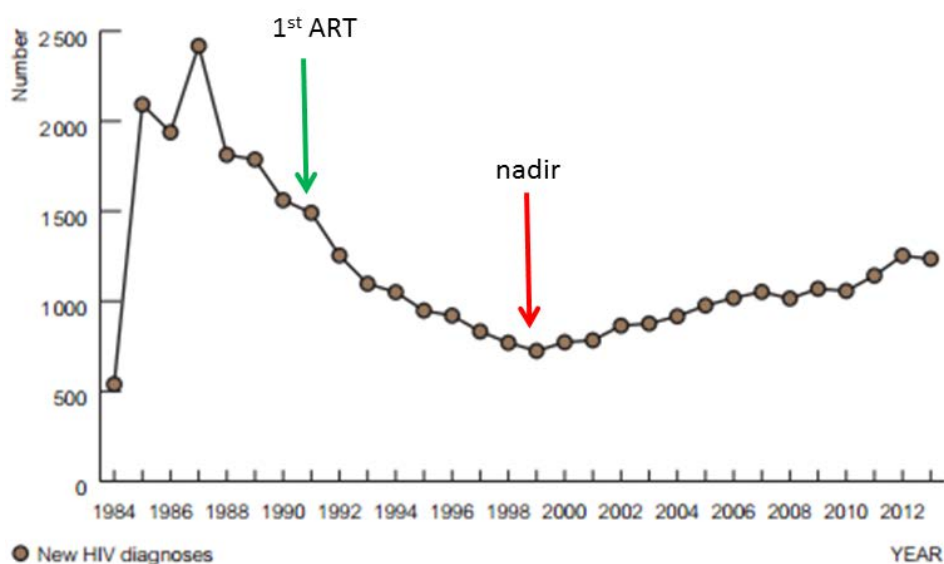


Figure 1: New HIV infections diagnosed, per year, since 1984. From the Kirby institute report [3]. Antiretroviral therapy was introduced in 1991 after which the number of diagnoses declined to a nadir in 1999. There has been a steady increase of newly diagnosed HIV infections in Australia since 1999, going against the current world-wide trend.

Chapter 5 presents a focus on monitoring the HIV epidemic within Western Australia through our investigation of the link between HIV-1 clinical parameters ($CD4^+$ T cell counts and HIV-1 RNA levels), HIV-1 viral subtypes and HIV-1 sequence relatedness with an aim to uncover the contribution of travel and migration, as well as local transmission networks, towards the recent evolution of the HIV epidemic in Western Australia. This approach has also been applied to Australia as a whole, through the formation of the Australian Molecular Epidemiology Network (AMEN), which represents a collaborative network of HIV-1 sequencing laboratories involving all Australian states. This network involves experts in the field of HIV and epidemiology and was formed to establish an ongoing connection throughout Australia with the aim

of assessing HIV phylogenetic characteristics, on a larger scale, as detailed in Chapter 6.

The HIV phylogenetic approach employed in both the Western Australian and Australian analyses is widely accepted [188]. The BioEdit program [189] was used to align sequences and Molecular Evolutionary Genetics Analysis Version 6 (MEGA V6) Phylogenetic tool [190] was utilised to construct the phylogenetic tree used to infer clustering and network patterns. This statistical analysis approach determines HIV-1 sequence similarities by inferring maximum likelihood trees with 100 “bootstrap” runs while the substitution model (general time reversible, GTR) was used to estimate evolutionary distance. The “bootstrap” percent infers how reliable it is that HIV-1 sequences will cluster together, statistically, in 100 repeated and consecutive analyses (Figure 2A). The substitution model used in the phylogenetic analysis determined the genetic similarity of the sequences. In keeping with previous analyses [188], the phylogenetic trees were searched for patterns that defined transmission clusters according to the following conservative approach (Figure 2B);

- 1) paired transmission where two sequences only cluster together according to the criteria where the bootstrap value (BS) was $\geq 98\%$ and the genetic distance (GD) was $\leq 1.5\%$ and
- 2) a “network” where more than two sequences had a BS value of $\geq 98\%$ and a GD $\leq 1.5\%$.

The stability of sequence clustering within both cohorts was also assessed by the removal of drug resistance sites while determining whether there was intrusion of “geographically distant HIV-1 subtype B sequences” within a pair or network was performed only in the Western Australian analysis. This approach provides a conservative estimate of HIV-1 sequence clusters, limiting the likelihood of false assignment of sequences into a pair or network where they do not belong.

There are several challenges faced when performing HIV-1 phylodynamics. Since HIV-1 is a genetically variable and rapidly mutating virus and because of the influence of host factors, phylogenetic analysis is most suited to sequences obtained during early HIV infection. For this reason baseline or the earliest obtained HIV-1 sequences will be used throughout the analysis. The substitution model estimates evolutionary distance and therefore the sequence quality and the length of sequence impacts final clustering results. The quality of sequence was assessed and confirmed

in the Bioedit alignment step in the analysis before the phylogenetic tree construction step.

Finally, a recent report from UNAIDS proposed an ambitious 2020 target of 90% of people living with HIV knowing their status, 90% of people diagnosed with HIV on treatment, and 90% of people on treatment with suppressed HIV-1 RNA. Achieving this target within Australia can be facilitated by knowledge gained through the surveillance of HIV-1 subtype diversity and sequence network characteristics within Australia. The analyses and results obtained in Chapters 5 and 6 will potentially assist in updating world-wide HIV epidemiology and provide insight into the design of prevention strategies and clinical monitoring techniques in Australia.

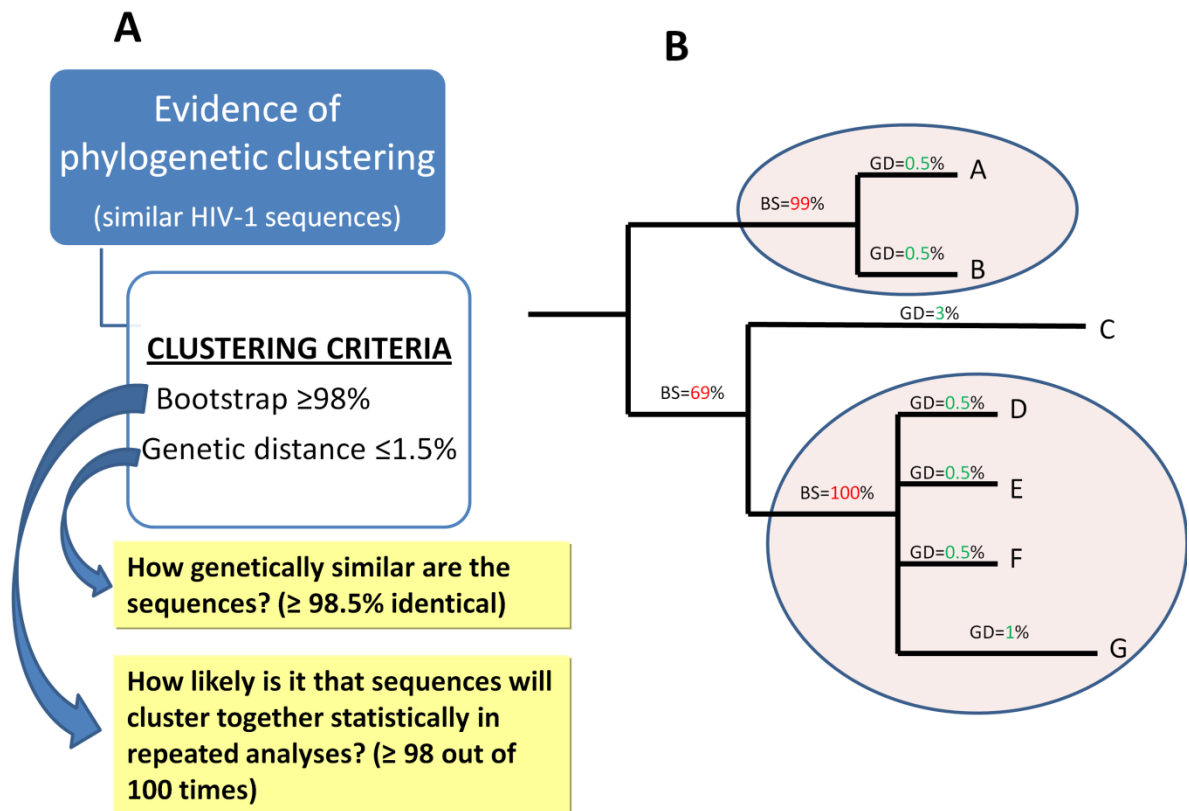


Figure 2: The concept behind the phylogenetic analysis approach. A). A schematic showing the criteria to provide evidence of phylogenetic clustering including “bootstrap” and “genetic distance” definitions. B). A possible phylogenetic tree result showing a pair of similar sequences (A+B) with GD=0.5% and BS=99%, a single sequence (C) genetically removed from the others (GD=3%) and a network of four similar sequences with low genetic variation (all GD \leq 1%) and with a BS of 100%.

Chapter 5:

Longitudinal trends in Western Australian HIV-1 sequence diversity and viral transmission networks and their influence on clinical parameters: 2000 – 2014.



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Attributions:

AC proposed and developed the design of the study, performed part of the HIV genotyping, accumulated and performed SPSS analysis on the results, reviewed literature, designed the tables, figures and compiled the manuscript. SG and MJ guided AC in the phylogenetic analysis and and critically reviewed the manuscript. IJ helped with the statistical analysis of data throughout the manuscript. LG was integral in the development of the HIV genotyping assay. GG compiled the HIV fasta sequence results into usable files and reviewed the manuscript. DN proposed and designed the study, supervised the research and critically reviewed the manuscript.

AC: 75%

Longitudinal Trends in Western Australian HIV-1 Sequence Diversity and Viral Transmission Networks and Their Influence on Clinical Parameters: 2000–2014

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Abstract

We examined baseline HIV-1 protease and reverse transcriptase sequences and HIV clinical parameters from 1,021 consecutive patients (814 male, 207 female) through the Royal Perth Hospital HIV service to investigate HIV-1 subtype diversity and local phylogenetic networks from 2000 to 2014. HIV-1 subtype B virus sequences were demonstrated in 619 (61%) of cases, with increasing non-B HIV-1 subtypes from 23.2% (2000–2003) to 48% (2008–2011) and 43% (2012–2014) ($p < 0.001$), including the CRF01_AE subtype [6.6% (2000–2003) to 21.5% (2008–2011)] and HIV-1 C subtype [9.5% (2000–2003) to 20.2% (2008–2011)]. More HIV-1 B subtypes were assigned to phylogenetic clusters compared to non-B subtypes (34% vs. 18%; $p < 0.001$), with larger clusters identified (cluster size >2 : 135/211; 64% vs. 13/69; 19%; $p = 0.001$), including one cluster of 53 HIV-1 B subtype sequences that evolved from 2008 to 2014. Non-B subtype HIV-1 was associated with lower baseline CD4 T cell count ($p = 0.005$) but not plasma HIV-1 RNA levels ($p = 0.31$), suggesting relatively delayed diagnosis. Baseline viral load was strongly associated with calendar time [mean 18,620 copies/ml in 2000–2003; 75,858 copies/ml in 2012–2014 ($p < 0.001$)], and was also associated with larger phylogenetic clusters (size >2) in adjusted analyses ($p = 0.03$). This study identifies a number of temporal trends over the past 15 years, including an increasing prevalence of non-B subtype HIV-1 that highlights the growing influence of migration and travel on the Australian HIV-1 epidemic and the associated increased role of heterosexual HIV-1 transmission in this context. At the same time, these data indicate that local transmission within predominantly male networks remains a challenging issue for HIV-1 prevention.

Introduction

THE DYNAMIC NATURE OF THE GLOBAL HIV-1 pandemic is indicated by social and behavioral factors that influence the risk of transmission, as well as by biological factors that influence host–viral interactions. In Australia, as in many developed countries, early implementation of free diagnostic and treatment services as well as public health strategies including needle-sharing programs and promotion of safe sex practices have contributed to a low overall national HIV-1 prevalence of ~ 158 per 100,000, with $\sim 26,800$ people currently living with HIV in Australia.¹ Despite this laudable

history the number of new diagnoses in Australia has steadily increased over the past 13 years with a 26% increase in the population rate since 2003, including 1,236 new cases in 2014 representing a 10% increase over the numbers diagnosed in 2011.¹ This is set against an overall downward global trend in new HIV diagnoses, most notably in sub-Saharan Africa and also evident in the Asia Pacific region.²

In this context, we have utilized routinely generated HIV-1 partial *pol* sequences to characterize the changes in HIV-1 genetic diversity from 2000 to 2014 to determine whether local transmission pairs or networks are increasing and to identify changing epidemiological patterns within Western

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Australia that could reveal the relative contributions of overseas travel and migration as well as local risk factors. This investigation is predicated on known characteristics of HIV-1 genetic diversity including historical associations between geographic location and distinct HIV-1 subtypes (A, B, C, D, F, G, H, J, and K) of which the B and C subtypes account for approximately 60% of HIV-1 infections.³

The C subtype is prevalent in sub-Saharan Africa and Asia, A is dominant in east Africa, while the B subtype accounts for the majority of infections within white populations that are typically also characterized by transmission via male-to-male sexual contact as previously documented in 2002 for the Western Australian HIV population.⁴ Genetic diversification of HIV-1 is also promoted by high rates of recombination events⁵ that generate stable circulating recombinant forms (CRFs), which together account for 20% of global HIV-1 infections, as well as unique recombinant forms (URFs).³ While these recombinant forms are increasing in number within the Los Alamos National Laboratory repository (there are currently 72 HIV-1 CRFs compared with 10 in 2003), it is also relevant to this study that CRF01_AE is the dominant viral subtype in southeast Asia, while CRF02_AG originates in west and central-west Africa.³ More recent reports support the idea that some HIV subtypes may have differing biological outcomes, including clinical implications relating to disease progression,⁶ transmissibility,⁷ and susceptibility to HIV therapy.⁸

In addition to identifying likely geographic origins of local HIV-1 infections, this investigation of viral sequence variation also allows for the estimation of transmission networks,⁹ in which there is sufficient viral sequence similarity relative to other local sequences to suggest a common source. This methodology has been applied in previous studies of domestic and international transmission networks at a global scale,¹⁰ as well as in studies within Europe,^{11,12} the United Kingdom,¹³ the United States,¹⁴ and eastern Australia.¹⁵ These studies have highlighted the complex interplay between travel, migration, and domestic factors that contributes toward new HIV-1 infections, changing global epidemiological profiles, and growing viral diversity. We were particularly interested in this aspect of phylogenetic analysis, given Western Australia's role as a mineral and energy resource economy with close links to Asia and Africa and a relatively mobile local population, and to identify clinical parameters (CD4 T cell counts or HIV RNA levels) potentially linked to HIV diversity, transmission pairs, or networks.

Materials and Methods

Study population

This study was conducted within the Department of Clinical Immunology at Royal Perth Hospital, which is the sole provider for diagnostic HIV laboratory services (including HIV-1 RNA quantitation, lymphocyte subsets, and HIV-1 sequencing for drug resistance testing) in Western Australia. Data collection and analyses were conducted on all plasma samples genotyped between the years 2000 and 2014 ($n=1,021$). In cases in which multiple sequences were performed during the study period, the earliest sequence was drawn on to represent the baseline sequence for the analysis. The sequences were then stratified into four periods of state

department of health notifications (2000–2003, 2004–2007, 2008–2011, and 2012–2014). Written ethics committee approvals for this investigation were also received from the Royal Perth Hospital (EC2012/170, REG-14/112) and Murdoch University (2012/216). All viral HIV-1 sequences and clinical parameters were collated and linked through the routine HIV laboratory service at Royal Perth Hospital. This information was subsequently deidentified by assigning a unique anonymized assessment number used throughout the study. To determine the effectiveness of identifying highly related sequences we applied 173 HIV B subtype sequences from a geographically distinct cohort,¹⁶ included multiple time point sequences from patients within the Western Australian cohort ($n=30$) and removed drug-resistant sites as previously reported.¹²

Sample collection, sample processing, and clinical assessments

Data collected at the time of HIV-1 sequence analysis included gender and age at notification, “earliest” plasma HIV-1 RNA level, and “earliest” CD4 T cell count where the patients' first HIV-1 RNA result and CD4 T cell count were defined as the “earliest” one. From 2000 all HIV-1 viral load tests were performed using the Cobas Ampliprep/Cobas TaqMan HIV-1 test (version 1 and version 2 implemented in 2010) with a lower detection limit of 1.6 log₁₀ copies/ml. CD4 T cell counts were performed using a BD FACSCanto (II) Flow cytometer instrument. All assay runs were conducted according to NATA accreditation standards, subject to quality control procedures and RCPA or alternative quality assurance programs.

For HIV-1 sequencing the protease (PR) gene (amino acid positions 1–99 in HXB2) and the reverse transcriptase (RT) gene (amino acid positions 100–235 in HXB2) were amplified by separating the plasma samples from EDTA whole blood within 6 h of collection by centrifugation at 2,000 rpm for 20 min and then storing at –20°C (24 h) or –80°C (>24 h). Viral RNA was extracted from plasma as per the manufacturer's instructions using the QIAGEN QIAamp Viral load RNA Mini Spin kit (Cat. #52906) and converted into cDNA using Oligo d(T) 20mer primers and RNase Out Ribonuclease Inhibitor (Invitrogen, Cat. #18418-020), Superscript III reverse transcriptase (Invitrogen, Cat. #18408-085). Two sets of primers were utilized in the first round cDNA amplification to account for potential primer site polymorphisms (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid). PCR products were electrophoresed on 1% Ultrapure Agarose gel (Invitrogen, Cat. #15510-027). Purification of positive PCR products for DNA sequencing was performed using the MioBio Ultra Clean purification kit (Geneworks: MB-12500-250). Sequencing was performed using the ABI Prism Big Dye terminator chemistry (ABI Cat. #4337457) using M13F and M13R universal primers, purified using CleanSEQ beads (Agencourt Cat. #AG000135), and electrophoresis was performed on the ABI 3730xl instrument. Analysis of PR and RT sequences was performed by using the ASSIGN editing program (Conexio Genomics). We obtained drug resistance profiles by submitting the fasta files to a calibrated population resistance tool linked to the Stanford HIV database (<http://hivdb.stanford.edu/>).

The HIV-1 phylogenetic analysis approach and definition of clusters

Phylogenetic analysis was performed utilizing an approach previously published.¹² In brief, the BioEdit tool¹⁷ and Molecular Evolutionary Genetics Analysis Version 6 (MEGA V6) Phylogenetic tool¹⁸ were utilized to construct the phylogenetic tree used to infer clustering patterns. In keeping with previous analyses, we defined transmission clusters according to the following conservative approach: (1) paired transmission where two sequences only group together according to the criteria in which the bootstrap (BS) value was $\geq 98\%$ and the genetic distance (GD) was $\leq 1.5\%$; (2) a “network” in which more than two sequences had a BS value of $\geq 98\%$ and a GD value of $\leq 1.5\%$; (3) cluster stability after removal of drug resistance sites; and (4) no intrusion of “geographically distant HIV-1 subtype B sequences” within a pair or network. This conservative criteria approach limits the likelihood of falsifying a sequence into a pair or network in which they do not belong.

Statistical analysis

Statistical data analysis of demographic and clinical data was performed using Statistical Package for the Social Sciences version 21.0 (SPSS v21: Armonk, NY: IBM Corp.). Data distribution was assessed for normality with some variables requiring log or square root transformation based on their distributional profiles. Statistical analysis utilized ANOVA and post hoc tests with correction for multivariate regression comparisons for multivariable analyses. Results were considered statistically significant when p -values were < 0.05 .

Results

Demographic and clinical features of the 1,021 study participants, who provided sequence data between 2000 and 2014, including 814 males (80%) and 207 females (20%), are described in Table 1. Overall, 619 (61%) of the HIV-1 patients were infected with subtype B virus while 402 (39%) were infected with non-B viruses including A, C, D F, G, CRF01_AE, and CRF02_AG subtypes and other inter-

subtype recombinant forms. Females were more likely to be infected with non-B subtype viruses compared with males (70% vs. 31.6%, $p < 0.001$) and were on average 6 years younger than males (33 years of age for females with both B subtype and non-B subtype HIV-1 infection, compared with 39 years for both B and non-B subtype males; gender difference $p = 0.0007$; viral subtype difference $p = 0.52$).

