Prevention and pathogenesis of vertically acquired HIV infection in a high-income setting

by

Bipasha Choudhury

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Abstract

Background

Human immunodeficiency virus (HIV) continues to be a major public health issue globally. An estimated 150,000 infants are infected annually through vertical transmission (VT). The prevention of vertical transmission of HIV can be mitigated through HIV screening in pregnancy; however, prevention remains challenging for mothers who seroconvert after the antenatal screening, later in pregnancy, or while breastfeeding. Despite public health interventions, VT, although rare, continues to occur in these groups and may be difficult to prevent entirely with current strategies. Moreover, children living with HIV (CLWH) face lifelong exposure to HIV and may be at risk of developing premature cardiovascular disease (CVD) despite achieving viral suppression with combination antiretroviral therapy (cART). Endothelial activation is one mechanism that may increase the risk of CVD.

This thesis has two objectives: (1) to describe pregnancy outcomes and the prevalence of risk factors for HIV transmission in HIV-serodiscordant couples (chapter 2); and (2) to examine the correlation between markers of the pathologic pathways involved in endothelial activation in CLWH (chapter 3).

Methods

In chapter 2, we report a case of maternal primary HIV infection during the postpartum period and VT to a nursing infant in our high-income setting. We then document a case series of 47 serodiscordant pregnant couples identified through a nurse-led public health program to follow HIV serodiscordant couples.

In chapter 3, we present results of a cross-sectional study with measurement of selected biomarkers of microbial translocation, systemic inflammation, and endothelial activation in the Early Pediatric Initiation-Canadian Child Cure Cohort (EPIC^4), a well-characterized cohort of CLWH across Canada with detailed longitudinal clinical and laboratory data.

Results

In chapter 2, we found that no cases of HIV seroconversion occurred in 47 HIV-serodiscordant couples. However, high viral load (VL) among some seropositive males was observed during the period of pregnancy and breastfeeding. Among HIV-infected male partners, 15 had detectable viral load (VL) during their partner's pregnancy, with median (IQR) peak VL of 3,800 (IQR 370-14,000) copies/mL, and 16 males had a detectable VL during breastfeeding, with median (IQR) peak VL was 5,000 (210-30,000).

In chapter 3, in a sample of 90 CLWH, we found that 16% of Angiopoietin-2 (Ang2), 15% of soluble vascular endothelial growth factor-1 (sVEGFR1), and 23% of soluble endoglin (sEng) levels were elevated relative to healthy historic controls. Pairwise rank-correlations between the three markers of endothelial activation were statistically significant ($\rho = 0.69$, $\rho = 0.61$, and $\rho = 0.65$, $p<0.001$ for all correlations respectively). An endothelial activation index (EAI), derived by factor analysis of the three endothelial biomarkers, correlated with inflammatory markers tumour necrosis factor (TNF, $\rho = 0.47$, $p<0.001$) and interleukin-6 (IL-6, $\rho = 0.60$, $p<0.001$). The EAI also correlated with a marker of gut barrier injury, intestinal fatty acid binding protein (I-

FABP1, $\rho = 0.67$, $p<0.001$). Current or past treatment with ritonavir-boosted lopinavir (LPV/r) was associated with endothelial activation (OR 5.0 (95%CI 1.7-17), p=0.0020).

Conclusions

This thesis addresses VT of HIV in a high-risk group (serodiscordant couples) and one of its consequences for CLWH (endothelial activation). These findings may contribute to continuing efforts to eliminate VT of HIV in Canada and may help understand and prevent long-term cardiovascular complications in CLWH.

Preface

Some of the research conducted for this thesis forms part of a national research collaboration, the Early Pediatric Initiation-Canadian Child Cure Cohort (EPIC^4), led by Dr. Hugo Soudeyns at the Université de Montréal. Data from Chapter 3 has been submitted for publication to the journal HIV Medicine.

In chapter 2 and chapter 3, the contribution of collaborators appears in the section "Contributions".