HIV-1 subtype trends between 2000 and 2014

As shown in Fig. 1, there has been a substantial increase in the proportion of non-B HIV-1 subtypes rising from 23.2% in 2000–2003 to 43% in the period 2012–2014 ($p < 0.001$) with a peak in 2008–2011 (47.7%). This was associated with an increase in HIV-1 subtype diversity with a notable increase of the CRF01_AE subtype from 6.6% in 2000–2003 to 17.1% in 2004–2007, followed by a smaller increment to 21.5% in the 2008–2011 notification period, staying stable through 2012–2014 at 20.4%. There has also been a marked increase of HIV-1 C subtype infections over time, from 9.5% in 2000–2003 and 2004–2007 to 20.2% in 2008–2011 and then finally to 15.2% in 2012–2014. Infections with a CRF02_AG subtype were $< 4\%$ throughout the study eras.

Analyzing these results in terms of gender (Fig. 2), there has been a clear increase in the number of cases of non-B subtype HIV-1 from 2000 to 2012 in both males and females (Fig. 2A and B respectively) while the proportion of B subtype infections has decreased from 2000–2003 to 2012–2014 among both males (83% to 64%) and females (53% to 22%). As expected, the proportion of males acquiring HIV-1 B subtype infection has remained consistently high throughout the study period (with a 1.5-fold increase over time: Fig. 2C) while non-B subtypes increased at a higher rate (4-fold), largely accounted for by CRF01-AE cases followed by HIV-1 C subtype infections. Among females there was a significant contribution of HIV-1 C subtype infections, with a 4-fold increase from nine infections (28%) in 2000–2003 to a peak of 36 infections (47%) in 2008–2011, while CRF01_AE subtype infections increased from two (6%) in 2000–2003 to 19 in 2008–2011 (29%: Fig. 2D).

TABLE 1. PATIENT CHARACTERISTICS AND CLINICAL PARAMETERS AT TIME OF HIV NOTIFICATION ($N = 1,021$)

Subtype	B	Non-B	Intersubtype recombinants
Patient (n)	619	364	38
Gender**			
Male	557	232	25
Female	62	132	13
Age (years)			
Male	38.8 (12–76)	39.6 (1–74)	38.4 (4–68)
Female	33.1 (1–74)	32.7 (1–68)	38.2 (21–59)
Viral load (\log_{10} copies/ml)			
Male	4.57 (1.6–7)*	4.76 (1.6–7)*	4.79 (2.6–6.63)
Female	4.33 (1.6–6.23)	4.40 (1.6–7)	4.51 (2–6.13)
CD4 T cell count (cells/ μ l)			
Male	445 (3–1792)**	362 (2–1710)**	379 (84–1302)
Female	436 (6–1020)	357 (6–2024)	374 (77–736)
CD4:CD8 ratio			
Male	0.46 (0.01–2.57)	0.41 (0.01–1.92)	0.36 (0.07–0.73)
Female	0.50 (0.04–2.6)*	0.39 (0.02–1.21)*	0.53 (0.12–1.77)

* $p < 0.05$, ** $p < 0.001$.

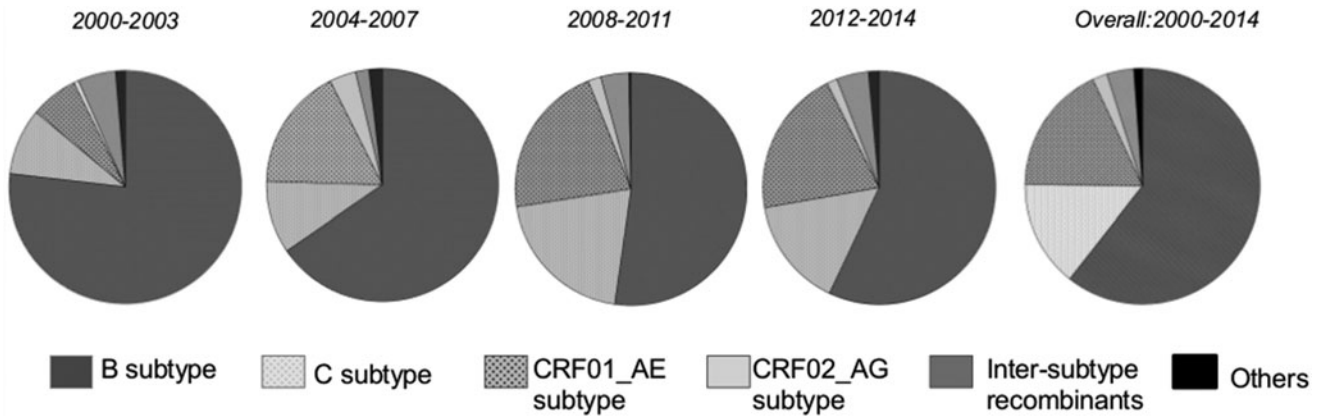


FIG. 1. The HIV-1 subtype diversity distribution and proportions identified in Western Australia during 2000–2014, represented according to four notification eras and an overall proportion.

Phylogenetic analysis of subepidemic transmission networks

We then undertook phylogenetic analysis of the 1,021 available HIV-1 *pol* sequences to investigate clusters of highly related sequences likely to reflect linkage through transmission networks. A radial representation of the phylogenetic analysis for all HIV-1 B ($n = 619$) and non-B subtypes

($n = 402$: including intersubtype recombinants) from 2000 to 2014 is shown in Fig. 3, following maximum likelihood tree construction utilizing MEGA v6. All phylogenetic clusters, defined in this analysis by a bootstrap value $\geq 98\%$ and genetic distance $\leq 1.5\%$, are also represented in Table 2.

For the HIV-1 B subtype phylogenetic analysis (Fig. 3A) paired transmissions, networks with more than two patients, the subepidemic network of 53 patients and the subepidemic

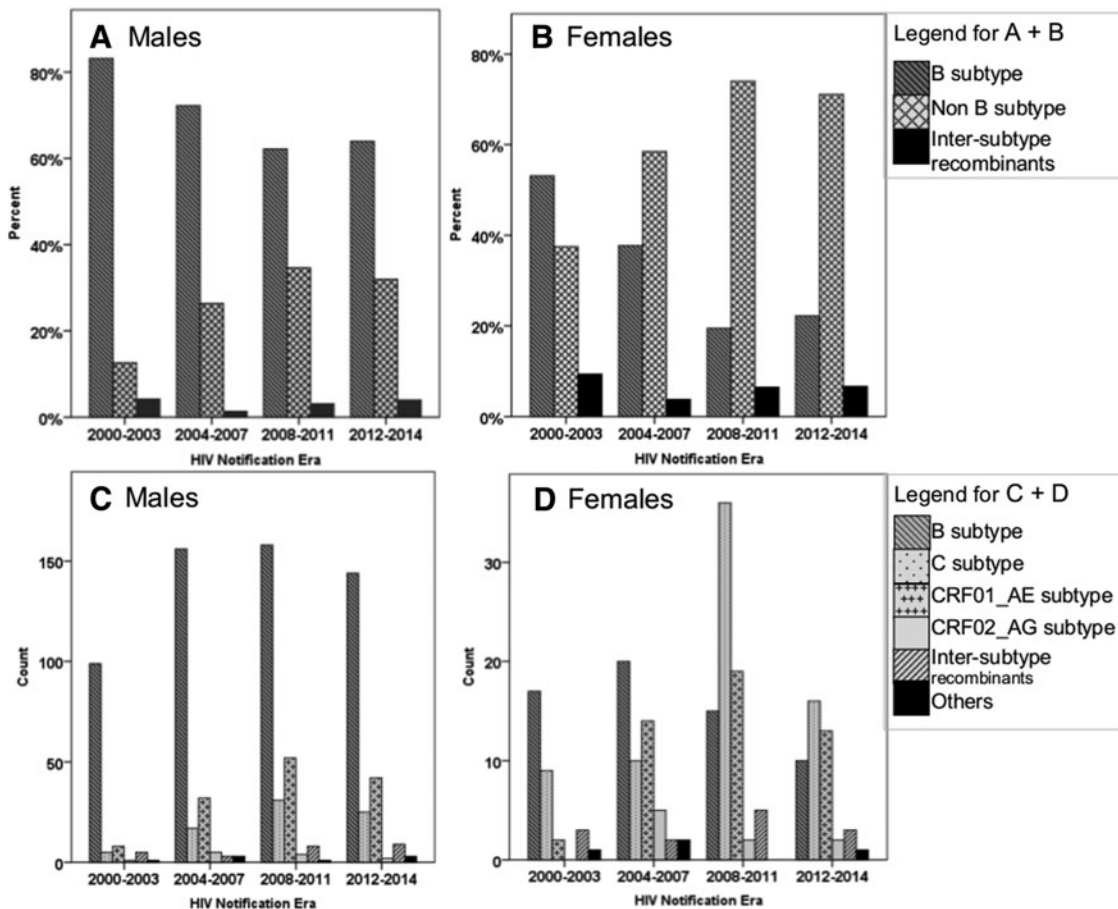


FIG. 2. The proportion of males (A) and females (B) within HIV-1 B-subtype, non-B subtype, and recombinants represented by the HIV notification era and the number of HIV sequences assessed by specific HIV-1 subtypes for males (C) and females (D).

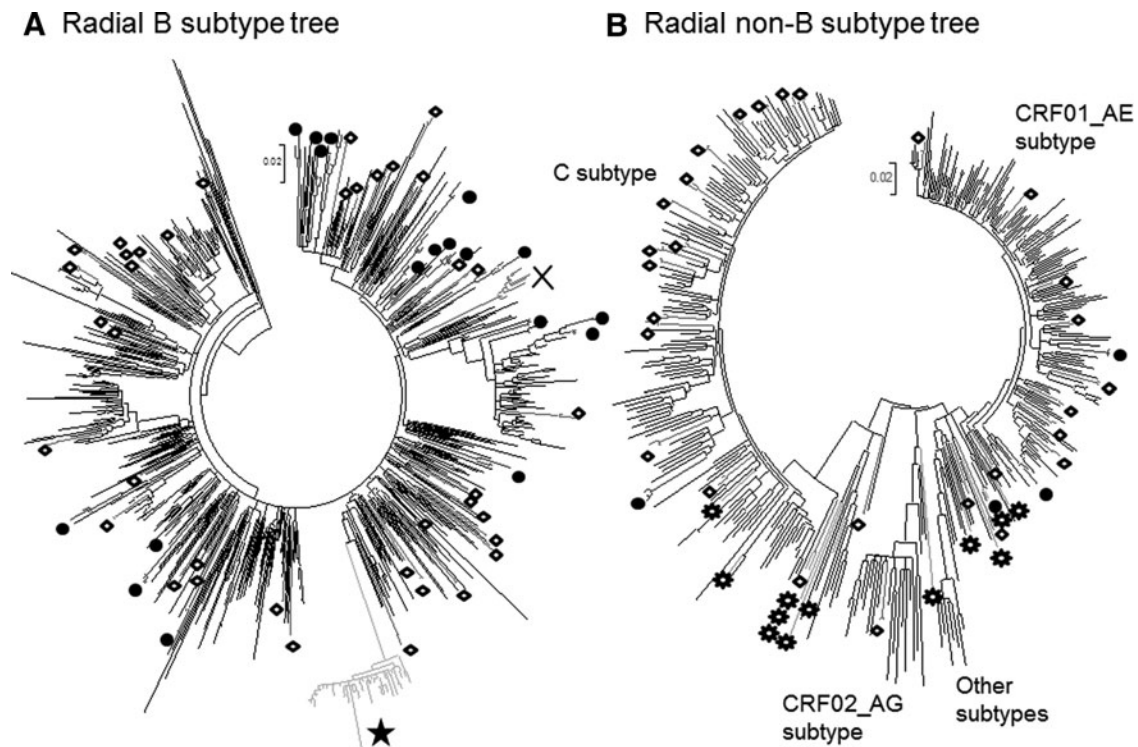


FIG. 3. A radial representation from the phylogenetic analysis performed on HIV-1 partial *pol* sequences (protease and reverse transcriptase) for all HIV-1 B (A) and non-B (B) subtype samples genotyped during 2000–2014 [diamonds = pairs ($n=66$, 132 patients), star = a cluster of 53 patients, cross = a cluster of 12 patients, circles = clusters > 2 ($n=22$, 82 patients), suns = intersubtype recombinants and singletons].

network of 12 patients are defined by the “diamonds”, “circles”, “star” and “cross” respectively. For the non-B analysis (Fig. 3B) intersubtype recombinant forms are represented by the “suns”.

Of the 619 HIV-1 B subtype sequences analyzed, 56 clusters were identified incorporating 211 (34%) individuals (Table 2). Among the 402 non-B sequences, 69 (18%) fell into one of 32 clusters. Non-B subtype phylogenetic clusters were characterized by a larger proportion of pairs (28/32 clusters; 87.5%) compared to B subtype clusters (38/56 clusters; 68%; $p < 0.05$). Conversely, more individuals with B subtype HIV-1 infection were identified within larger networks (cluster size > 2) than non-B-infected patients (135/211; 64% vs. 13/69; 18.8%; $p = 0.001$).

Including gender in this analysis (Supplementary Table S2), we found 53 male only clusters (60.2%), 31 male and female clusters (35.2%), and four clusters represented by females only (4.6%). The majority of B subtype clusters were composed of males only (45/56, 80.4%) while the majority of C subtype clusters included males and females (11/15, 73%).

Within the CRF01_AE subtype we noted both male-only clusters (6/15, 33%) as well as a larger proportion of female and male clusters (8/15, 53%). Larger phylogenetic clusters of males (cluster size ≥ 3) were identified predominantly within the B subtype (15/56 clusters: 26.8%), while only two male-only clusters with ≥ 3 subjects were identified within non-B subtypes (CRF01_AE, 13%). These findings are consistent with HIV-1 B subtype transmission within networks of men who have sex with men (MSM), while the HIV-1 C subtype was predominantly associated with heterosexual transmission. For CRF01_AE clusters the mode of transmission was either MSM or heterosexual contact.

The largest subepidemic network was represented by a group of patients with B subtype infections. This network was established in 2008 with three patients and evolved to 53 patients by 2014 (Fig. 4), including one female and 52 males, with a median age of 40 years (range = 19–61 years). This network is defined by the presence of a minor non-nucleoside reverse transcriptase inhibitor (NNRTI) drug resistance mutation K101Q (present in all sequences) and the

TABLE 2. HIV-1 SUBTYPE TRANSMISSION CLUSTERS IDENTIFIED 2000–2014

HIV-1 subtype	Number of clusters (n, %)	Number of pairs (n, %)	Number of clusters with ≥ 3 subjects (n, %)	Number of clusters 2008–2014 (n, %)
B	56 (64)	38 (68)	18 (32)	30 (54)
CRF01_AE	15 (17)	12 (80)	3 (20)	12 (80)
C	15 (17)	14 (93)	1 (7)	10 (67)
Others	2 (2)	2 (100)	0 (0)	0 (0)
Total	88	66 (75)	22 (25)	52 (59)

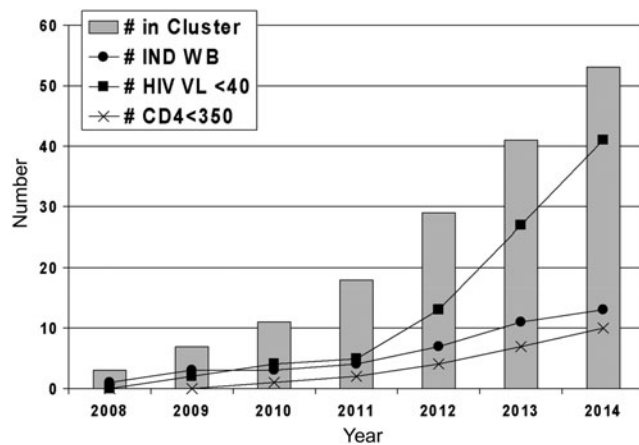


FIG. 4. A large WA cluster ($n=53$) showing clinical dynamics over time including indeterminate 4 Western blot, HIV-1 RNA <40, and CD4 T cell counts <350.

minor PR drug resistance mutation, L10F, which emerged during the end of 2011 into 2012 in 6/53 patients. Interestingly, the network of 53 patients is characterized by a higher baseline viral load (Fig. 5A: $p=0.01$) and CD4 count (Fig. 5B: $p=0.001$) than ungrouped patients. This network expanded in 2014 (12 new cases) despite high proportions of early diagnoses (25% with acute HIV-1 serology) and treatment uptake (76% with viral load <40 copies/ml by 2014), although three patients (two notified in 2013 and one in 2014) had a CD4 T cell count <55 cells/ μ l and HIV-1 viral load >750,000 copies/ml at the time of notification, indicating delayed HIV-1 diagnosis.

A second large subepidemic, involving males only, emerged in 2008 as two similar sequences, increasing to four patients by 2010 and then to 12 cases by 2014 (median age: 31.5 years, range: 20–67 years). The minor PR drug resistance mutations L33I and A71T are seen in all sequences for the subepidemic network with 12 patients. We have identified only four smaller networks within the non-B subtypes (CRF01_AE and C subtype; three to five patients/network) established after 2010.

To investigate temporal patterns for the formation of clusters overall, we determined the notification date of the second sequence into the cluster. This revealed a more recent evolution of clusters within the CRF01_AE HIV-1 subtype (80% of clusters since the beginning of 2008) compared to C subtype (67%) and B subtype clusters (54%). To explore any potential bias associated with HIV-1 drug resistance profiles we removed drug resistance sites from all sequences. We additionally included sequences from the Swiss HIV cohort study ($n=173$) to determine if geographically and temporally distant sequences would cluster with local sequences. Both approaches did not significantly alter the results of the underlying clustering analysis. Finally, we also included a number of multiple time point sequences from 30 patients. The samples with the same identifier clustered within the conserved criteria approach of BS $\geq 98\%$ and GD $\leq 1.5\%$ (data not shown).

HIV-1 intersubtype recombinants

Of the 1,021 samples analyzed in this study 38 (3.4%) were intersubtype recombinants (Supplementary Table S3). Of

these 18 (47.4%) have been identified during the period 2011–2014. Of all recombination events present in our cohort, half of them (19/38) are intersubtype recombinants between non-B HIV-1 subtypes. The main HIV-1 recombinant forms represented in the Western Australian cohort were A/CRF01_AE (6/38; 15.8%) and D/B (5/38; 13.1%). Three patients with an A/CRF01_AE subtype clustered tightly together (BS 100%, GD <1.0%).

Influence of HIV-1 subtype and phylogenetic clusters on viral load and CD4 T cell count at diagnosis

When we assessed CD4 T cell counts and plasma HIV-1 RNA levels for C and CRF01_AE subtypes within each notification period we found an association with reduced CD4 T cell counts (Fig. 5C, $p<0.001$) and CD4:CD8 ratios at notification ($p=0.02$) compared to HIV-1 B subtypes. In contrast, HIV-1 subtype was not associated with differences in plasma HIV-1 RNA levels ($p=0.31$).

We also observed a progressive increase in the plasma HIV-1 RNA level at diagnosis over calendar time, increasing from 18,620 copies/ml in 2000–2003 to 75,858 copies/ml in 2012–2014 (Fig. 5D: $p<0.001$). This was not associated with any evidence of a trend in CD4 T cell count ($p=0.1$) or the CD4:CD8 ratios ($p=0.2$) over time. Examining these results in terms of gender and HIV-1 subtype, we found no significant associations between baseline viral load and HIV-1 subtype or age in females ($p>0.1$), although males with non-B HIV-1 subtype were found to have significantly higher baseline viral load results than males with B subtype (57,550 copies/ml vs. 37,150 copies/ml: $p=0.028$) regardless of age.

Overall multivariate regression analysis revealed that baseline plasma HIV-1 RNA levels were positively associated with calendar time ($p<0.001$; β 0.20), male gender ($p=0.003$; β 0.23), age ($p=0.04$; β 0.01), membership of a phylogenetic cluster of size >2 ($p=0.03$; β 0.06), and lower CD4 T cell count ($p<0.001$; β -0.01). In contrast, a higher baseline CD4 T cell count was associated with HIV-1 B subtype ($p=0.005$; β -0.09), lower HIV viral load ($p<0.001$; β -0.31), and younger age ($p<0.001$; β -0.12) as well as membership of a cluster of size >2 ($p<0.001$; β 0.12) (Fig. 6).

Discussion

This study has identified increasing genetic diversity of HIV-1 in Western Australia over the past 15 years, with significant increases in HIV-1 non-B subtype sequences over the study period, although HIV-1 B subtypes remain marginally dominant. These findings correspond to previous studies that have noted increasing heterosexually acquired HIV notifications in the Western Australia cohort including a 50% increase in HIV notifications among heterosexual women since 2006.¹⁹ The relative increase of HIV-1 C and CRF01-AE subtypes in the Western Australian HIV cohort has also been observed in other Australian studies.^{15,20} These non-B subtypes are known to be from regions where HIV-1 is highly prevalent and corresponds with the increased migration from sub-Saharan African regions (subtype C) and overseas travel to Southeast Asia (subtype CRF01_AE).

The phylogenetic analysis performed in this study, based on a previously described approach,¹² provides a conservative estimate of likely transmission networks within the

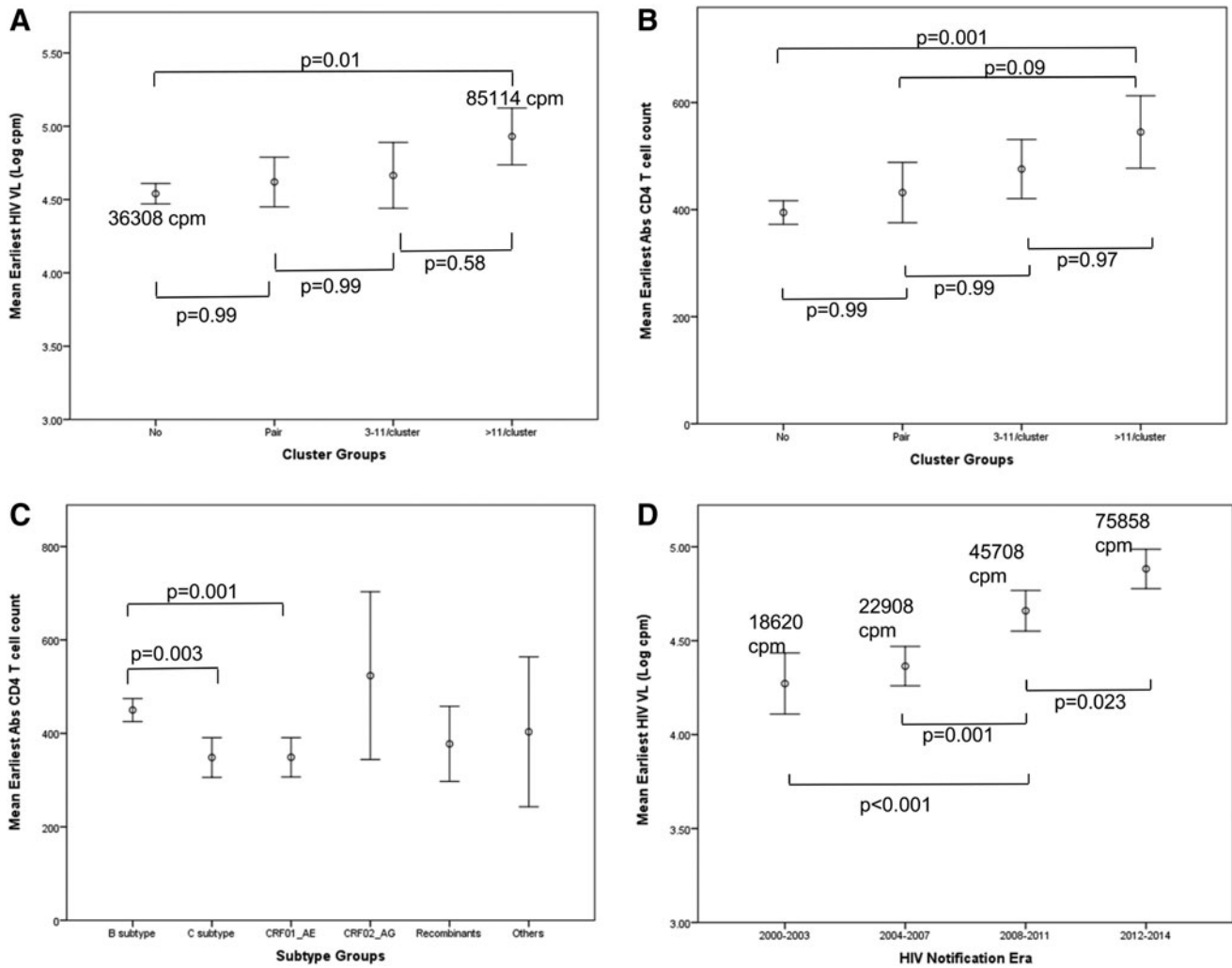
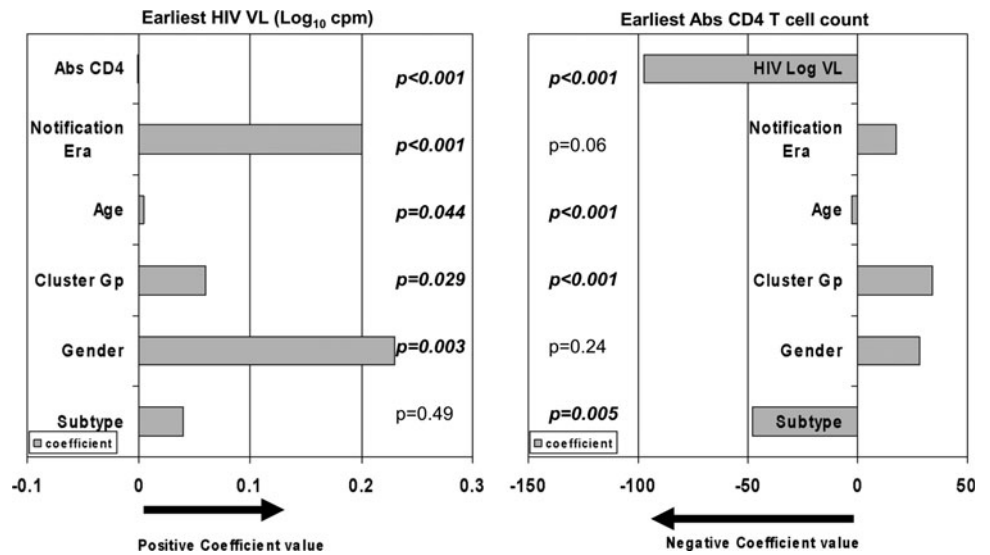


FIG. 5. Dynamics of the earliest HIV-1 RNA (A, D) and earliest CD4 T cell count (B, C) for sequences within a cluster group (A, B) or defined by HIV subtype (C, D).

FIG. 6. Multivariate regression results showing significant associations of HIV clinical parameters (HIV-1 RNA and CD4 T cell counts) with notification era, age, cluster group, gender, and HIV subtype.



Western Australian cohort. The results reveal more pairs and large networks within the B subtype analysis, which remain largely confined to male groups. In contrast, phylogenetic clusters within the C subtype analysis predominantly consisted of female/male pairs, while in the CRF01_AE analysis there were both male-only groups as well as female/male groups. Larger transmission networks are largely confined to the HIV-1 B subtype, and show significant associations with higher HIV RNA levels and higher CD4 T cell counts at the time of diagnosis but not with age or gender. These findings suggest that larger networks are expanding despite early diagnosis (many in the acute phase of infection) and high treatment uptake, in keeping with a U.S. study in which large cluster size was associated with higher CD4 counts (>350 cells/ μ l) and HIV RNA levels (>10,000 copies/ml). This U.S. study also reflects the network patterns identified here, in which a small number of large networks exists along with small defined networks and pairs.¹⁴

These data suggest that local HIV-1 transmission is still dominated by B subtype viral strains, mostly within male networks. The introduction of increasing non-B subtypes into Western Australia, in keeping with increasing immigration and overseas travel, does not appear to be associated with a noticeable rise in local transmission of non-B HIV-1, apart from an emerging pattern of onward transmission of CRF01_AE within male networks. We were also interested to observe a pronounced temporal trend in viral load measurements at the time of diagnosis in this study, irrespective of HIV-1 subtype. A similar trend has been identified in a meta-analysis of >30,000 individuals spanning the years 1984–2010,²¹ raising the possibility that HIV-1 replicative capacity ('viral fitness') is increasing over time, and that higher viral load levels represent a virological risk factor for membership of larger transmission clusters.

While the HIV-1 subtype does not influence viral load measurements in this study, we did note that baseline CD4 T cell counts were lower among those with non-B HIV-1 subtypes, including both C and CRF01_AE subtypes, with a mean difference of ~ 80 cells/ μ l in both males and females compared to those with B-subtype HIV-1. It is conceivable that this reflects the increased virulence of non-B HIV subtypes with greater loss of CD4 T cells as a consequence. For example, CXCR4 tropism, which has been associated with rapid disease progression,^{22,23} is associated with the CRF01_AE subtype^{23,24}; however, in contrast, C subtype HIV-1 has a lower CXCR4 virus than B subtype²⁵ with a slower CD4 T cell decline in patients with a C subtype²⁶ unless failing HIV therapy.²⁷ In the context of this study, a more likely explanation is that diagnosis of HIV-1 is delayed among those with non-B HIV-1 infection in Western Australia, due in part to the fact that HIV-1 screening is not undertaken among migrants entering Australia with temporary visas, and diagnosis is therefore often delayed until a permanent visa application is undertaken or until medical care is required for symptomatic disease or pregnancy.²⁸ In this regard, a study involving the provision of HIV treatment to Medicare-ineligible migrants in Australia has also noted reduced CD4 T cell counts within this population compared to the overall Australian HIV Observational Database.²⁹

This study has the advantage of full assessment of baseline HIV-1 *pol* sequencing performed within the Royal Perth Hospital laboratory between the years 2000 and 2014, in-

cluding 1,021 HIV-positive individuals. With regard to phylogenetic analysis and the classification of HIV-1 transmission networks, we acknowledge that this approach has limitations associated with viral divergence over time as well as uncertainty regarding the appropriate bootstrap values and genetic distance thresholds that are utilized to define clusters. We therefore applied relatively stringent clustering criteria (bootstrap value $\geq 98\%$, genetic distance $\leq 1.5\%$) in order to provide a conservative estimate of phylogenetic clusters as utilized in other studies.^{9,10,12} We also undertook sensitivity analyses including the incorporation of a group of sequences from a geographically distinct B subtype cohort, and removal of drug resistance sites from all sequences to remove any potential bias associated with HIV-1 drug resistance profiles. These approaches did not alter the results of the underlying clustering analysis.

Overall, this study identifies a number of temporal trends that are relevant to the evolution of the Australian HIV-1 epidemic over the past 15 years. We have identified an increasing prevalence of HIV-1 non-B subtypes over this period, from 23% in 2000–2003 to more than 40% from 2012 onward. This reflects an increased "importation" of HIV-1, particularly from sub-Saharan African and southeast Asian countries, including migrants^{28,29} as well as Australians who have travelled to these destinations for business and tourism.^{30,31} These findings are not unique to Western Australia, and it is notable that similar observations have been made in Victoria¹⁵ and South Australia.²⁰ These data also provide reassurance that onward transmission of non-B subtype HIV-1 within local networks remains uncommon, despite evidence for delayed diagnosis of HIV-1 in this population. On the other hand, we have observed the evolution of large local transmission networks of B-subtype HIV-1 despite relatively high rates of early diagnosis and treatment within local networks. The observation of increasing baseline viral load measurements over calendar time suggests that viral factors may be influencing this trend, within an overall framework that argues for a range of preventive measures beyond "test and treat" approaches to reduce HIV-1 transmission within Australia.³²

The overall findings of this study highlight the cooperative approach that is required to effectively prevent new HIV cases in Australia, involving an increased appreciation of the role played by travel and immigration (and immigration policy), and the associated increased role of heterosexual HIV-1 transmission in this context. At the same time, these data indicate that local transmission within MSM networks remains a challenging issue for HIV-1 prevention.

Sequence Data

The sequences used in this study have been deposited into GenBank under the following accession identifications: Western Australian sequences KT228338–KT229359.

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Author Disclosure Statement

No competing financial interests exist.

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Additional supplementary data for the manuscript in Chapter 5.

Supplementary Table S1: HIV-1 Protease and Reverse Transcriptase primer sequences.

1st Round PR-RT amplification primers:

PR2056- 5'TGAAAAAGTGTACTGAGAGACAGGR

RT3.2Outer- 5'CAGGAAACAGCTATGACCACTTCTGTATGTCATTGACAGTCC

Alternative 1st Round PR and RT amplification primers:

PR5A- 5'TGTAAAACGACGGCCAGTCAGAGCCAACAGCCCCACCA

PR3A- 5'CAGGAAACAGCTATGACCTTTCCCCACTAACTTCTGTATGTCATTGA

RT5'Outer- 5'CCATTAGCCCTATTGAGACTGTACCAG

RT3.2Outer- 5' CAGGAAACAGCTATGACCACTTCTGTATGTCATTGACAGTCC

Second round RT primers:

RT5'Inner94-M13F- 5'TGTAAAACGACGGCCAGTTAAAGCCAGGAATGGATGGCCC

RT3'Inner94-M13R- 5'CAGGAAACAGCTATGACCGATGGAGTTCATAACCCATCC

Second round PR primers:

PR5A-M13F -5'TGTAAAACGACGGCCAGTCAGAGCCAACAGCCCCACCA

PR3B-M13R- 5'CAGGAAACAGCTATGACCGGGCCATCCATTCCTGGCTTTAATTT

Supplementary Table S2: The distribution of HIV-1 clusters according to subtype, cluster size and gender.

<i>Subtype</i>	<i>Clusters (# of subjects)</i>	<i>Male only cluster #</i>	<i>Male+Female cluster #</i>	<i>Female only cluster#</i>	<i>Total # of clusters</i>
B	2	30	7	1	38
	3-5	12	1	0	13
	>6	3	2	0	5
CRF01_AE	2	4	7	1	12
	3-5	2	1	0	3
	>6	0	0	0	0
C	2	2	10	2	14
	3-5	0	1	0	1
	>6	0	0	0	0
Others	2	0	2	0	2
	3-5	0	0	0	0
	>6	0	0	0	0
Total		53 (60.2%)	31 (35.2%)	4 (4.6%)	88

Supplementary Table S3: HIV-1 inter-subtype recombinants identified 2000-2014 (n=38) via Stanford HIV drug resistance database.

Reverse Transcriptase	Protease							
	<i>B</i>	<i>CRF01_AE</i>	<i>C</i>	<i>A</i>	<i>CRF02_AG</i>	<i>D</i>	<i>K</i>	<i>F</i>
<i>B</i>		3	3			5		3
<i>CRF01_AE</i>	2			6	1	1		
<i>C</i>	2			1			3	
<i>A</i>		3			2		1	
<i>G</i>					1			
<i>F</i>	1							

Summary for Chapter 5:

The identification of extensive HIV-1 diversity within the Western Australian HIV cohort, along with the recent evolution of increased numbers of clusters and large networks has highlighted several challenges in HIV-1 prevention, including delayed diagnosis among HIV-1 non-B-subtypes, namely migrants and overseas visitors. Although HIV-1 B subtype remains the dominant viral strain, this study highlights the more recent evolution of clustering pairs within the C and CRF01_AE subtypes since 2008, which are highly prevalent in sub-Saharan Africa and south east Asia respectively, reflecting a role of overseas travel to these areas and the importation of HIV-1 into Western Australia through heterosexual and MSM contact. We have also identified a substantial increase in baseline HIV-1 RNA over time, including higher viral load levels within a large B subtype transmission cluster (identified through stringent phylogenetic analysis) that continues to expand despite frequent early diagnosis and high treatment uptake.

HIV infection in Western Australia was once thought of as predominantly affecting young gay men. This analysis clearly shows a shift from this perception as indicated from the wide age range at the time of HIV diagnosis within the Western Australian cohort, from infants to 76 year olds regardless of subtype groups (Figure 1). HIV-1 B subtype infection is still the predominant subtype among men while women are more likely to be infected with a non-B subtype. This also reflects an ageing Western Australian HIV cohort which is approaching a median age of 50 years. As indicated in Chapters 3 and 4, there are a number of important patient care considerations, namely the impact of age related comorbidities - particularly CVD, adding to the complication of HIV management.

As stated in the preamble, by the end of 2013, 13 children within Australia had been infected with HIV through perinatal transmissions [3]. The age distribution plots below represent a high proportion of children, who reside in Western Australia, are infected with non-B subtypes, suggesting a link between imported HIV-1 viruses potentially through migration.

Overall, the results shown in Chapter 5 identify a number of temporal trends that are relevant to the evolution of the Western Australian HIV-1 epidemic over the past 15 years. For this reason, in Chapter 6, HIV-1 subtype diversity and phylogenetic network characteristics within B and non-B subtypes will be investigated within an Australian HIV cohort. Keeping in line with Chapter 5, baseline HIV-1 partial *pol* sequences will be phylogenetically mapped using techniques outlined in Chapter 5 with the aim of highlighting any new challenges in

HIV-1 prevention that may inform strategies to treat HIV and to end HIV-1 transmissions in Australia and potentially world-wide.

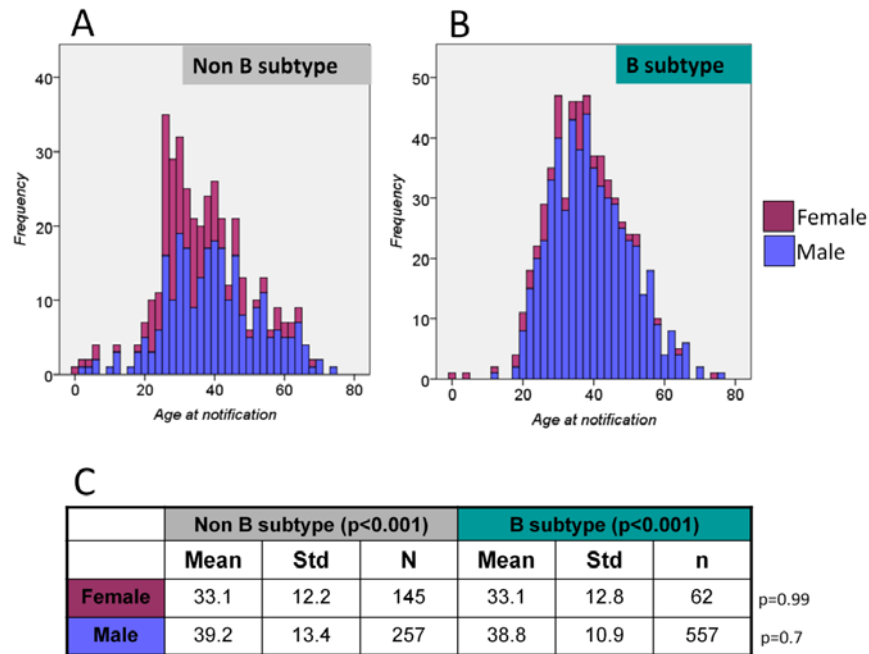


Figure 1: The age distribution plots for patients in the Western Australian HIV cohort. This is stratified by HIV-1 non-B subtypes, including inter-subtype recombinants (A) and HIV-1 B subtype (B). The mean age (std, n) and significance is represented in (C).

Chapter 6:

Molecular epidemiology of HIV-1 in Australia 2005-2012: a national collaborative study.



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Additional comments: This manuscript has been compiled in the format required for publication and is currently undergoing review with the AMEN collaborators. References are included at the end of the manuscript.