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AIDS acquired immune deficiency syndrome
Ang2 angiopoietin 2
ATV/r ritonavir -boosted atazanavir
cART combined anti retro viral therapy
CLWH children living with human immunodeficiency virus
CMV cytomegalo virus
CVD cardiovascular disease
DBS dry blood spot
DRV/r ritonavir-boosted darunavir
EAI endothelial activation index
ELISA enzyme linked immune sorbent assay
eNOS nitric oxide synthase
EPIC^{4} early pediatric initiation-canadian child cure cohort
FADD Fas-associated death domain
GALT gut-associated lymphoid tissues
HIV human immunodeficiency virus
ICER incremental cost-effectiveness ratio
I-FABP intestinal fatty acid binding protein
IFN Interferon
IL-6 interleukin-6
INSTIs integrase strand transfer inhibitors
IQR inter quartile range
IUGR intra uterine growth retardation
IVDU intra venous drug user
JAK Janus kinase
KT knowledge translation
LBP LPS-binding protein
LMICs low- and middle-income countries
LPS lipopolysaccharide
LPV/r ritonavir-boosted lopinavir
MAPK mitogen-activated protein kinase
MMP-14 membrane-type metalloprotease-14
MT microbial translocation
mTOR mammalian target of rapamycin
NAP Northern Alberta Program
NF-κb nuclear factor kappa-light-chain-enhancer of activated B cells
NK-cells natural killer cells
NNRTIs non-nucleoside reverse transcriptase inhibitors
NO nitric oxide
NRTIs nucleoside reverse transcriptase inhibitors **OR** odds ratio
PI3-kinase phosphoinositide 3-kinase
PIs protease inhibitors
PLWH people living with human immunodeficiency virus
POC point-of-care
PrEP Pre-exposure prophylaxis
PYY peptide tyrosine tyrosine
sEng soluble endoglin
STAT3 signal transducers and activators of transcription
STI sexually transmitted infection
sVEGFR1 soluble vascular endothelial growth factor 1
SVS sustained viral suppression
TGF-β transforming growth factor-β
Th17 T helper 17
Tie2 tyrosine kinase with immunoglobulin-like and EGF-like domains-2
TKIs molecule tyrosine kinase inhibitors
TNF tumor necrosis factor
TNF-RI tumor necrosis factor receptor type I
TNF-RII tumor necrosis factor receptor type 2
TRADD TNF receptor-associated death domain
Treg T regulatory
UNAIDS united nations program on HIV/AIDS
VL viral load
VT vertical transmission
WHO world health organization

CHAPTER 1 – Introduction to Human Immunodeficiency virus

1.1 Etiology

Human immunodeficiency virus (HIV) is the cause of the spectrum of disease known as HIV/AIDS. HIV is a retrovirus (subfamily lentivirus) and is grouped into two distinct species that infect humans, HIV-1 and HIV-2. HIV-1 is the leading cause of HIV/AIDS globally and in Canada. Throughout this thesis, for simplicity, we refer to HIV-1 as HIV.

HIV targets $CD4^+$ T-lymphocytes of the adaptive immune system by binding the CD4 receptor and chemokine co-receptors CCR5 and CXCR4. Infected individuals gradually become immunodeficient, resulting in increased susceptibility to a wide range of infections, cancers and other diseases [1].The most advanced stage of HIV infection is acquired immunodeficiency syndrome (AIDS), which can take from 2 to 15 years to develop in untreated individuals [2].

1.2 Epidemiology

HIV/AIDS is a global pandemic, a major public health issue, and an important contributor to the global burden of disease. According to the World Health Organization (WHO), around 37.9 million people globally were living with HIV in 2018 and of these, 1.8 million were children. The global incidence of pediatric HIV is estimated at 150,000 per year, most cases being vertically acquired. Approximately 2,110 people die from an AIDS-related infection every day (770,000 annually). This is declining from an estimated 1.7 million deaths at the peak of the global pandemic in 2004 [3].

Globally, HIV epidemics may be classified as “concentrated” or “generalized.” In concentrated epidemics, defined vulnerable groups such as sex workers, men who have sex with men, and people who use injection drugs are mainly susceptible to transmission. In generalized epidemics, the transmission persists by sexual behavior in the general population and is likely to continue despite effective programs for vulnerable groups. North America has a concentrated epidemic whereas sub-Saharan Africa deals with a generalized epidemic [4]. Low- and middle-income countries (LMICs) experience the heaviest impact of HIV [5]. The most severely affected region is WHO Africa, with nearly 1 in every 25 adults (3.9%) living with HIV. This region accounts for more than two-thirds of the people who are living with HIV worldwide [6].