Authors include; *Castley A, Sawleshwarkar S, Varma R, Herring B, Thapa K, Chibo D, Nguyen N, Hawke K, Ratcliff R, Dwyer D, Kelleher A, Nolan D: **The Australian Molecular Epidemiology Network-HIV (AMEN-HIV).***

Attributions:

AC proposed and developed the design of the study, accumulated the data into the Bioedit format, performed the phylogenetic and SPSS analysis, reviewed literature, designed the tables, figures and compiled the manuscript. Scientists and clinicians overseeing the HIV services within each state contributed the HIV-1 sequencing data to the AMEN group and are currently critically reviewing the manuscript (KH, RR-South Australian, SS, RV, KT, BH, AK, DD- western New South Wales, NN- Queensland, DC-Victoria). DN assisted with the design the study, supervised the research and critically reviewed the manuscript.

AC: 75%

ABSTRACT

Introduction: Rates of new HIV-1 diagnoses are increasing in Australia, with evidence of an increasing proportion of non-B subtypes reflecting a growing impact of migration and travel. This present study aims to define HIV-1 subtype diversity patterns and investigate HIV-1 transmission networks within Australia.

Methods: The Australian Molecular Epidemiology Network (AMEN) HIV collaborating sites in Western Australia, South Australia, Victoria, Queensland and western Sydney, provided baseline HIV-1 partial *pol* sequence, age and gender information for a total of 4,873 patients during 2005-2012. HIV-1 phylogenetic analyses utilised MEGA V6, with a stringent classification of transmission clusters (bootstrap $\geq 98\%$, genetic distance $\leq 1.5\%$).

Results: HIV-1 B subtype represented 74.5% of 4,873 sequences (WA 59%, SA 68.4%, w-Syd 73.8%, Vic 75.6%, Qld 82.1%), with similar proportion of clusters compared to non-B subtypes (23% vs 24.5% of sequences, $p=0.3$), significantly more larger cluster size within B subtype (45.0% with >2 sequences vs 24.0% of non-B clusters, $p=0.021$) and more male-only groups (88%). HIV-1 subtype C networks (38 groups) included more female/male clusters (74%) and a smaller proportion of groups >2 (16%), while CRF01_AE networks (44 groups) included 59.1% male-only clusters, with groups >2 accounting for 22.7%. Factors associated with being in a cluster of any size include; being sequenced in a current time era and being younger ($p<0.001$), being male ($p=0.023$) and having a B subtype ($p=0.02$). Being in a larger cluster (>3) is associated with being sequenced in a current time era ($p=0.05$) and being male ($p=0.008$).

Conclusion: This nationwide study of HIV-1, involving 4,873 patient sequences, highlights the increased diversity of HIV-1 subtypes within the Australian epidemic, as well as differences in transmission networks that are associated with these HIV-1 subtypes. These findings provide epidemiological insights not readily available using standard surveillance methods and can inform the development of effective strategies for prevention of new HIV-1 diagnoses within Australia.

INTRODUCTION

HIV-1 is a genetic variable and rapidly mutating virus, with ongoing high rates of mutation and recombination associated with an error-prone and non-proofreading reverse transcriptase activity [1]. There are four distinct HIV-1 groups (M, N, O and P) of which the M group accounts for 90% of infections. Within the M group there are nine phylogenetically distinct subtypes (A-D, F-H, J and K) along with an increasing number of inter subtype recombinant forms, with 72 identified to date. The main HIV-1 subtypes have distinct geographical associations that can provide useful epidemiological information [2], although there is growing evidence of increasing subtype and inter-subtype HIV-1 genetic diversity in regions previously characterised by specific HIV-1 subtypes [3-6]. Globally, HIV-1 subtype C infection has the highest prevalence with strong associations with sub-Saharan Africa and India, followed by A subtype (east Africa) and B subtype (western Europe, United States and Australia); these jointly account for 70% of HIV infections [2,7]. Other major subtypes (F, H, J and K) have remained stable at low levels, accounting for around 1% of infections worldwide, whilst HIV-1 D-subtype has decreased over time [2]. Circulating recombinant forms of HIV-1 account for 20% of infections worldwide and have increased in proportion by 17% during 2000-2007, although unique recombinant forms (URFs) have diminished by 3.1% over time [2].

Although HIV-1 subtype diversity is known to exist in Africa, where HIV infection has been long established [2,8,9], there is recent evidence of migration of previously geographically-restricted HIV-1 subtypes to broader regions of the world [2,4-6,10,11]. This increasing HIV-1 diversity may have important clinical implications given that HIV-1 subtypes have been associated with differences in disease progression [12-14], transmissibility [15,16] susceptibility to antiretroviral therapy [12, 17], HIV-1-specific immune responses relevant to *in vivo* infection and vaccine design [18], as well as risk of age-related diseases [19]. Importantly, increased HIV-1 subtype diversity including inter-subtype recombinant forms pose a challenge in HIV diagnostic laboratories, particularly pertaining to HIV-1 RNA assays where the accuracy of results may be influenced by sequence variation among non-B HIV-1 subtypes [20].

Australia has a history of strong public health and clinical management strategies that have contributed to a low national HIV-1 prevalence of ~158 per 100,000, with ~26,800 people currently living with HIV [21]. Despite this, the number of new diagnoses in Australia has steadily increased over the past 13 years with a 26% increase in the population rate since

2003, including 1,236 new cases in 2014 representing a 10% increase over the numbers diagnosed in 2011 [21]. This is set against an overall downward global trend in new HIV-1 diagnoses, most notably in sub-Saharan Africa and also evident in the Asia Pacific region [22].

The HIV epidemic in Australia has previously been characterised by a high prevalence of HIV-1 B subtype in all risk categories [23-25]. Recently however, there has been a reported increase of migration by travellers [26] to and from areas where known HIV-1 diversity has been established or from areas where HIV-1 infections are increasing or highly prevalent [22,27,28] and among risk categories other than men who have sex with men (MSM) [27]. Given the ongoing rise in new HIV infections in Australia, investigating the distribution of HIV-1 subtypes in Australia may provide valuable information that can inform prevention strategies, while also ensuring that laboratory monitoring is appropriate for the local epidemic. This concept is supported by recent epidemiological studies of HIV-1 sequences that have shown marked increases in the prevalence of HIV-1 non-B subtypes and circulating recombinant forms (CRFs) [3,4,10].

Sequence analysis of the HIV-1 *pol* region remains the gold standard for drug resistant mutation classification due the class of antiretroviral therapies used to treat HIV-1, however, the same sequence can be utilised for HIV-1 subtype determination as well as phylogenetic analysis. Phylogenetic analysis of HIV-1 can be used to monitor sequence similarities, follow the introduction of new subtypes, and has increasingly been utilised to characterise transmission clusters and networks in order to trace the mobility of HIV-1 globally [29-31] including over time analyses [32-34] and within different risk groups [35,36]. This methodology has been applied at a global scale to study transmission networks [29] and to investigate the role travel plays in the spread of HIV-1 within Europe [6, 33], the United States [32] and the United Kingdom [35]. These approaches are useful to gain a better understanding of sequence dynamics which is proving essential for real time HIV-1 surveillance and potentially suitable to prevent further HIV infections.

In order to provide a national population-based platform for these analyses, we have established a collaborative network of all Australian HIV-1 sequencing laboratories, which together provide accredited HIV-1 sequencing for clinical management throughout all Australian states and territories. This network has been identified as the Australian Molecular Epidemiology Network (AMEN). In this study, we have performed a retrospective analysis of predominantly baseline (pre-treatment) HIV-1 sequences to determine the subtype distribution and phylogenetic structure of HIV-1 within Australia during the period 2005-

2012, with the aim of supporting rational, evidence-based approaches to prevent, treat and monitor HIV-1 infection within Australia and its linked transmission networks.

MATERIALS AND METHODS

Sample population and dataset collation: This study was conducted within the framework of the Australian Molecular Epidemiology Network (AMEN). The AMEN is a collaborative network formed to include HIV laboratory services across Australia (Western Australia, South Australia, Victoria, Queensland and New South Wales: represented in this study by western Sydney). Samples notified in the Northern Territory and in Tasmania were also represented in the dataset as sequencing was conducted through Victoria. State and national ethics and governance frameworks were established to include assessment of de-identified HIV-1 sequences where a unique identification number was assigned, along with the notification of each state, the year the sequence was performed, the gender and the age of the patient at the time of sequence. Data collection and analysis was performed on base line HIV-1 plasma RNA samples analysed throughout Australia from 2005-2012 with the exception of Queensland where sequences from 2007-2012 were provided. In the case where multiple sequences were identified, the earliest sequence was determined to be the base line sequence for the analysis. A total of 4,873 sequences were assessed and stratified over 4 time eras based on the year of sequence (2005-2006, 2007-2008, 2009-2010 and 2011-2012).

HIV-1 subtype determination: Protease (PR, positions 1-99 in HXB2) and reverse transcriptase (RT, positions 20-240 in HXB2) sequences were amplified according to each state using in-house techniques (Western Australia [37], South Australia [38], Victoria [39], and western Sydney) or commercially available methods (Queensland; Viroseq HIV-1 Genotyping System, Abbott Celera). The HIV-1 subtype determinations were assigned by each state by submitting the fasta files to the calibrated population resistance tool linked to the Stanford calibrated HIV database (<http://hivdb.stanford.edu/>) with the exception of Victoria who submitted to the Los Alamos database (<http://www.hiv.lanl.gov>). For the assessment of inter-subtype recombination sequences, all sequences were submitted to four alternative phylogenetic analysis tools including COMET, SCUEAL, pgHMM and REGA. A consensus was assigned when 3 or more tools (including Stanford) obtained the same HIV-1 subtype result.

All laboratories sequence assays were monitored for quality according to the National Association of Testing Authority (NATA), Australia accreditation standards and subjected to

quality control procedures and the Royal College of Pathologists of Australasia (RCPA) or alternative quality assurance programs.

HIV-1 phylogeny approach: Sequence alignments and processing: Data collation and analysis was performed on all de-identified samples at one site (Western Australia). This process involved utilising the BioEdit tool [40], for sequence alignments and assessing sequences quality, while the Molecular Evolutionary Genetics Analysis Version 6 (MEGA V6) phylogenetic tool [41], was used to construct the phylogenetic tree and infer clustering patterns of similar sequences as previously described [33].

Characterisation of sequence similarities pertaining to clusters: In keeping with previous analyses we removed drug resistance sites then defined clustering patterns of similar sequences according to the following conservative approach: (1) paired sequences where two sequences group together according to the criteria where the bootstrap (BS) value was $\geq 98\%$ and the genetic distance (GD) was $\leq 1.5\%$; and (2) a “cluster or network” where more than two sequences had a BS value of $\geq 98\%$ and a GD $\leq 1.5\%$.

Statistical analysis: Statistical data analysis of demographic was performed using Statistical Package for the Social Sciences version 21.0 (SPSS v21: Armonk, NY: IBM Corp). Data distribution was assessed for normality then subjected to statistical analysis using T tests, ANOVA and post hoc tests with correction for multiple comparisons in multivariable analyses. Results were considered statistically significant when P-values <0.05 .

RESULTS

Data distribution within the AMEN HIV-1 cohort: The baseline HIV-1 sequence contribution from each Australian state from 2005-2012, and the corresponding subtype diversity for 4,873 sequences are shown in Table 1. Samples submitted by Victoria (n=1,668, 34%) and Queensland (n=1,579, 32%) account for 66% of the total samples for the study while Western Australia (15.5%), Western New South Wales (11%) and South Australia (7.5%) account for 44% of the sequences. Overall, 3631 (74.5%) of HIV-1 patients were infected with subtype B virus while 1,242 (25.5%) were infected with non-B viruses including A, C, D F, G, CRF01_AE, CRF02_AG subtypes and other inter-subtype recombinant forms. Males were more likely to be infected with HIV-1 (n=4,203, 86%) than females (n=648, 13%), as described in Table 2 however females were more likely to be infected with non-B subtype viruses compared with males (females: 436/648, 67% versus

males: 796/4,203 19%, $p < 0.0001$). Females with a non-B subtype were on average six years younger than non-B males (females: 32.5 years vs males: 38.5 years) and 3.8 years younger than females with a B subtype (gender difference $p < 0.001$; viral subtype difference $p = 0 < 0.001$) while females with a B subtype were approximately three years younger than males with a B subtype (females: 36.3 years vs males 39.2 years, gender difference $p < 0.001$).

HIV-1 subtype diversity distributions from 2005-2012: As shown in Figure 1, a substantial increase in the proportion of HIV-1 non-B subtypes has been demonstrated within each state over time, with Western Australia having higher baseline non-B sequences in the earliest time era (32.3% in 2005-2006), rising to approximately 48% in the period 2011-2012. Interestingly, the substantial increase in HIV-1 non-B subtypes observed in South Australia, western Sydney and Queensland occurred during 2009-2010 while Victoria showed a steady increase from 2007-2012. Overall Queensland had the lowest proportion of non-B subtypes in the study (21%) while Western Australia had the most (48%).

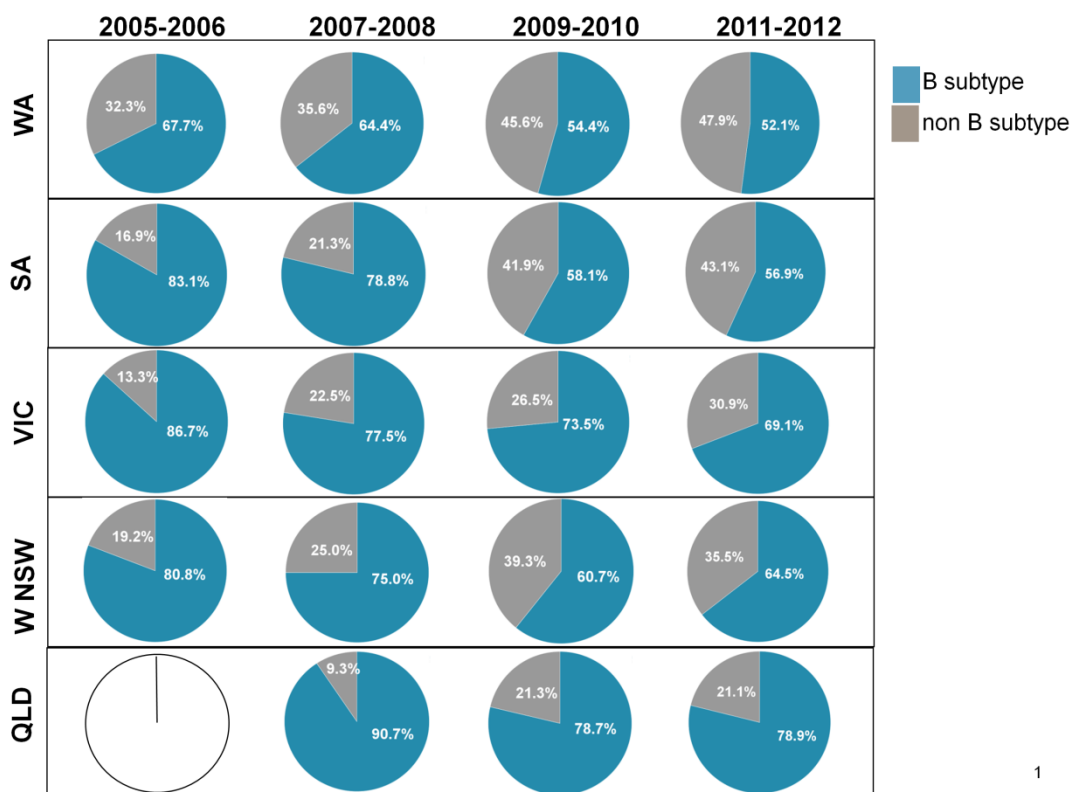


Figure 1: HIV-1 subtype distribution in Australia over time. Four eras, 2005-2006, 2007-2008, 2009-2010 and 2011-2012 show the changing subtype distribution for 4,873 sequences.

Table 1: Contribution toward the AMEN epidemiological and diversity study, by state, showing HIV-1 subtype characteristics and baseline sequence information.

<i>State</i>	<i>Method</i>	<i>Year</i>	<i>Sequence (n)</i>	<i>SUBTYPE ID</i>							<i>Hospital/ Service</i>
				B Subtype	CRF01_AE Subtype	C Subtype	CRF02_AG Subtype	D Subtype	Recombinant Forms	Other Subtypes	
WA	Stanford dB	2005- 2012	724	427	139	109	17	5	23	4	RPH-DCI
SA	Stanford dB	2005- 2012	351	240	38	35	20	1	9	8	SA Health
VIC	Los Alamos dB	2005- 2012	1668	1261	174	153	10	4	49	17	VIDRL
W- NSW	Stanford dB	2005- 2011	551	407	43	54	21	3	13	10	Westmead
QLD	Stanford dB	2007- 2012	1579	1296	90	124	20	8	34	7	Pathology QLD HIV Reference Lab
Total			4873	3631	484	475	88	21	128	46	

Each state was represented by a range of HIV-1 subtypes including B, C, D, CRF01_AE, CRF02_AG subtypes and other inter-subtype recombinant forms as shown in Figure 2. There are proportionally more C (15.1%) and CRF01_AE (19.2%) subtype sequences in Western Australia, more B subtypes (82%) sequences in Queensland and more CRF02_AG (5.7%) subtype sequences in South Australia than other states while inter subtype recombinant forms are represented in all states (discussed below).

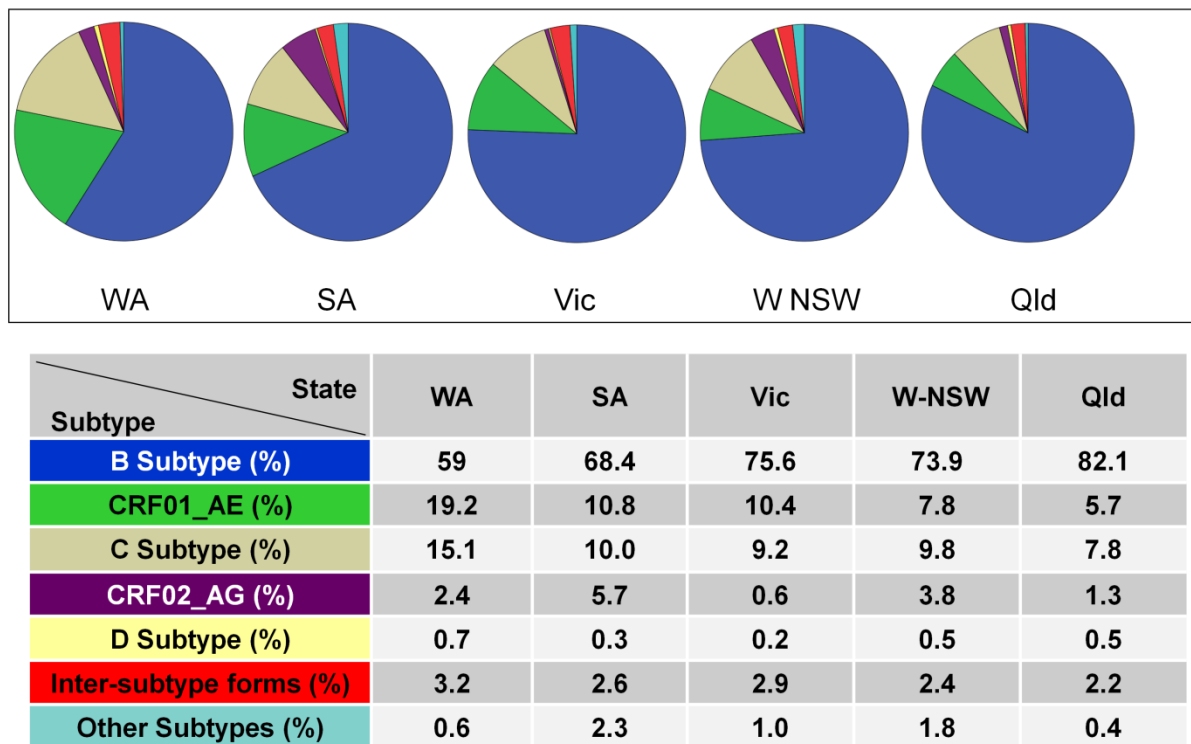


Figure 2: The distribution and proportion of HIV-1 subtypes present in each Australian state. The pie graphs represent the proportions while the table shows the proportion for each state per HIV-1 subtype.