In Canada, ongoing HIV transmission remains an issue of concern, with a total of 2,402 new HIV cases were reported in 2017. HIV incidence increased between 2014 (5.8 per 100,000) and 2017 (6.5 per 100,000) [3]. Among Canadian provinces, Saskatchewan had the highest per capita HIV infection rate at 15.5 per 100,000 population, accounting for 7.5% of total new HIV cases [3].

The 90–90–90 targets were launched in 2014 by the Joint United Nations Program on HIV/AIDS (UNAIDS) and partners in order to benchmark progress toward controlling the HIV/AIDS pandemic. The aim is to diagnose 90% of all HIV-positive individuals, provide combination antiretroviral therapy (cART) for 90% of those diagnosed, and achieve viral suppression for 90% of those treated on treatment. As of 2016, an estimated 86% of Canadians living with HIV were

diagnosed, 81% of Canadians diagnosed with HIV were on treatment, and 91% of HIV positive Canadians on treatment had achieved viral suppression [7].

1.3 Transmission

Established modes of HIV transmission are: (1) sexual contact; (2) percutaneous blood exposure; (3) mucous membrane exposure to contaminated blood or other body fluid; (4) vertical transmission; and (5) contaminated blood products transfusion. Since highly effective screening methods are in place, transfusion of blood products has been nearly eliminated as a cause of HIV transmission in North America [8]. Behaviors and conditions that put individuals at greater risk of contracting HIV include: unprotected anal or vaginal sex; co-infection with another sexually transmitted infection (STI) such as syphilis, herpes, chlamydia, gonorrhea and bacterial vaginosis; sharing contaminated needles, syringes and other injecting equipment and drug solutions when injecting drugs; unsafe injections, blood transfusions and tissue transplantation, and medical procedures that involve unsterile cutting or piercing; and accidental needle stick injuries [8].

1.3.1 Sexual transmission

Heterosexual transmission in generalized HIV epidemics is an important method of sustained HIV transmission globally. Seminal and endocervical viral load are major determinants of HIV-1 sexual transmission, even after adjustment for plasma viral load [9]. Other behavioral factors that increase HIV-1 sexual transmission include multiple sexual partners and concurrent partnerships.

Self-reported condom use reduces the per-coital act risk of HIV-1 transmission by approximately 78% [10].

In early 2016, the Undetectable=Untransmissible (U=U) slogan was launched by HIV activists (Prevention Access Campaign). U=U means that PLWH with an undetectable viral load cannot sexually transmit the virus to others [11-13]. Undetectable VL in blood typically requires three to six months of consistent adherence to an effective cART regimen. Awareness of U=U encourages patients to adhere to their cART regimen in order to achieve and maintain viral suppression [14].

Social factors affect sexual behavior and are important upstream determinants of the global HIV pandemic. Gender inequality plays a role, especially in sub-Saharan Africa, where women account for 57% of people living with HIV [15]. Women who are victims of intimate partner violence have an increased incidence of HIV infection [16]. Stigma against PLWH, discrimination, and punitive laws against high-risk groups (e.g., men who have sex with men, people who inject drugs, and commercial sex workers) are obstacles for people to seek HIV testing, access care, and adhere to preventive measures [17].

The probability of male-to-female sexual transmission is increased in late pregnancy and the postpartum period. In a prospective analysis of 2,751 African HIV-serodiscordant couples, in which 686 pregnancies and 82 incident HIV infections occurred, the probability of HIV acquisition per coital act was estimated at 0.0011. The relative risk in late pregnancy was

approximately three-fold and in the postpartum period four-fold higher than the nonpregnant period [18]. In this study, the transmission risk increased linearly with increasing male VL [18].