Phylogenetic analysis: Overall we identified 1,135/4,873 sequences (23%) that were classified within a phylogenetic cluster, with no differences in the proportion of B versus non-B subtype sequences in a phylogenetic cluster (B subtype 829/3,631 (23%); non-B subtype 306/1,242 (24.5%), $p=0.3$). The phylogenetic trees based on HIV-1 partial *pol* sequences of B subtypes and non-B subtypes are represented in Figure 3A and 3B respectively. From the total number of pairs and clusters with more than two sequences ($n=419$) there were 286 paired sequences, 115 clusters with 3-5 sequences, 17 clusters with 6-13 sequences and one outlying cluster consisting of 29 related sequences.

Table 2: Demographics for HIV-1 B subtype, non-B subtypes and sequences in a network including age, gender, AMEN centre and sequencing era.

Characteristic	All sequences	B subtype	Non-B subtype	Sequences in a network (%)	Sequences not in a network (%)
All Sequences	4873	3631	1242	1135 (23.1)	3738 (76.9)
Gender					
— Female	648	212	436	142 (21.9)	506 (78.1)
— Male	4203	3407	796	991 (23.6)	3212 (76.4)
— Other	22	12	10	2 (13.0)	20 (87.0)
Age (Years)					
— Male	38.3	39	36.4	36.5	38.9
— Female	39.2	39.2	38.5	39.6	37.2
— <18yo (n)	33.8	36.3	32.5	31.7	34.3
— ≥18yo (n)	80	23	57	26	54
	4773	3595	1178	1103	3645
AMEN centre					
— WA	724	427	297	178 (24.6)	546 (75.4)
— SA	351	240	111	70 (19.9)	281 (80.1)
— VIC	1668	1261	407	439 (26.3)	1229 (73.7)
— WM NSW	551	407	144	84 (15.2)	467 (84.8)
— QLD	1583	1296	283	364 (23.1)	1215 (76.9)
Sequence Era					
— 2005-2006	809	651	158	119 (14.7)	690 (85.3)
— 2007-2008	1231	989	242	256 (29.8)	975 (70.2)
— 2009-2010	1424	1016	408	359 (25.2)	1065 (74.8)
— 2011-2012	1409	975	434	401 (28.4)	1008 (71.6)

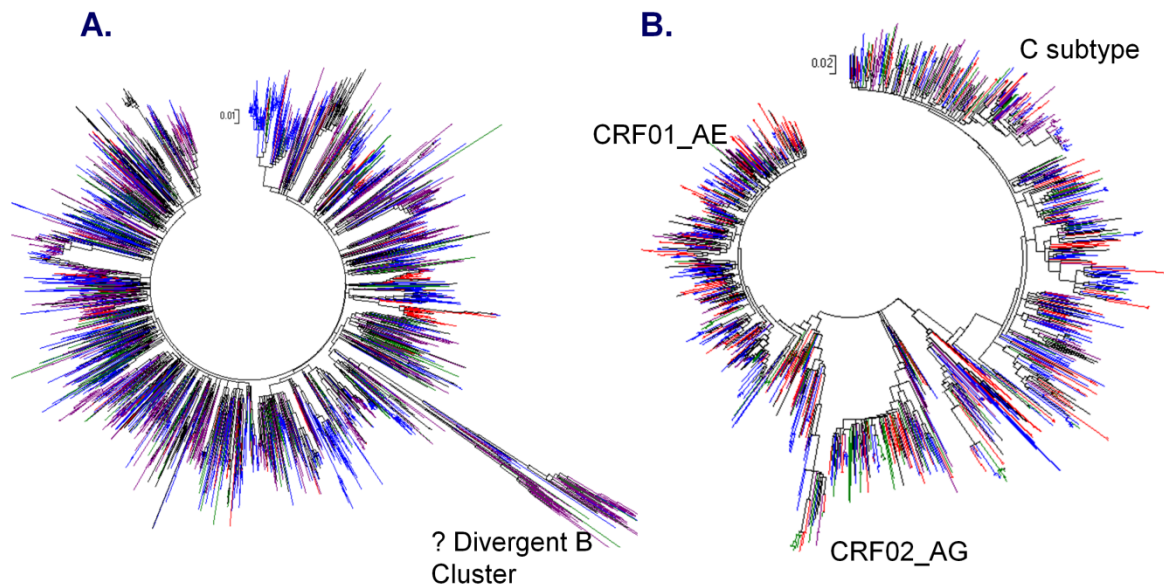


Figure 3: The phylogenetic tree constructions based on HIV-1 *pol* sequences for (A) HIV-1 B and (B) non-B subtypes assessed in the AMEN study. Each branch colour represents a state (Western Australia (black), South Australia (red), Victoria (blue), western Sydney (green) and Queensland (maroon)).

Analysing these results further in terms of subtype distribution, we found proportionally more pairs (two similar sequences) within the C subtype analysis (77%; Figure 4A) compared to those within the CRF01_AE or B subtype analysis (64%; Figure 4B and 47%; Figure 4C), along with significantly more non-B pairs than non-B larger clusters (>2 similar sequences: 94 vs 29, $p=0.003$) and significantly more larger clusters with a B subtype than a non-B subtype (45% vs 24% $p=0.021$).

At a national level, the dynamics of gender distribution within clusters are remarkably different between HIV-1 B, CRF01_AE and C subtypes (Figure 5A, Anova: $p<0.001$). We show, as expected, a higher proportion of B subtype sequence clusters include only males (88%) compared to CRF01_AE and C subtype (53% and 17% respectively). Significantly more C subtype clusters include both males and females (74%) compared to CRF01_AE (39%) and B subtype clusters (11%).

The analysis of B and non-B subtype clusters according to gender can be further assessed by a “state by state” approach. Within the HIV-1 B subtype (Figure 5B), most states have high proportions of male-only clusters (>84%), with Victoria registering the highest proportion

(95%), however South Australia and western Sydney have lower proportions of male-only B subtype clusters (64% and 68% respectively) due to higher proportions of male/female clusters (36% and 18% respectively).

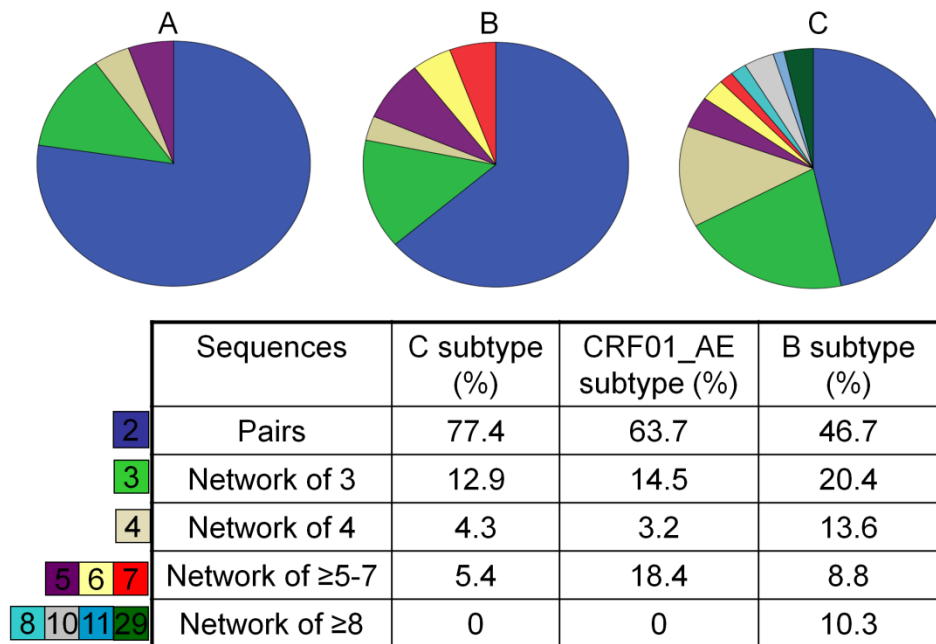


Figure 4: Differences in the distribution and proportions of pairs and larger cluster size for HIV-1 subtypes; HIV-1 C subtype (A), CRF01_AE subtype (B) and B subtype (C) (excludes singletons).

When assessing the non-B cluster dynamics, surprisingly, Victoria has a larger proportion of male only non-B clusters (63%) followed by Queensland (44%) than other states (Figure 5C). Conversely, western Sydney, Western Australia and South Australia experienced the highest proportion of non-B males clustering with females (73%, 67% and 65% respectively) followed by Queensland and Victoria (49% and 32% respectively). More non-B female only clusters were represented in South Australia (13%) than in any other state.

Analysis of large sub-epidemic HIV-1 clusters: In the large sub-epidemic cluster analysis we found five distinct clusters with more than nine similar sequences represented per cluster. The largest sub-epidemic cluster was represented by sequences within HIV-1 B subtype analysis. This cluster was initially established in 2008 by three similar sequences then evolved to 29 patients by 2012. All sequences were from males, including 27 sequences from Western Australia and two from Victoria, with a median age of 47.5 years (range 23-70). The overall mean genetic difference for this cluster was 0.3% (range, 0.2%-1.2%) with a

bootstrap value of 99%. Other B subtype clusters include two clusters of 10 sequences, one cluster of 11 sequences (all male only clusters and all from Victoria) and one male/female cluster of ten sequences which included sequences from three states (Western Australia, South Australia and Victoria), with 80% of the sequences notified in South Australia.

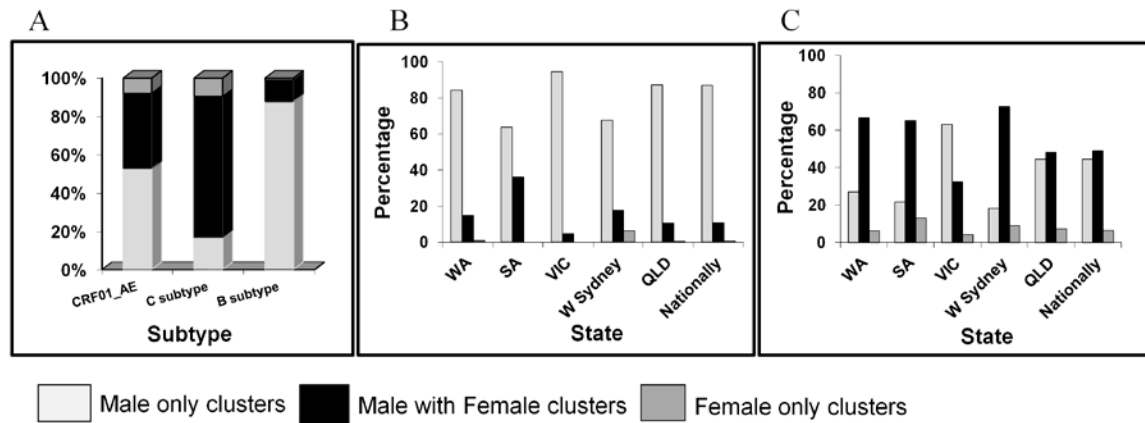


Figure 5: Gender distribution within clusters according to HIV-1 subtypes (A) and for clusters in each state with B subtype (B) or a non-B subtype (C).

Factors influencing large HIV-1 networks of similar sequences: We employed a multivariate regression analysis to show factors associated with being in a cluster of two or more similar sequences and furthermore, factors associated with being in a large cluster (>3 sequences), with both analyses excluding children <18 years of age.

The analyses identifying factors with being in a cluster (regardless of size) are presented in Figure 6A and show, overall at a national level, associations with being younger (β -0.003; $p < 0.001$), having a sequence performed during later era (β 0.05; $p < 0.001$), being male (β -0.01; $p = 0.023$) and having a B subtype (β -0.05; $p = 0.021$) however no associations with Australian states were found (β 0.01 $p = 0.4$). Differences within each state revealed age was associated with being in a cluster for sequences from Western Australia, western Sydney and Queensland, while being sequenced in the current era was a significant factor for being in a cluster for all states besides western Sydney. Being male was a significant factor for being in a cluster in Victoria while, interestingly, having a non-B sequence was associated with being in a cluster in Queensland but not in a large cluster size (>2).

When we assessed factors associated with being in a larger cluster only (>3 sequences in a cluster) compared with smaller clusters of two or three sequences (Figure 6B), we found a weak association with having a sequence performed during later era (β 0.03; $p = 0.05$) and a strong association with being male (β -0.12; $p = 0.008$). In Western Australia, having an HIV-1

B sequence (β -0.34; $p < 0.001$) and being sequenced in a current era (β 0.09; $p = 0.004$) were associated with larger cluster size. Age was associated with large cluster size in western Sydney (β -0.01; $p = 0.04$) and Queensland (β 0.006; $p = 0.01$) while being male was a significant factor in Victoria (β -0.4; $p < 0.001$). There were no factors associated with large cluster size in South Australia.

A	Overall	WA	SA	VIC	W Sydney	QLD
AGE	-0.003 <i>p</i> <0.001	-0.005 <i>p</i> <0.001	-0.002 <i>p</i> =0.3	-0.001 <i>p</i> =0.34	-0.008 <i>p</i> <0.001	-0.003 <i>p</i> =0.003
SEQ ERA	0.05 <i>p</i> <0.001	0.05 <i>p</i> <0.001	0.07 <i>p</i> <0.001	0.03 <i>p</i> =0.002	0.02 <i>p</i> =0.13	0.04 <i>p</i> =0.001
Gender	-0.01 <i>p</i> =0.023	-0.06 <i>p</i> =0.17	0.02 <i>p</i> =0.69	-0.09 <i>p</i> =0.03	-0.02 <i>p</i> =0.59	-0.02 <i>p</i> =0.54
Non-B vs B	-0.05 <i>p</i> =0.021	-0.07 <i>p</i> =0.058	-0.08 <i>p</i> =0.11	0.05 <i>p</i> =0.08	-0.02 <i>p</i> =0.67	0.07 <i>p</i> =0.02
State	0.01 <i>p</i> =0.41	-	-	-	-	-

B	Overall	WA	SA	VIC	W Sydney	QLD
AGE	0.002 <i>p</i> =0.2	-0.001 <i>p</i> =0.72	-0.001 <i>p</i> =0.8	0.001 <i>p</i> =0.77	-0.01 <i>p</i> =0.04	0.006 <i>p</i> =0.01
SEQ ERA	0.03 <i>p</i> =0.05	0.09 <i>p</i> =0.004	-0.003 <i>p</i> =0.96	0.003 <i>p</i> =0.88	0.02 <i>p</i> =0.7	0.03 <i>p</i> =0.4
Gender	-0.12 <i>p</i> =0.008	-0.02 <i>p</i> =0.86	0.03 <i>p</i> =0.8	-0.4 <i>p</i> <0.001	0.13 <i>p</i> =0.17	-0.04 <i>p</i> =0.6
Non-B Vs B	-0.03 <i>p</i> =0.34	-0.34 <i>p</i> <0.001	-0.07 <i>p</i> =0.6	0.09 <i>p</i> =0.12	-0.07 <i>p</i> =0.51	0.03 <i>p</i> =0.7
State	0.003 <i>p</i> =0.7	-	-	-		

Figure 6: Multivariate regression analysis reveals factors associated with a sequence being classified into (A) a cluster regardless of cluster size or as a singleton or (B) only sequences in larger networks where the sequence size equals 2-3 or >3. All results are for >18yo (Coefficient and significance are shown).

Phylogenetic clusters within and between Australian states: Monitoring sequence similarities within and across state boundaries was assessed within both the HIV-1 B and non-B subtype analysis (data not shown). Sequences from Victoria were more likely to cluster with sequences from other states regardless of being a B or non-B subtype (72/190 sequences (38%) and 18/52 sequences (35%) respectively), followed by Western Australia (B subtype 49/190, 26%) and Queensland (non-B subtype 13/52, 25%). Overall we showed the least migration of non-B sequences from Western Australia and South Australia and B sequences from South Australia and western Sydney.

Analysis of HIV-1 inter-subtype recombination sequences: Inter-subtype recombination sequences were identified from partial *pol* sequences using the Stanford HIV database tool for all states as shown in Figure 2. The highest proportions were observed in sequences from Western Australia (3.2%) followed by Victoria (2.9%). By applying 4 alternative phylogenetic subtype assigning tools (COMET, SCUEAL, jpHMM and REGA) to the 128 inter-subtype recombinants and five pure subtypes identified using Stanford and the large branch of divergent sequences (n=69) highlighted in Figure 3A, a consensus subtype was generated (Table 3). The results show we identified 45.5% (92/202) of the sequences differing to the results generated from Stanford while for 41% (83/202), no consensus was reached when applying the alternative tools. Only 13.5 % (27 sequences) generated the same results as Stanford.

The divergent branch of B subtype sequences (generated via Stanford tool) had disparate results when compared the consensus results including 39/69 (56.5%) having a C, CRF01_AE or A subtype, 25/69 (36.2) did not reach consensus and 5 were assigned as an inter-subtype recombination.

DISCUSSION

The outcomes of this study highlight an HIV-1 epidemic in Australia that is characterised by an increasing prevalence of non-B HIV-1 infections as well as increasing overall HIV-1 subtype diversity. The dominant non-B subtypes, C and CRF01_AE, identified here reflect the main subtypes represented among the sub Saharan African and south east Asian populations, with phylogenetic analysis indicating that these non-B HIV-1 subtypes are more likely to involve heterosexual HIV-1 transmission networks. Similar proportions of HIV-1 C subtype infections among males and females in this study is consistent with the distribution of heterosexual transmissions documented in the sub Saharan Africa region [42] while the

increase proportions of females and males infected with CRF01_AE subtype correspond to patterns of heterosexual and increasing MSM transmissions and injecting drug use (IDU) present in south east Asia [43].

Although in the overall analysis we identified 25.5% of non-B subtypes, which is lower than observed in North America [4], Europe [6] and Belgium [44], the impact of time on the change of non-B HIV-1 subtype proportions for each Australian state highlights interesting patterns. Western Australia recorded higher baseline non-B sequences early in the analysis reflecting an earlier increase of overseas migration patterns than in other states, however the other states in Australia soon followed the pattern albeit at lower proportions. This could be attributed to by the economic surge in Western Australia during 2005-2012 and also due to a noticeable increase in overseas tourism to Asian countries. During 2006-2011 many overseas migrants that settled Western Australia were from South Africa [45] where C subtype dominates [9]. More recent supporting data identified Thailand, Malaysia, China and India as being in the top 10 main countries where increased visitor arrivals into and resident departure from Australia [26] were recorded. A recent report also documented human mobility of CRF01_AE subtype into countries previously identified with low rates of CRF01_AE transmissions, has traced and confirmed these from Asian countries [30]. By the end of the study period the proportions of HIV-1 non-B subtypes identified in Western Australia reflected the increased diversity seen in other countries [4,44].

This study highlights the growing contribution of migration and travel – both within Australia and overseas – to increased HIV-1 subtype diversity within Australian states and at a national level. This is consistent with evidence of sharp increases in HIV-1 subtype diversity in the United States [4,32,46] and Europe [3,33-35]. Several previous investigations from Australia have also recognised the evolution from an early epidemic characterised by predominantly B subtype HIV-1 with limited genetic diversity [23] to recent studies showing increased HIV-1 non-B subtype diversity in Australia [38,39].

HIV-1 phylogenetic analysis provides an important objective resource for dynamically assessing new HIV infections and monitoring geographical changes in the global HIV epidemic. In this study, in which we classified phylogenetic clusters using a conservative approach that required low genetic distance between sequences ($\leq 1.5\%$) as well as high bootstrap values ($>98\%$), distinct transmission network patterns for B, C and CRF01_AE subtypes were revealed.

Table 3: A comparison of Stanford HIV-1 subtyping results compared to the consensus reached from COMET, SCUEAL, jpHMM and REGA for pure subtypes and inter-subtype recombination.

	Results from Stanford			Total
	Same as the Consensus (n%)	Different to the Consensus (n,%)	No consensus reached (n,%)	
Pure HIV-1 subtypes	7 (3.5)	40 (20)	27 (13.5)	74 (37)
Inter-subtype recombination	20 (10)	52(25.5)	56 (27.5)	128 (63)
Total	27	92	83	202

As expected, more pairs and large networks were identified within male-only groups in the B subtype analysis, with higher proportions of male-and-female clusters in the C subtype analysis than in the CRF01_AE analysis.