1.3.2 Vertical transmission.

The risk of infection for an infant born to an HIV seropositive mother who did not receive interventions to prevent transmission is approximately 16% in Canada [19]. Most vertical transmission occurs during the intrapartum period, with fewer transmission events occurring *in utero* and postnatally through breast feeding. Risk factors for vertical transmission of HIV can be categorized as follows: (1) maternal viral load; (2) duration of exposure; and (3) factors that facilitate transfer of the virus. High maternal viral load may be associated with advanced maternal clinical disease, a lower maternal $CD4^+$ T-lymphocyte count, or with recent seroconversion during the pregnancy. The duration of ruptured membranes, and vaginal route of delivery (*versus* cesarean delivery) increase the risk of intrapartum transmission [20].

Breastfeeding is the usual route of post-natal transmission for neonates and young infants. HIV genomes have been detected in both cell-associated and cell-free fractions of human milk. Cell-associated HIV can be detected in the milk of lactating women receiving cART [21]. Transmission through breastmilk still occurs among a small percentage of women with suppressed VL [22]. Therefore, replacement (formula) feeding continues to be recommended for Canadian mothers receiving cART [23]. In low-resource settings, the advantages of breastfeeding (e.g., prevention of diarrheal disease) may outweigh the small risk of HIV transmission [24]. The duration of breastfeeding increases the risk of vertical transmission.

Maternal breast lesions or infant oral candidiasis may facilitate transfer of virus from mother infant [22].

1.4 Pathogenesis

1.4.1 Primary HIV infection

After infection, there is a period of rapid viral replication, leading to a high level of viremia, which may reach several million virus particles per milliliter of blood. Viral proliferation is accompanied by a drop in the number of $CD4^+$ T-lymphocytes. Host adaptive immune mechanisms are engaged, including cytotoxic ($CD8^+$) T-lymphocytes and B-lymphocytes. B-cell activation results in antibody production (seroconversion). The $CD8^+$ T-lymphocyte response causes lysis of infected cells and controls virus levels, which peak and then decline. A robust $CD8^+$ T-lymphocyte response is associated with slower disease progression and a better prognosis, though it does not eliminate the virus [25].

Most $CD4^+$ T-lymphocyte loss occurs during the first weeks of HIV infection; however, the symptoms of immune deficiency may not appear for years after a person is infected. T-cell loss is especially pronounced in the intestinal mucosa, which harbors the majority of the body's lymphocytes [26]. The reason for the selective loss of mucosal $CD4^+$ T-lymphocytes is that the majority express CCR5, a co-receptor for HIV entry [27]. In contrast, only a small fraction of $CD4^+$ T-lymphocytes in the bloodstream express CCR5 [28].

1.4.2 Chronic HIV

Ultimately, HIV causes AIDS by depleting $CD4^+$ T-lymphocytes. This results in profound defects in adaptive immunity and allows for opportunistic infections [29]. After the primary infection is controlled by a vigorous immune response, the clinically latent phase is initiated. Ongoing HIV replication causes a state of generalized immune activation persisting throughout the chronic phase. Various innate and adaptive immune cells remain activated and release pro-inflammatory cytokines [28].

1.4.3 Cardiovascular Complications of HIV-1 infection

Thanks to highly effective cART regimens, PLWH previously burdened by opportunistic infections are now living significantly longer. As a consequence, they increasingly experience non-infectious complications, including cardiovascular disease (CVD), stroke, and cancer. CVD has now surpassed opportunistic infections as the leading cause of mortality in PLWH [30].

The spectrum of CVD in PLWH is broad and includes pericardial effusion, myocarditis, dilated cardiomyopathy, endocarditis, coronary artery disease, pulmonary hypertension, vasculitis, aneurysm formation, and cardiac tumors [31]. However, atherosclerosis, particularly coronary artery disease, is the most important cardiovascular complication of HIV. CLWH demonstrate an earlier evidence of some CVD precursor conditions such as dyslipidemia, increased arterial stiffness, increased carotid intimal media thickness, and coronary arteriopathy, as well as complications such as myocardial infarction that suggest accelerated progression of atherosclerosis [32-34].

A combination of microbial translocation, systemic inflammation, and endothelial activation may contribute to the pathogenesis of cardiovascular complications in HIV (Figure 1.1).