A state by state approach revealed distinct dynamics among transmission groups with higher proportions of male-only non-B subtype networks represented in Victoria indicating the likely establishment of local MSM transmission networks, while the large proportion of male-female pairs within non-B transmission networks in western Sydney, South Australia and Western Australia suggest limited forward transmission into local networks.

The observation of a distinctively large and expanding B subtype male network, established in 2008 with three patients and evolving to 29 by 2012, is in line with other epidemiological studies [6,44] showing one distinct large population among smaller pairs and networks. The impact of this requires further exploration particularly in terms of assessing clinical parameters, viral fitness and targeting strategies given a recent US study showed large cluster size was associated with CD4⁺ T cell counts >350 cells/ μ l and HIV-1 RNA levels >10,000 copies/mL [32]. This observation is also supported by a recent study within Western Australia [37], identifying challenges as well as opportunities to prevent local transmissions within the MSM networks.

The analysis of inter-subtype recombination sequences along with the divergent B subtype sequences using COMET, SCUEAL, pgHMM and REGA programs revealed similar findings to previous studies, suggesting that no single online tool could accurately subtype 100% of the sequences [47]. Each tool performed slightly differently due to unique methods and algorithms including differences in access to up-to-date reference sequences. The findings from our study support this, as we obtained an overall consensus result of 59.2% which diminished to 13.5% when comparing results to the Stanford calibrated tool only. Phylogenetic, statistical and similarity-based tools worked well for the identification of more common HIV-1 subtypes present in Australia such as B and C and CRF01_AE [47-49], however they may be problematic when identifying super infections [50].

Understanding patterns associated with HIV-1 subtypes within Australia is of significant interest as increased HIV-1 diversity pose new challenges to molecular laboratories as well as potentially having unfavourable clinical implications. Inaccurate HIV-1 RNA quantitation on non-B subtypes, mostly due to genetic variation at primer sites, has previously been reported [20] and this can have adverse outcome on disease progression, effective monitoring of treatment and potentially risk of transmissions from mother to child. For these reasons thorough evaluation of HIV-1 RNA viral load assays in light of local HIV-1 sequence

diversity is imperative for laboratories to offer an optimal service. Increased risk of drug resistance has been shown in non-B subtypes when treated with integrase inhibitors [51], while concerns regarding the potential failure of NNRTI/NRTI treatment within HIV-1 C subtype infection has also been described [17]. Overall there are many major drug resistant mutations observed in different HIV-1 subtypes and these require careful consideration in the context of increasing non-B subtype diversity [52]. HIV-1 D subtype, inter-subtype recombinants [13,14] and CXCR4 tropism [16] have been shown to be associated with rapid disease progression, noting that CXCR4 tropism has been associated with CRF01_AE subtype [53], while HIV-1 A subtype may have slower disease progression. All these factors may be important to HIV surveillance and patients care in developed regions where HIV-1 is becoming more diverse.

In this current investigation it is important to recognise each Australian state contributed differing numbers of HIV-1 sequences to the analysis, therefore overall proportions was used to analyse the data. To the best of our knowledge all the sequences submitted for assessment, with the exception of those submitted by Queensland, represented newly acquired HIV-1 infection. Due to the very strict criteria used to assign networks there is the potential to underestimate the number of pairs and networks identified in the study however it would prevent the inclusion of unrelated sequences into the results. This present study utilised a sizeable collection of sequences obtained from Australian routine clinical laboratories, represents a large portion of new HIV-1 diagnosis identified in Australia during 2005-2012 and using strict phylogenetic approaches demonstrates a comprehensive and updated analysis of the changing HIV-1 epidemic across Australia.

To our knowledge this analysis is the first longitudinal, large-scale assessment of HIV-1 subtypes and phylogenetic network patterns in Australia over an eight year period. The study highlights the key influences of migration and overseas travel on increasing rates of non-B infections, and reinforces the importance of Australia's engagement with regional and global aspects of the HIV epidemic, as well as the value of molecular epidemiology surveillance in the future.

The establishment of the Australian Molecular Epidemiology Network, and the results that have now been obtained from routine laboratory HIV-1 sequencing from 4,873 individuals, provide the basis for a greater understanding of the Australian HIV-1 epidemic that is complementary to existing national surveillance methods. The information presented can assist in the development of effective strategies for laboratories, as well as inform prevention

and treatment strategies that can favourably influence the HIV epidemic within Australia and beyond.

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Summary Chapter 6

The analysis of 4873 national de-identified sequences has provided a broad representation of the contemporary HIV-1 epidemic in Australia. Chapter 6 “Molecular epidemiology of HIV-1 in Australia 2005-2012: a national collaborative study”, identified the effects of migration and travel on the increasing HIV subtype diversity observed throughout Australia with particular emphasis on, 1) monitoring HIV-1 subtype diversity across Australia; 2) identifying HIV-1 migration patterns, with respect to time, across jurisdictions, and 3) recognising distinct dynamics within sequences that form HIV-1 clusters and networks.

The results indicate that new notifications of HIV-1 infections in Australia remain dominated by HIV-1 B subtype virus with increased proportions of HIV-1 non-B subtypes presenting over time. When monitoring this by state, the differences in the proportions of non-B subtypes present are fascinating. As shown in Chapter 5 and confirmed in Chapter 6 the HIV-1 epidemic in Western Australia has been confirmed as a national “hotspot” for HIV-1 subtype diversity, accounting for the largest proportions recognised in Australia. Migrating across Australia, higher proportions of non-B subtypes have been shown in South Australia and New South Wales (western parts of Sydney) followed by Queensland and Victoria.

6.1 Analyses of age distribution in the Australian HIV-1 cohort

The age distribution of the HIV-1 population in Australia is of growing interest particularly with respect to increased age-related complications in this cohort. A wide age distribution at the time of diagnosis within the Australian HIV-1 cohort is noted, as shown in the Western Australia analysis in Chapter 5, including differences among HIV-1 B and non-B subtypes as well as males and females (Figure 1) confirming HIV-1 in Australia does not only occur in young gay men. The analysis in Chapter 6 clearly shows a shift from this perception within the Australian cohort ranging from infants to 86 year olds, regardless of HIV-1 subtype including 105 children under eighteen (at notification date). Once again the results also suggest more children have been infected with HIV-1 non-B subtypes and again as previously noted, this is thought to be through perinatal transmissions [3]. From the patients in the analysis there are currently 40 children 18 years of age or younger as many have now entered adulthood. The age distribution plots from Chapter 5 represent a high proportion of children within Western Australia HIV-1 cohort having a non-B subtype. The current Australian analysis has confirmed that a greater proportion of children in the Australian HIV-1 population also have a non-B subtype suggesting the link between imported virus through

migration. Tying this in with the results from Chapter 5, it can be concluded many reside in Western Australia (13/40, 32%) and Queensland (12/40, 30%).

In terms of age according to network size, females and males in a large B subtype network are significantly younger than those in a singleton (females - 30.4 vs 37.0; males - 37.9 vs 39.7, $p < 0.01$), while males in a large non-B network are significantly younger than those in a non-B subtype singleton (34.7 vs 39.4, $p < 0.01$) and males in a B subtype network (34.7 vs 37.9, $p < 0.01$). HIV-1 B subtype infection is still the predominant subtype amongst men while women are more likely to be infected with a non-B subtype. Overall, the trend is that younger males and females are presenting in larger networks regardless of having an HIV-1 B and non-B subtype.

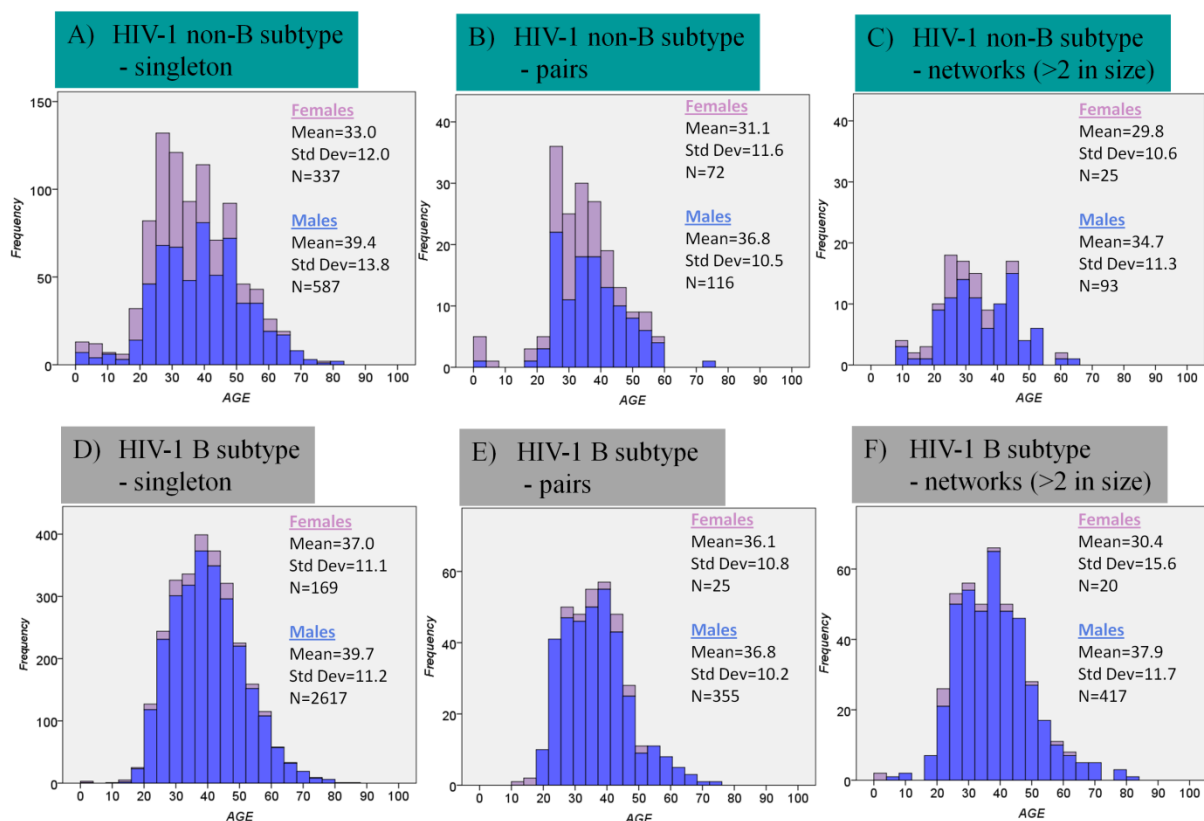


Figure 1: Age and gender according to HIV-1 subtype and network size. The age distribution plots for males (blue) and females (pink) with HIV-1 non-B subtypes (A-C) and B subtype (D-F). The ages for patients in a singleton (A+C), a pair (B+E) or network (C+F) are also represented.

6.2 The analysis of networks within the Australian HIV-1 cohort

The analysis of HIV-1 subtypes, phylogeny and network size has revealed distinct patterns within some states (as summarised in Chapter 6) however this can be further defined by

assessing the network size within the HIV-1 B or non-B subtypes for each state (Figure 2). The results illustrate a large distribution of network size for Victoria within both the HIV-1 B and non-B subtypes. The proportion of larger B subtype networks is also evident in the Queensland cohort while HIV-1 sequences from the Western Australia cohort were well represented in a B subtype cluster of 29 sequences. Likewise, HIV-1 sequences from South Australia were well represented in smaller HIV-1 B subtype clusters of 10.

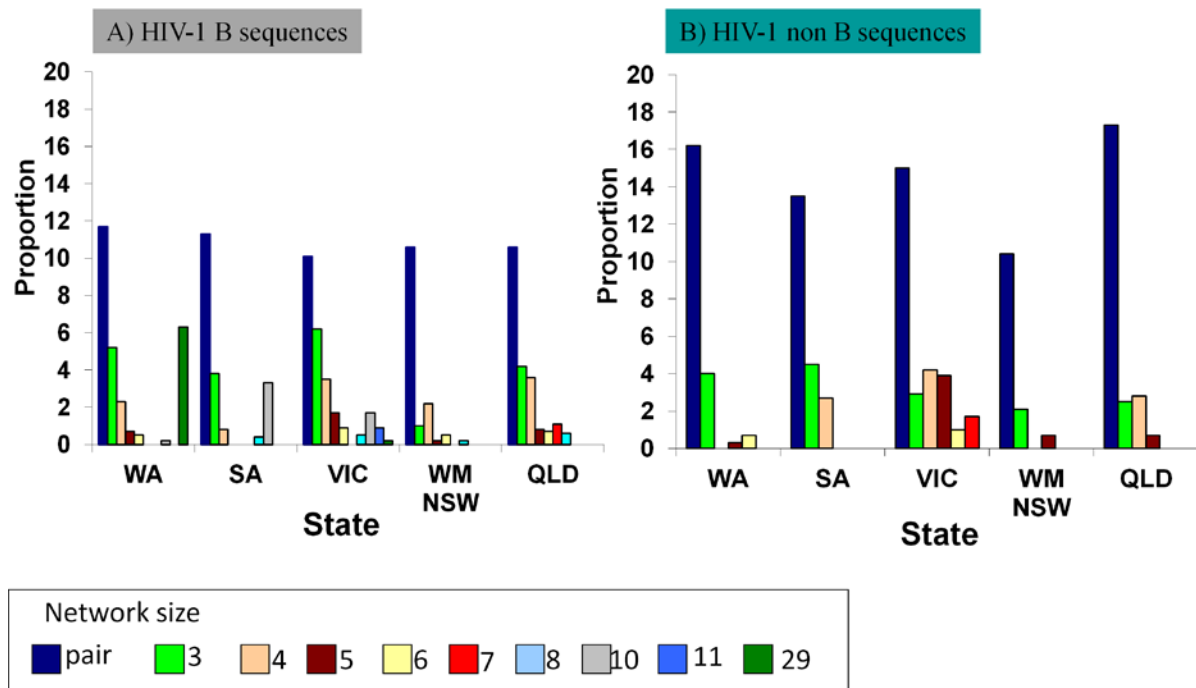


Figure 2: An Australian, state by state approach on network size within HIV-1 B (A) and non-B subtypes (B).

With the increasing rate of HIV-1 diagnoses in Australia continuing against global trends, an analysis of HIV-1 subtype pairs and networks detected over time is warranted to determine the impact on the HIV epidemic. To identify the era a pair or network appeared, the date of the 2nd or 3rd sequence in the pair or network (regardless of size) respectively, defined the time era nationally (Figure 3A).

Remarkably, both pairs and networks have grown over time with the largest proportion noted in the most current time era (2011-2012). Defining this further for HIV-1 non-B and B subtypes shows non-B subtype pairs and networks continue to appear over time, with strikingly large proportions for networks evident in the latest time era (Figure 3B; 2011-2012, 65%). Many of these larger, non-B subtype networks reside in Victoria and highlight the transmission of non-B subtypes into the MSM community. This suggests non-B networks are

being established in Australia and are being diagnosed and sequenced in recent time. The study also established that large networks exist between states (interstate networks) indicating across jurisdiction travel exists within the HIV-1 population.

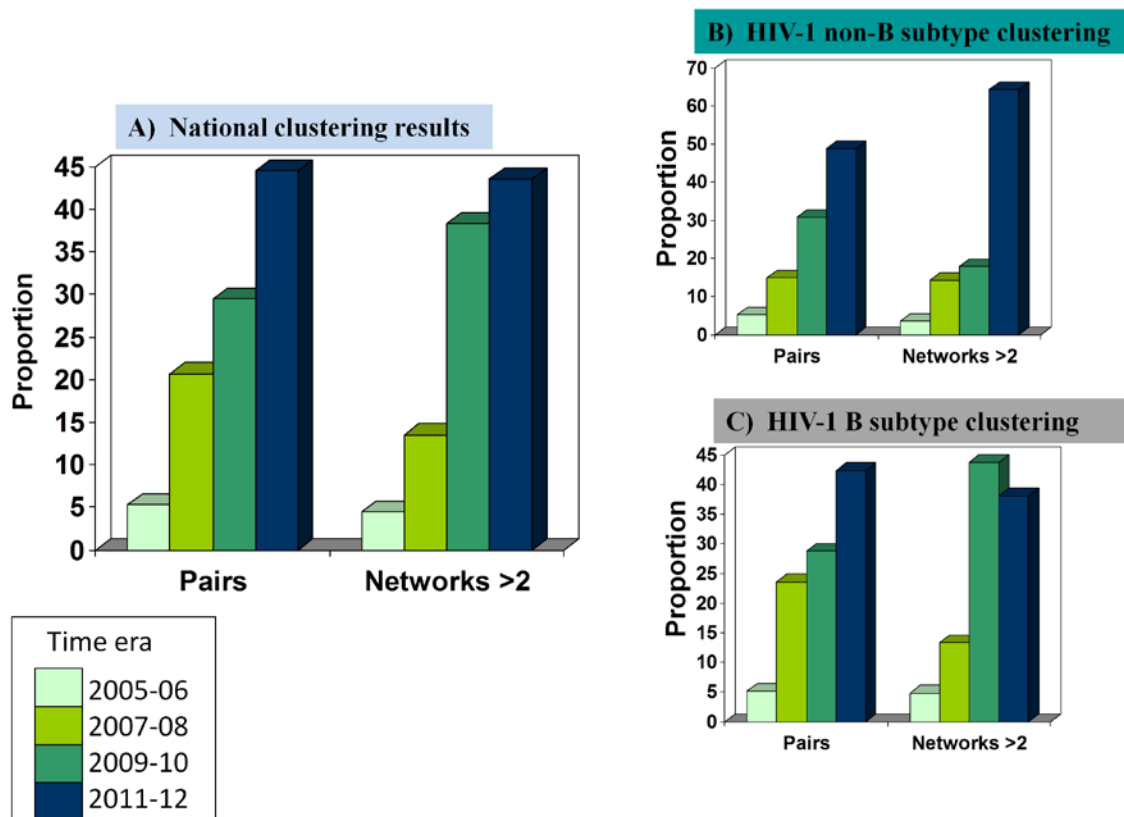


Figure 3: The proportion of HIV-1 subtype pairs and networks established nationally (A), for HIV-1 non-B (B) and B subtypes (C) over time.

The proportion of HIV-1 B subtype pairs was also deemed largest in the latest time era, while the largest proportion of networks within the B subtype analysis were present in the 2009-2010 time era with a slight reduction in the following time era (Figure 3C).

The mobility of HIV-1 infections within Australia was also studied. Interestingly, we have shown across-jurisdiction migration with HIV-1 B subtype sequences in a network size of 5 or 6 with larger networks more likely to be “intrastate only” (Figure 4A). As the network size increases, HIV-1 non-B “interstate” networks are less common, however larger network sizes are more likely to include sequences from one state only (Figure 4B). These results suggest that there is evidence for imported HIV-1 virus into Australia however, although “interstate” networks exist within both HIV-1 B and non-B subtypes, it is generally at a lower proportion and occurs more frequently within the HIV-1 B subtype.

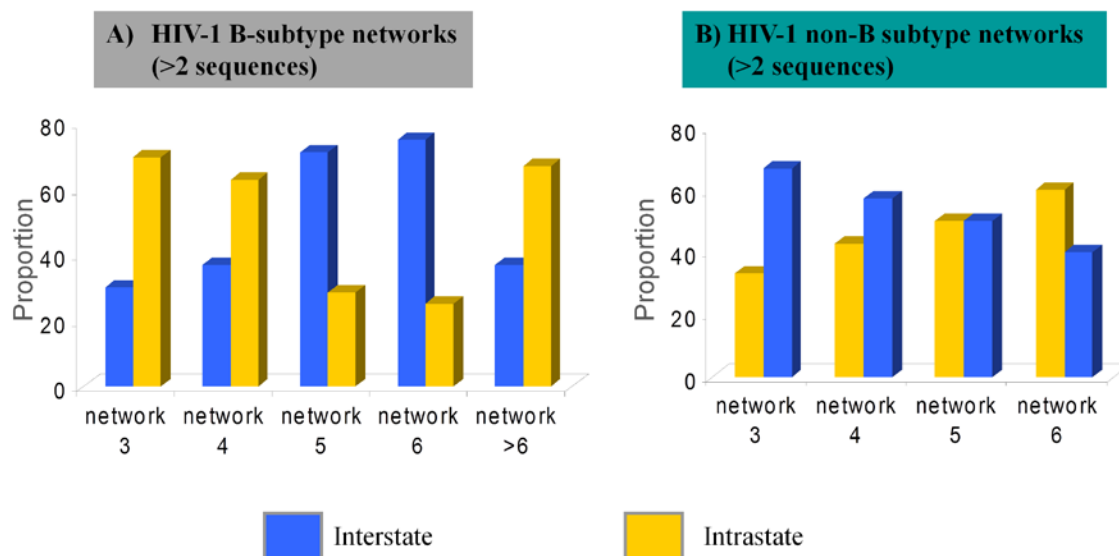


Figure 4: The proportion of “interstate” or “intrastate” networks based on the number of sequences in the network (>2 in size) and either having an HIV-1 B (A) or non-B (B) subtype.