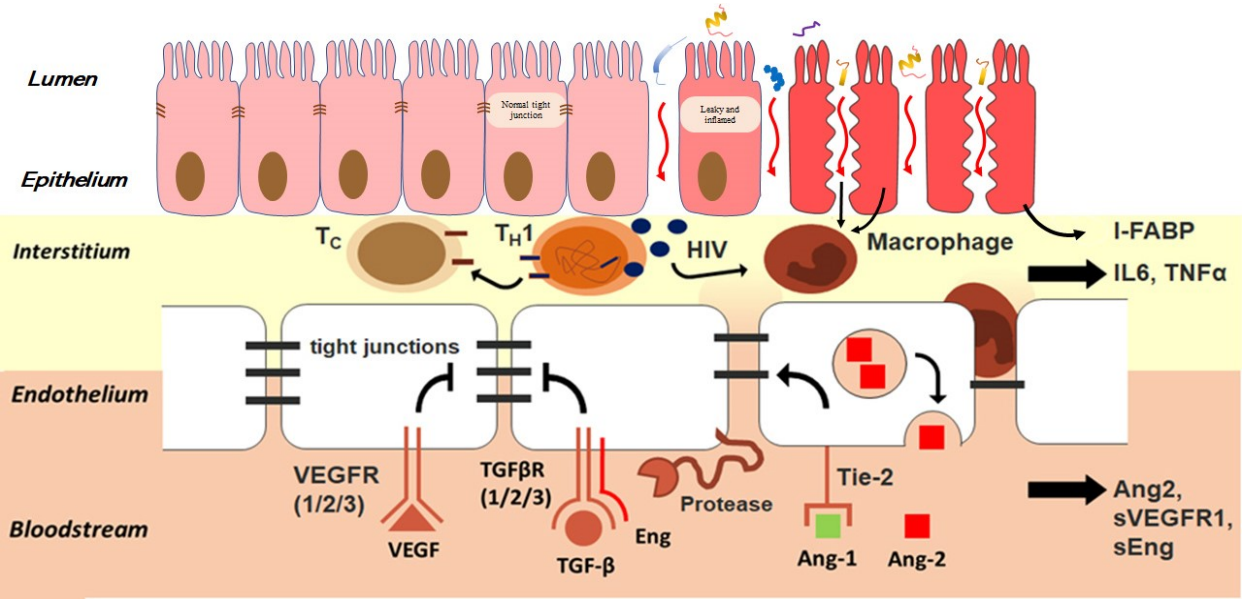

Figure 1.1 Diagram of processes of microbial translocation, systemic inflammation, and endothelial activation in HIV. The intestinal epithelium is shown in health and disease states, illustrating leakage of gut lumen contents. One specific biomarker of microbial translocation is shown: including intestinal fatty acid binding protein (I-FABP). Inflammation, driven by microbial products and the innate and adaptive immune responses can be measured using biomarkers such as tumor necrosis factor (TNF) and interleukin-6 (IL6). The endothelium is activated during systemic inflammation, and can be interrogated using the quantitative biomarkers soluble vascular endothelial growth factor receptor -1 (sVEGFR1), soluble endoglin (sEng) and angiopoietin 2 (Ang2).

1.4.4 Microbial translocation in HIV infection

Microbial translocation (MT) refers to the passage of gastrointestinal microflora or microbial products through the damaged gastrointestinal mucosa and lamina propria, reaching the

mesenteric lymph nodes and finally the peripheral circulation. This results in systemic immune activation without overt bacteremia [35]. Under physiologic conditions, translocating microbes and microbial products are phagocytosed at the site of lamina propria and the mesenteric lymph nodes. However, these defense mechanisms fail under certain conditions where the host immune system is compromised, such as chronic HIV infection [36].

MT is recognized in other pathologies, such as inflammatory bowel disease, graft- versus-host disease (GVHD), and post-gastrointestinal surgery. MT in the context of HIV was described only 10 years ago [37]. Since then, MT has been repeatedly described in several cohorts and now it is established as a general phenomenon in HIV [38].

HIV infection causes a profound depletion of $CD4^+$ T lymphocyte in blood, lymphoid organs, and mucosal tissue. The gut-associated lymphoid tissues (GALT) are an ideal target for HIV infection since they contain a high density of $CD4^+$ T-lymphocytes [39]. Therefore, the gastrointestinal tract is an important anatomical locus of HIV infection, irrespective of the route of infection [39]. The massive and irreversible damage of gut lymphocytes occurs in the initial phase of acute HIV infection and continues throughout the course of chronic disease [40].