To conclude, for the first time a retrospective, nation-wide HIV-1 phylogenetic analysis was performed to monitor HIV-1 subtype diversity and network dynamics within Australia. This analysis was not a contact tracing study but more about highlighting the changing dynamics of the HIV-1 epidemic within Australia utilising de-identified baseline viral sequences from 2005 which accounts for a high percentage of new HIV-1 notifications.

There are clear state similarities including a wide age distribution, general increases in HIV-1 non-B subtypes and similar HIV-1 subtype diversity, however there are state differences in network size for major subtypes. The analyses confirm the mean age of the Australian HIV-1 cohort at diagnosis is 38 years, however this only accounts for new notifications since 2005 and does not include patients notified before 2005 and currently living with HIV. Including these patients into the analysis would reveal an ageing population fast approaching a median of 50 years. Interestingly however, 16% of patients in this current analysis were older than 50 years of age (797/4873), which gives rise to a number of important patient care considerations, including the impact of age related illnesses and co-morbidity all of which add to the complications of current HIV management.

The impact of this multistate phylogenetic analysis requires further exploration particularly in terms of assessing clinical parameters, drug resistance, viral fitness and targeting strategies given a recent US study showed large cluster size was associated with CD4 counts

>350cell/ μ l and HIV-1 RNA levels >10,000 copies/mL [187] and confirmed with results presented in Chapter 5. These studies highlight an ongoing role of research using phylogenetic approaches linked in with HIV notification data to inform a prevention response in real time. This study also recognises challenges faced to prevent local and across jurisdiction transmissions within the MSM networks, imported HIV infections from overseas migrants and imported infections from Australian overseas tourism. The changing HIV-1 subtype diversity demonstrates the requirement for updated prevention strategies to include targeting messages for key populations regardless of age.

A collective summary for Chapters 5 and 6

The spread of HIV-1 variants in Australia has been largely driven by sexual contact within MSM and dominated by the HIV B-subtype. Although this remains evident within the current analysis, changing trends have been revealed in Chapters 5 and 6 with the main conclusions being:

- 1) The analysis of the HIV-1 epidemic in Western Australia highlighted a number of temporal trends over 15 years specifically revealing it as a “hotspot” for HIV-1 diversity, with an increased prevalence of HIV-1 non-B subtypes.
- 2) In Western Australia patients with an HIV-1 B subtype generally have earlier diagnosis than patients with a non-B subtype representing delayed diagnosis in this group.
- 3) Large networks are expanding despite early diagnosis and high uptake of HAART.
- 4) Local transmissions remain dominated by HIV-1 B subtype viral strains, mostly within male only networks, therefore targeting strategies towards MSM networks remains a challenging issue for HIV-1 prevention in Australia.
- 5) A distinct relationship between gender and HIV-1 subtypes revealing more females are infected with non-B viral strains along with more heterosexuals infected within non-B subtype networks.
- 6) As revealed in the age distribution plots, HIV-1 is not an infection only observed in young men. It is infecting both young and old within various risk groups.
- 7) Recognised impact of migration into Australia and Australian overseas tourism on HIV-1 notifications.
- 8) It remains interesting that only one very large cluster of 29 patients has formed within Australia over time and, also of interest, is that this cluster consists of sequences from only two states (Western Australia and Victoria) highlighting an important role for intra-state travel. Analysis of the same cluster in the Western Australia analysis revealed this cluster has escalated to 53 genetically similar sequences.
- 9) These studies highlight the cooperative approach that is required to effectively prevent new HIV cases in Australia, involving an increased appreciation of the role played by travel and immigration including associations with increased heterosexual HIV-1 transmission.

6.3 The challenges surrounding changes in HIV-1 subtype diversity

When a virus initially enters a host, the HIV viral population adapts rapidly by altering its genetic sequence at specific regions [191, 192]. This can potentially result in the inhibition of the host's combat mechanisms (i.e. ISGs), promoting escape from the immune system and increasing virus efficiency to enter immune cells. It is therefore critical to identify the HIV-1 strains circulating among the Australian population as this will aid ongoing HIV-1 surveillance and phylogenetic analysis that is required to explore biological differences between HIV-1 subtypes including differences to highlight what is driving the generation of large networks. Differences in HIV-1 genetic sequences among subtypes may carry functional differences, with subsequently increased spread through the population, leading to important clinical implications relating to the rate of disease progression, particularly in non-B subtypes [193-195], HIV-1 transmissibility [196], differences in drug-resistant mutations and susceptibility to HIV therapy within non-B subtypes [197, 198] and increased virulence of HIV-1 as shown by others [199] and in Chapter 5. These highlight the importance of monitoring the emerging HIV-1 diversity in real-time to assist in the maintenance of public health prevention strategies as well as individual patient care. Including additional data that acknowledges and addresses potential confounding influences (Medicare status, mode of transmission and host genetics-CCR5 Δ 32 mutation, HLA) may assist in clarifying clinical implications further.

6.4 Impact of HIV-1 inter-subtype recombination

HIV-1 inter-subtype recombination has contributed to the diversity of HIV-1 variants within the Australian epidemic. Currently 72 different inter subtype recombinants exist in the Los Alamos National Laboratory repository, with the likelihood that more of these stable circulating recombinant forms will be identified over time. These variants may have unique properties that could enable them to adapt within a host leading to increased prevalence [5] and replication capacity as previously shown in the spread of the CRF01_AE subtype in Thailand [200]. The genetic differences identified with inter subtype recombinant forms may have important clinical implications relating to the rate of disease progression as shown with the evolutionary fit CRF19_cpx strain [201] and CXCR4 tropism association with rapid disease progression [202] and CRF01_AE subtype [202, 203]. Increasing HIV-1 surveillance within Australia could potentially identify unique recombinants which will assist patient care

(especially in the current epidemic where CRF01_AE subtype is common in Australia) and in the development of an effective vaccine and microbicides.

6.5 Strength and limitations of phylogenetic approaches

Assessing sequence similarities has both strengths and limitations. Phylogenetic analysis approaches are more successful with large cohorts allowing for accurate identification of the overall patterns of HIV epidemiology within a region and have been particularly useful in assessing sequences and clustering dynamics from Western Australia and at a national level. By employing stringent clustering criteria, phylogenetic analysis provides insight into local transmission network patterns including identifying onward transmission of non-B subtypes and the evolution of large networks over time. It also provides a robust link between HIV-1 sequences available in an analysis, making it a useful tool in laboratory practices provided that mechanisms are in place to ensure that patient confidentiality is maintained. Although not assessed in this dissertation, phylogenetic analysis can also be used to identify HIV superinfections.

The analysis approach used throughout Chapters 5 and 6 does not clearly identify direct transmission. This is important given it is estimated that 14% of HIV infections in Australia remain undiagnosed therefore there is the possibility of not including genetically similar sequences in the analysis. Phylogenetic analysis does not identify when HIV transmission occurred or the duration of infection. Added to this, the AMEN analysis included HIV-1 viral sequences from western New South Wales, which account for a small set of HIV-1 infections diagnosed in New South Wales. Incorporating more viral sequences from this region would add valuable data in terms of subtype diversity and network dynamics. The analysis does not include the large sequence dataset of baseline sequences notified before 2005 and not all HIV-1 patients have a viral sequence. This is due to the constraints surrounding the performance of HIV-1 viral sequencing on plasma samples retrieved from patients whose HIV-1 RNA level is <1,000 copies/mL, therefore missing these HIV-1 sequences adds to the limitations of this study.

Another limitation of the AMEN study was the lack of clinical data, which would offer added value given the significant associations shown in Chapter 5 and other studies [187]. This would require partnerships with Ministries of Health at the state and national level, to assist in the incorporation of HIV notification and enhance surveillance information, together with the phylogenetic data, which would greatly improve public health strategies.

6.6 Overview of automated HIV-1 subtype sequence tools

The current laboratory practice in most Australian capital cities is to use the Stanford HIV database tool to assign HIV-1 subtypes generated from sequences used in the routine monitoring of drug resistant mutations. The AMEN-HIV study revealed problems associated with this database when assigning some inter-subtype recombinant sequences. The Stanford database assigned a group of 69 samples with a B subtype. These sequences were visually divergent from the main HIV-1 B subtype analysis and therefore warranted further investigation. Utilising four alternative assigning tools, a consensus was achieved for many of the samples most of which were not HIV-1 B subtype. Analysis of HIV subtyping tools has been previously addressed and showed similarity and that statistical based tools (COMET and jpHMM) were highly reproducible when compared to phylogenetic based tools (REGA and SCUEAL) [204-206]. The results presented in Chapter 6 were not as conclusive but confirmed the limitation with the Stanford tool. For this reason laboratories should adopt an alternative procedure to assign subtypes which will be discussed further in Chapter 7.

6.7 Implications for successful vaccine development

The ultimate control of the HIV-1 epidemic in the future is to eliminate transmission by developing an effective vaccine. This has become very challenging since traditional vaccine development approaches to date have failed and one approach raised the concern of possible increased risk to HIV-1 transmission due to pre-existing immunity [207]. One of the greatest challenges standing in the way of this achievement is HIV-1 diversity. The ability to identify HIV-1 genetic differences, to produce a broad neutralising antibody response [208] that is present at the time of HIV-1 infection and to generate strong T cell responses [209] could be important to the success of a vaccination program, however all these approaches will need to be assessed in a population where HIV-1 diversity is evident.

6.8 Updating strategies to assist in reducing the rate of HIV transmissions and end HIV in Australia

It is known that high rates of forward transmissions can occur during acute and early HIV infection [210, 211] and that diagnosing and treating individuals early can potentially reduce HIV transmissions [212] along with having additional benefits including improve health and long term outcomes [141, 213]. It is thought that HIV transmission risk is highest in early HIV infection due to the advantages of HIV founder viruses during early transmission [214]

and high HIV-1 RNA levels [215], as well as the fact that the assessment of transmission risk is not appropriately informed by knowledge of HIV status.

For these reasons new strategies focusing on reducing HIV transmissions should be adopted and include;

1. Recognising high risk groups have expanded to include heterosexuals and MSM of a wide age range, along with migrants and short term visitors from areas where HIV rates are on the rise and where HIV is prevalent.
2. Increased rollout of testing to all risk groups to increase the rate of diagnosis and to identify those with early stage infection. Identifying those in early HIV infection may also assist in contact tracing, in turn identifying increased numbers of people at risk.
3. Implement HAART immediately after diagnosis so patients achieve an “undetectable” HIV-1 RNA level during earlier infection to reduce the risk of onward transmission and to reduce detrimental immune activation.

Early HIV treatment and increased treatment coverage has been successful in reducing the rate of new infections around the world however it appears some countries, including Australia would benefit greatly by introducing early treatment strategies to all patients.

In conclusion, the studies within Chapters 5 and 6 demonstrate the changing nature of HIV-1 diversity within Australia, indicating the increasing influence of migration and travel including local transmission network dynamics linked to specific gender patterns. This up-to-date HIV-1 subtype and phylogenetic analysis allows for a greater understanding of the Australian HIV-1 epidemic that is not readily attainable under existing national surveillance methods. The analyses not only deepen the understanding of the epidemic in Australia and Western Australia, it also directs attention to the paramount importance of continuous surveillance and phylogenetic analysis of HIV-1 variants to assist in adapting transmission, adherence, treatment and vaccine strategies. The information presented in Chapters 5 and 6 can assist in the development of effective strategies for laboratories, clinical requirements, improve population target strategies, preventing new HIV-1 infections and improving the lifestyle of those living with HIV across Australia.

Chapter 7:

Final Discussion and concluding comments; Human Immunodeficiency Virus (HIV) in Western Australia, Australia and the impact on current era clinical care.



7.1 Final Discussion

In the current era of HIV-1 infection, monitoring HIV-1 progression and treatment response, at least within the developed world, has been successfully achieved due to the development of reliable HIV-1 RNA detection methods, the sequencing of HIV-1 *pol* region for the determination of developing drug resistant mutations, and the use of flow cytometry based assays to routinely monitor CD4⁺ T cells. Through the use of combination antiretroviral therapy, guided by these laboratory techniques to monitor HIV-1 disease progression, patients can experience a near normal level of lifestyle comparable to uninfected persons. Modelling studies, however, have suggested that life expectancy could improve further if there was increased uptake of HIV testing, better antiretroviral regimens and treatment strategies, and the adoption of healthier lifestyles by those living with HIV. In particular, earlier diagnosis is one of the most important factors associated with better life expectancy [216] as is earlier treatment initiation [141].

This final chapter summarises the results presented throughout the dissertation and finalises discussions regarding the outcomes relating to the;

- 1) Implications of achieving HIV-1 RNA levels below the detectable range of routine laboratory tests (Chapter 2);
- 2) Role monocyte activation markers play in innate immune dysfunction during chronic HIV infection and determining the potential roles that soluble markers of monocyte activation play in contributing to cardiovascular disease risk in these patients (Chapter 3 and 4); and
- 3) Temporal trends relevant to the evolution of the Western Australian and Australian HIV-1 epidemic (Chapter 5 and 6).

Each of these studies is linked to the main theme driving this dissertation being “*Filling the gaps; towards improved surveillance and monitoring of immunological status relevant to long term co-morbidities in HIV infection*”.

This thesis began by examining the determinant of HIV-1 RNA below the threshold of the routinely used method to determine the long term consequences that persistent low level viraemia has throughout HIV disease progression. The results extrapolated from the Western Australian experience presented in Chapter 2 support results from modelling studies and are summarised in Box 1. Taken together, the results from the study have interesting implications for the timing of treatment (supporting the SMART study and now reinforced by the START study findings) and identified treatment strategies to potentially further improve the

management of people living with HIV. Unfortunately there are limitations when using HAART as their clinical utility can be restricted due to drug resistance, toxicity and adherence, and antiretroviral regimens cannot eliminate HIV from infecting some cells leading to persistent chronic HIV infection.

Box 1: Key findings from chapter 2;

- 🚫 During 2007-2010, around 75% of patients had well controlled HIV RNA to undetectable levels (<40copies/ml) whilst a remarkable percentage (70%) had no detectable HIV RNA target.
- 🚫 A strong association of pre-treatment HIV-1 RNA with the detection of persistent HIV RNA among patients with HIV-1 RNA <40 copies/ml.
- 🚫 Findings suggest earlier treatment initiation based on level of HIV viraemia rather than CD4 T cell count may have long term benefits on viral response.
- 🚫 Choice of antiretroviral treatment regimen may influence the likelihood of achieving no target HIV-1 RNA, although pre-treatment viral load remains the dominant determinant of 'residual viraemia'.
- 🚫 Results support the concept of residual viraemia and potentially of long lived reservoirs of latently infected cells.

We have demonstrated that the average age of patients in the Royal Perth Hospital HIV cohort is approaching 50 years of age and they are at risk of developing age related disease, with high CVD risk (>10%; 5-year risk), suggesting that there is a significant risk of long-term illness in this population that is not fully explained by the increased uptake of treatment or HAART, therefore further studies are required to determine possible links between non AIDS defining illnesses and HIV infection.

During the early years of the HIV epidemic, the innate immune system was recognised as an important player in the progression of HIV-1 [217-219] however over the past 15 years this area has received less attention as an important factor associated with HIV disease progression. The main reasons behind this stem from studies focusing on the adaptive immune system as the CD4⁺ T cells are the main target of HIV-1 infection, replication and finally, destruction as well as the role T cells play in generating immune memory (important for vaccine design success). Recently, there has been mounting interest pertaining to the role

the innate immune system has during the chronic inflammatory component of HIV infection (which can be observed among those who have residual viraemia) by studying the role monocytes play throughout HIV-1 infection.

Chapter 3 addressed the potential biological relevance and clinical utility of performing assays to determine the level of monocyte activation during HIV infection. Innate immune cells, like CD16⁺ subsets of monocytes, have been shown to be highly pro-inflammatory leading to immune activation. Incorporating the monitoring of monocyte immune activation markers into a research cohort like the WA HIV cohort enables large scale assessments that may clarify whether monocyte activation serves as a better predictor of HIV disease progression than some current diagnostic approaches. The primary concern addressed in Chapter 3 was to evaluate methods relevant to monocyte activation which could be readily adopted into routine laboratory practice. A summary of the major findings are presented in Box 2.

Box 2: Key findings from Chapter 3.

- ⓧ The results support the concept that monocytes are phenotypically altered with HIV infection.
- ⓧ CD16⁺ monocytes showed increased expression of CD64, CD163 and CD143 that was not reduced to normal levels with HAART.
- ⓧ There are distinct associations between plasma biomarkers and HIV disease and HAART.
- ⓧ CD16⁺ monocytes and sCD14 did not correlate with gender, age or CD4⁺ T cell count and were not corrected by HAART therefore including them into HIV monitoring systems may provide additional prognostic information and also highlight opportunities for therapeutic intervention beyond the current antiretroviral treatment paradigm.

Exploring novel aspects of chronic HIV infection that remain currently invisible in routine care is an area of growing interest in the current era of treatment and monitoring of HIV-1 patients. This is particularly important since the availability of durably effective antiretroviral therapy has brought into question the prognostic value of performing CD4⁺ T cell counts when a patient achieves and maintains treatment goals and is able to maintain virological suppression [101].

Chapter 4 further explored the role that plasma biomarker levels of monocyte activation may play in the setting of cardiovascular disease risk during well controlled HIV infection. Circulating biomarkers of monocyte activation like sCD14 have been shown to be predictors of HIV disease progression [220] and mortality [28], while sCD163 has been shown to be significantly associated with non-calcified coronary plaques in HIV patients in a setting where HIV infection is well controlled [221] and all cause mortality [222]. Furthermore, CXCL10 has been associated with immune activation in HIV elite controllers [223], with rapid disease progression in acute HIV [121]. In the case of HIV and CVD, there are emerging concerns that patients are at risk even after adjusting for traditional risk factors [222]. It is evident, HIV patients have increased risk for coronary heart disease when compared to the general population and the risk may be present for males and females [224, 225]. The results from Chapter 4 indicate a complex array of associations between the three biomarkers, HIV and CVD. Box 3 outlines the key points from the study.

Box 3: Key findings from Chapter 4.

- 🚫 Three levels of effects played by plasma biomarkers in HIV infection.
- 🚫 CXCL10 was most readily explained by routinely monitored variables (ie. viral load, CD4⁺ T cell count or CD4:CD8 ratio).
- 🚫 sCD163 appears to reflect a deeper level of virological suppression and is also associated with traditional CVD risk factors.
- 🚫 sCD14 was the least associated with routinely monitored variables but was strongly associated with smoking.
- 🚫 Lower levels of sCD14 were associated with treatment using an integrase inhibitor.

Adding to the concept of “*making the invisible visible*”, the idea behind Chapter 5 was to identify temporal trends that are relevant to the evolution of the Western Australian HIV-1 epidemic over the past 15 years. Baseline HIV-1 partial *pol* sequences (protease and reverse transcriptase) and HIV clinical parameters were examined from 1,021 consecutive patients (814 male, 207 female) through the Royal Perth Hospital HIV service, to investigate HIV-1 subtype diversity and local phylogenetic networks from 2000-2014. The main findings, outlined in Box 4, highlight the changes in HIV-1 subtype distribution from the growing

influence of migration and travel and the challenges facing HIV-1 prevention in Western Australia.

Box 4: Key findings from Chapter 5.

- ✚ HIV-1 genetic diversity has increased over time with HIV-1 non-B subtypes accounting for approximately 40% of HIV-1 infections from 2000-2014.
- ✚ The non-B subtypes are known to be from regions where HIV-1 is highly prevalent and corresponds with the increased migration from sub-Saharan African regions (subtype C) and overseas travel to South East Asia.
- ✚ Local HIV-1 transmissions remain dominated by B subtype viral strains, mostly within male networks.
- ✚ The level of HIV-1 RNA has increased over time coinciding with minimal changes in CD4⁺ T cell counts, indicating earlier HIV-1 diagnosis.
- ✚ In non-B HIV-1 infections however, CD4⁺ T cell count levels were lower representing delayed diagnosis among this group.
- ✚ Despite being diagnosed early, large HIV-1 B subtype transmission networks are expanding and show significant associations with higher HIV-1 RNA levels and higher CD4⁺ T cell counts at time of diagnosis but not with age or gender, suggesting there are some “hard to reach” patients.

To date there have been large population-based studies that have investigated HIV-1 diversity including an international study [226], a study from the United States [187], the United Kingdom [227], Europe [188, 228] Africa [9] and Asia [8, 229] however a national perspective from Australia is lacking and would add to important information pertaining to the global epidemic. For this reason HIV-1 subtype diversity and phylogenetic network characteristics within HIV-1 B and non-B subtypes within an Australian HIV cohort were investigated in Chapter 6, with the aim of highlighting new challenges in HIV-1 prevention that may inform strategies to treat HIV and to end HIV-1 transmissions in Australia. The results highlight the increasing diversity of HIV-1 subtypes within the Australian epidemic, as well as differences in transmission networks within Australia that are associated with these HIV-1 subtypes. The main findings obtained from the national analysis are summarised in Box 5.

The extent of HIV-1 diversification continues within the M group due to mutations, nucleotide substitutions, insertions and deletions as high error rates of reverse transcriptase occur during HIV replication. Diversity also occurs as a result of the recombination events transpiring between two or more HIV viruses within a cell, however, regardless of the causes the recognition of extensive and increasing HIV-1 diversity identified within the Australian HIV cohort is particularly important in relation to successful prevention, treatment and vaccine development strategies with the goal of ending the global HIV epidemic.

Box 5: Key findings from Chapter 6.

- Ⓜ HIV-1 diversity is increasing in Australia with more non-B subtypes being recognised within all states.
- Ⓜ Similar HIV-1 subtypes are represented in all states however differences lie in the proportions of these subtypes present. Western Australia has the largest proportion of non-B subtypes.
- Ⓜ There is a wide age distribution across all states including juveniles and over 55's.
- Ⓜ Phylogenetic analysis revealed differences in network size among B and non-B subtypes.
- Ⓜ The data is valuable now and can be assessed in real time in future to assist with clinical care, educating and intervention strategies.

The findings in Chapters 5 and 6 provide epidemiological insights not readily available using standard surveillance methods and can inform the development of effective strategies for prevention of new HIV-1 diagnoses across Australian state boundaries.

7.2 Translating science into laboratory practice

This dissertation has highlighted the potential role of both monitoring immune activation and performing reliable HIV-1 subtyping and phylogenetic analysis within the laboratory to assist with improved monitoring of patient care and monitoring the HIV epidemic. A number of changes could possibly be implemented into standard laboratory practices to assist with this translation.

Monitoring monocyte activation markers, both plasma biomarkers and proportions of CD16⁺ monocytes by flow cytometry methods would be beneficial particularly as high sCD14 levels

are associated with increased mortality in HIV [26, 28] while sCD163 is associated with residual viraemia as shown in Chapter 4 and non-calcified coronary plaques [131, 221]. Higher proportions of CD16+ monocytes have been found to independently predict CVD in a non HIV setting [85, 230], are more permissive to HIV infection [23] and preferentially harbour HIV-1 [23, 231]. For these reasons, sCD14, sCD163 and a new 8 colour flow cytometry panel are warranted and should be incorporated into the routine monitoring of HIV infection. The new TBM (T cell, B cell and monocyte) 8 colour flow cytometry panel (Figure 1) would consist of CD14, CD16 and CD57 antibodies along with currently monitored antibodies (CD4, CD3, CD8, CD45, CD19). It will, however, require further validation before implementation into laboratory practice.

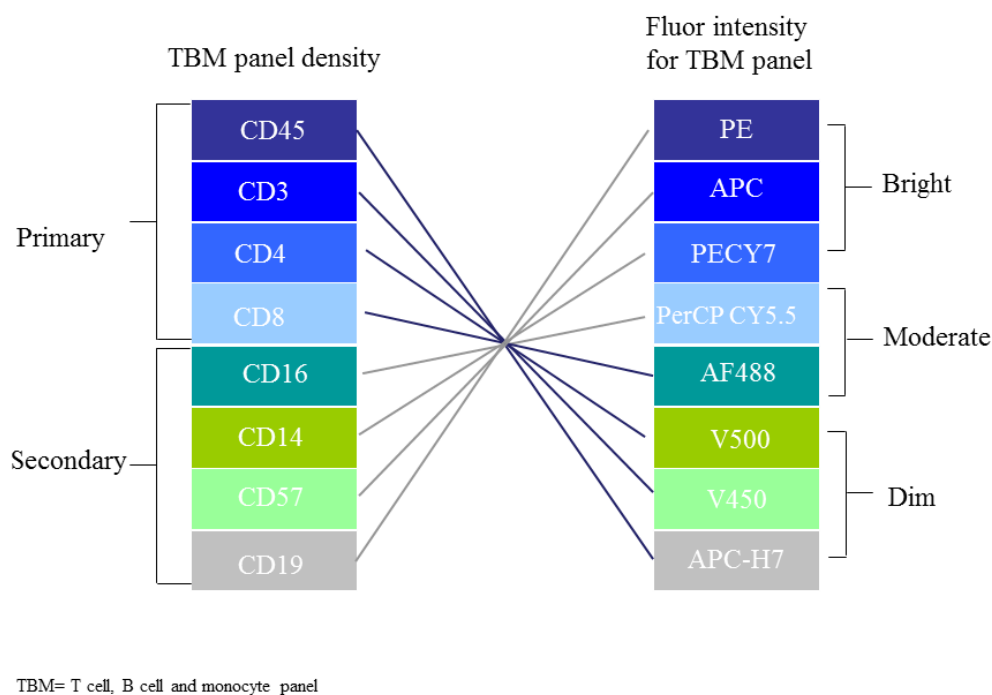


Figure 1: A potential T, B and monocyte (TBM) flow cytometry panel matching primary and secondary antigens to an acceptable fluorochrome based on intensity.

As highlighted in Chapter 6 the use of the Stanford HIV database for accurately assigning HIV-1 subtypes may not always be appropriate especially when assigning circulating inter subtype recombinants and unique forms of HIV-1. Implementing an alternative strategy into laboratory practice is warranted. Model I, proposed in Figure 2, utilises the Stanford database and MEGA analysis to determine subtype and HIV-1 maximum likelihood tree structure to identify any outlying and divergent branches. Sequences clustering with minimal divergence require no further action and the initial HIV-1 subtype would be reported. Sequences in any divergent branch undergo further analysis by submitting them to an alternative database

(COMET, REGA, jpHMM or SCUEAL) to determine subtype and breakpoints. If no consensus is reached a comment “unable to assign an HIV-1 subtype” should be reported.

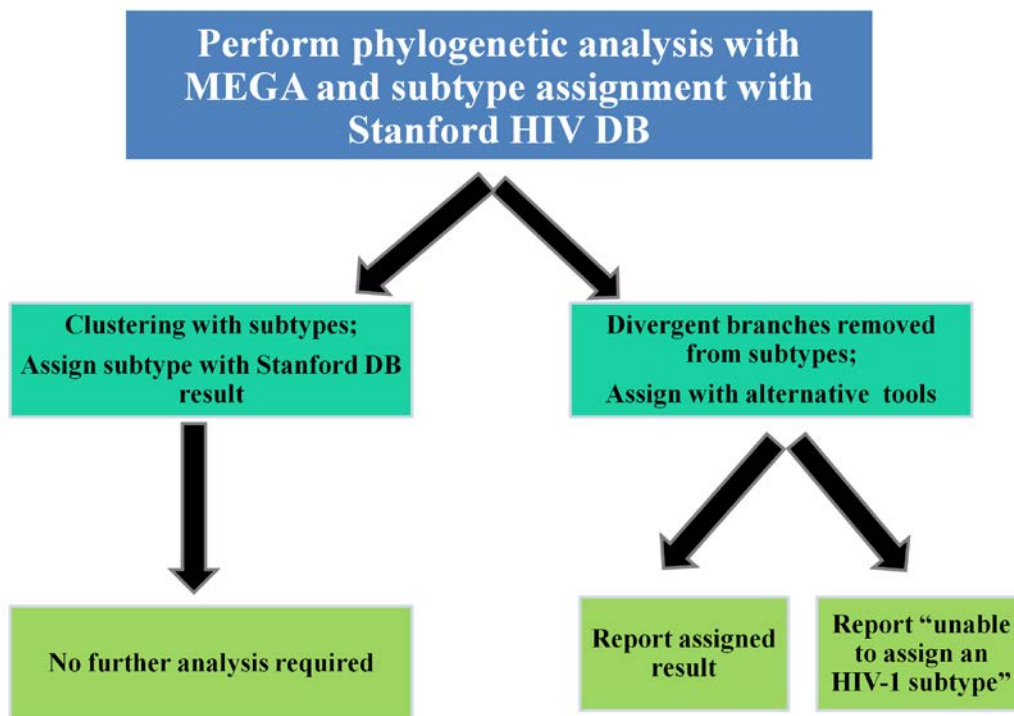


Figure 2: Model I - A proposed protocol for HIV-1 clinical laboratories to follow for the accurate assignment of HIV-1 subtypes.

Implementing a change to the procedure currently used for monitoring new HIV infections is also required. The proposed flow chart shown in Figure 3 identifies current (HIV-1 RNA levels, genotyping and the western blot assays) and new assays (2LTR, TBM panel and ELISAs; including sCD14 and sCD163) suitable for laboratory monitoring and based on results from this dissertation. Additionally, given the results presented in Chapter 2, the current reporting strategy (reporting absolute HIV-1 RNA level or <40 copies/mL should be adjusted to reflect the residual viraemia detected using the current Cobas Taqman. This change should include the comment “Residual viraemia was detected below 40 copies/mL” or “no residual viraemia was detected”. This minor change would be highly advantageous to clinicians. Furthermore, the assays presented in the proposed flow chart would not always be required at all patient visits therefore Table 1 shows the proposed assays required per visit and those to be determined by the clinician at the consultation.

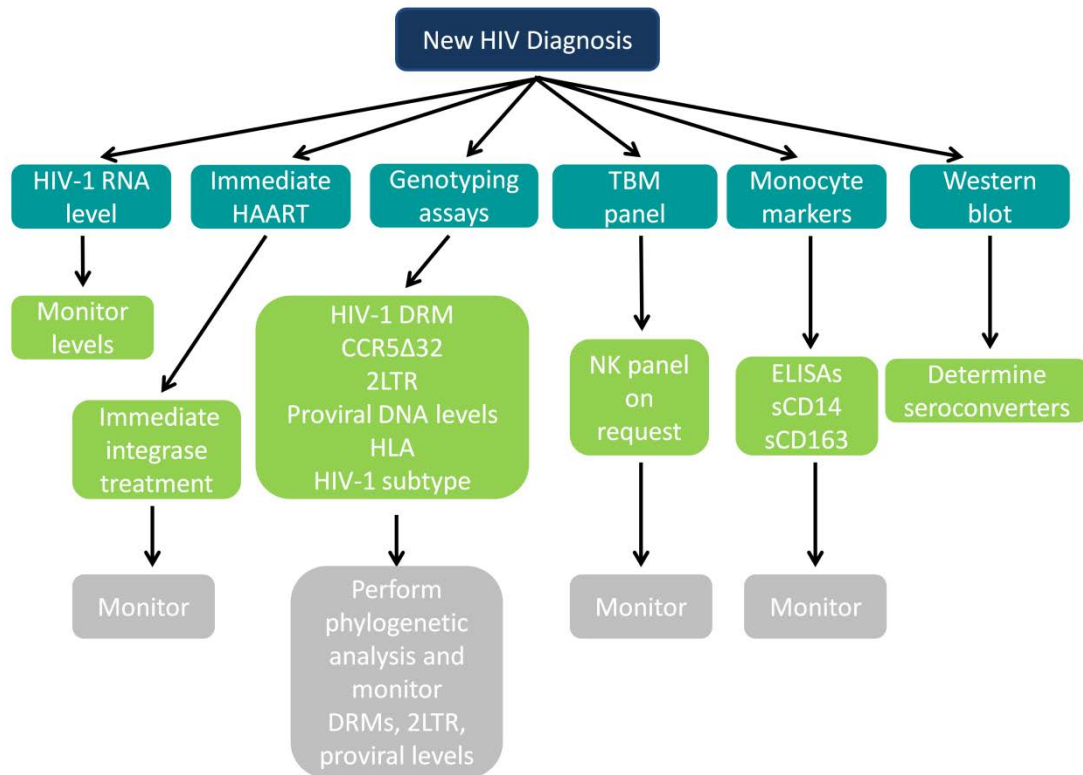


Figure 3: A proposed flow chart representing the possible laboratory assays to be requested upon new HIV diagnosis including new monitoring assays, TBM panel, ELISAs, 2LTR and phylogenetic analysis procedure.

7.3 Costs

Reduced CD4⁺ T cell monitoring would provide a substantial cost saving without impacting HIV clinical care. Data from the Royal Perth Hospital/Fiona Stanley Hospital Department of Immunology HIV laboratory show that in 2015, the TBNK test (which includes CD4⁺ T cell counts) was performed 2951 times for 1174 patients at an average of 2.5 tests per patient. This was at a total cost of \$88,530. Of these tests, 81.9% of patients always had an absolute CD4⁺ T cell count above 500, 8.1% never had an absolute CD4⁺ T cell count >500 and 10% had fluctuating CD4⁺ T cell counts. A saving of \$43,290 would be made if the frequency of the TBNK assay was reduced to an annual test for patients with CD4⁺ T cell counts maintained above 500 (81.9% of the cohort). The NK component of this test is rarely utilised in clinical practice, therefore eliminating NK monitoring from the current TBNK flow cytometry panel would allow for the inclusion of monitoring monocytes with no additional costs (when taking into account the savings on annual testing). Another benefit would be the potential to alleviate patient anxiety due to fluctuations in absolute CD4⁺ T cell counts due to

assay and laboratory variability. Allowing resources currently used for CD4⁺ T cell monitoring to be redirected to other clinical needs and replacing the TBNK panel with the proposed panel (Figure 1) including an antibody to detect senescence would be advantageous to patient care. The proposal would be to implement routine assessments for monitoring soluble monocyte activation markers (sCD14 and sCD163) at a cost of \$24.50 per patient (\$28,763.00 for 1174 patients/year).

Table 1: A representation of when HIV-1 monitoring assays should be performed within the first three years after diagnosis.

Visit	HIV-1 RNA	HAART	HIV-1 DRM	CCR5Δ32	2LTR	Proviral DNA	HLA typing	HIV-1 subtype	TBM panel	ELISA	Western blot
T0	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T4	✓	A	A	X	✓	A	X	X	✓	✓	X
T8	✓	A	A	X	✓	A	X	X	✓	✓	X
T12	✓	A	A	X	✓	A	X	X	✓	✓	X
T24	✓	A	A	X	✓	A	X	X	✓	✓	X
T36	✓	A	A	X	✓	A	X	X	✓	✓	X

A=assess, ✓=perform, X= no further action, T=time in months

7.4 Future studies

The work undertaken throughout this dissertation has highlighted interesting aspects of HIV pathogenesis that remain unresolved. Future research should build on work around reducing residual viraemia focusing on the role that unintegrated and integrated proviral DNA plays. Reducing residual viraemia would be a major step towards eliminating HIV-1 viral replication. For this reason future work should be continued towards HIV-1 treatment intensification, which could be informed by recent research that has highlighted the role of TREX1 in preparing viral DNA for integration into the host genome [156, 157]. Identifying ways to inhibit TREX1 during early HIV infection may offer an important immune based therapy, however improved understanding of how TREX1 pathways function is required. An interesting study would be the analysis of TREX1 gene sequences of high risk-uninfected subjects and HIV-infected subjects, to determine sequence differences associated with HIV infection, DNA integration and residual viraemia.

Additionally, with the ongoing identification of pathways leading towards monocyte activation, targeting these factors (i.e. ADAM17, IFNλ3, IFN-α, TNF-α) could lead to useful

therapeutic strategies to reduce immune activation. A recent study showed a link between the monocyte activation marker sCD163 to clinical outcomes in treated HIV cases [222]. This supports the work presented in this thesis but requires further investigation into the potential mechanisms driving the increased sCD163 levels. As described in the summary of Chapters 2-4, mechanisms providing plausible links between cell-associated HIV proteins (Nef in particular) and the release of sCD163 from its membrane-bound precursor have been identified however, ongoing work focusing on the impact that ADAM17, the Nef complex and exosomes have during HIV pathogenesis is required. Interestingly, monocyte ADAM17 facilitates endothelial migration by accelerating the rate of diapedesis [137] by cleaving Mac-1 therefore regulatory mechanisms of diapedesis may be important for the development of therapy to decrease CVD risk and for atherosclerosis; all of which warrants further investigation.

The future work surrounding the role monocytes play in promoting immune activation remains vastly unresolved and holds great interest. Continued studies focusing on the role CD14⁺⁺/CD16⁺ and CD14⁺/CD16⁺ monocytes play especially in the context of co-infections is worthy with specific focus on immune modulating approaches and how they impact monocyte activation in early acute and chronic HIV-1 infections. Also monitoring CXCL10 levels during primary infection could also be beneficial, as would monitoring the effect of early HIV treatment with HAART on immune activation and residual viraemia.

In terms of immune modulation, IFN λ 3 offers strong antiviral effects and the expression pattern of IFN- λ 3 receptors is very cell specific [181] therefore studying its use as a therapeutic option demands further attention especially as it may have fewer side effects over other IFNs hence making it more attractive to use. Another potential immune based therapy that has previously been assessed in SIV infected macaques but not in humans is IL-21 treatment [182]. This study revealed a link between immune activation and residual viraemia and showed that IL-21 effectively reduced both while infected macaques were on ART. Interestingly, plasma viraemia and cell associated SIV DNA levels remained low, for eight months after ART was ceased therefore monitoring IL-21 treatment in humans may prove to be advantageous.

Immune modulation could also be controlled with the use of aspirin as previously described [171]. The use of aspirin may have a place in effectively reducing immune activation and inflammation, although further studies are needed on a large scale to expand on the mechanisms and pathways.

Finally the data presented in Chapters 5 and 6 provided a solid foundation to propose continued HIV-1 surveillance in Australia. Adding clinical and notification data (as present in Chapter 5) and evaluating HIV-1 baseline drug resistant mutations in this cohort will only strengthen the analysis and provide a comprehensive HIV-1 epidemiological surveillance system, currently missing in Australia.

7.5 Final Conclusion

With more than 33 million deaths from HIV/AIDS worldwide and with the failure to reduce new HIV infections in Australia, to produce an adequate vaccine to curb new infections or to cure current infections, those infected who do have access to life changing HAART will experience persistent immune activation and immune dysfunction. This is largely mediated by the activation of monocytes, therefore alternative strategies must be adopted to prolong the health of patients ageing with HIV. Immunomodulating therapies and strategies with pharmacological or biological agents could be a way forward in the future to treat HIV patients. Adding to this the demonstration of increased HIV-1 diversity and increased HIV new infections in Australia is alarming. Safe sex campaigns should emphasise that HIV does not discriminate according to age, gender or sexual preference. Descriptive campaigning messages should reinforce the possibility and risk of becoming infected with HIV overseas regardless of sexual preference or age and the increased risk of acquiring HIV in Australia. From the information and results presented in this thesis, “filling the gaps” is a critical step towards achieving the goals of the UNAIDS current strategy of achieving “90, 90, 90” by 2020 and the 7th Australian National strategy by 2017 and further improving the quality of life of those already living with HIV in Australia.

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