Structural intestinal epithelial damage occurs via several mechanisms: (1) death or impaired function of enterocytes caused by HIV proteins; (2) apoptosis of enterocytes and disruption of tight junctions caused by inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β; (3) loss of T-cells that maintain the epithelial barrier; and (4) alteration of the microbiome toward a predominance of opportunistic bacteria [41]. Together, these changes allow increased entry of gut microflora and microbial products into the systemic circulation [38].

MT can be assessed by quantifying certain biomarkers circulating in the blood. Assays include direct detection of microbial products such as lipopolysaccharide (LPS, a component of the gram-negative bacterial cell wall) [42] or plasma bacterial 16SrDNA [43]. Innate and adaptive immune responses to microbial products such as soluble CD14 (sCD14) from macrophages [44], LPS-binding protein (LBP), anti-flagellin antibodies [43], and antibodies to LPS [45] have also been used as markers of MT. Finally, markers of enterocyte damage including intestinal fatty acid binding protein (I-FABP) [46] and zonulin [47] have been used [48].

In chapter 3 of the present thesis, we describe a cross-sectional study in which the circulating level of I-FABP in CLWH was used as a marker of epithelial damage and MT. The following section describes I-FABP structure, function, and utility as a marker of MT.

1.4.4.1 Intestinal fatty-acid binding protein (I-FABP)

Intestinal fatty-acid binding protein (I-FABP) is a cytosolic protein found in enterocytes of the gastrointestinal tract, most abundantly in the jejunum. In healthy individuals, its role is to

transport fatty acids from the apical membrane of enterocytes to the endoplasmic reticulum, where they are converted into triglycerides [49]. PYY (peptide tyrosine tyrosine), a hormone released by intestinal cells, in response to lipids in the gut regulates the expression of I-FABP in the intestine [50].

The molecular structure of I-FABP is a β-barrel comprised of 10 antiparallel β-sheets. The structure also contains a small helix–turn–helix motif that is interspersed between the first and second β-strands. The helical region of I-FABP is a crucial domain which maintains the membrane–protein interactions and participates in the mechanism of collisional transfer of fatty acids from I-FABP to membranes [51].

The small size (12–15 kDa) of I-FABP in the gut epithelium facilitates its leakage into the circulation from damaged enterocytes. Elevated levels of circulating I-FABP are associated with disease conditions where the intestinal wall is injured or compromised [49]. Thus, I-FABP has an established role as a non-invasive marker for evaluating gut wall integrity loss and inflammation [34, 52-54]. On the other hand, in HIV, there are several potential limitations of I-FABP as a biomarker of intestinal integrity. As a fatty acid transport protein, I-FABP may be affected by some antiretroviral medications that cause metabolic lipid changes [43]. I-FABP was reduced after treatment with statin therapy, without concomitant reduction in zonulin or LBP in one study [47]. I-FABP increased after initiation of effective cART in another study [43]. These findings suggest that metabolic changes independent of epithelial damage may confound the interpretation of I-FABP as a marker of MT.

1.4.5 Systemic inflammation

Chronic systemic inflammation is well-recognized in PLWH [34]. Markers of inflammation predict mortality independently of $CD4^+$ T-lymphocyte count and HIV viral load, illustrating the clinical importance of systemic inflammation. A large prospective cohort of CLWH initiating ART in 2 high-burden countries, demonstrated that inflammation is a major driver of adverse outcomes [55]. Children most at risk of this inflammation-associated mortality had preserved $CD4^+$ T-lymphocyte counts and therefore would not be identified by current routine monitoring [55].

Inflammatory responses in HIV infection may be explained by several mechanisms. First, HIV-1 products can directly trigger the immune cells. HIV accessory proteins Nef and Vpr stimulate monocytes and macrophages [56]. Similarly, detection of HIV-RNA by the pattern recognition receptors TLR-7 and TLR-9 induced the production of Interferon (IFN)-α by dendritic cells [57]. HIV-DNA in the cytoplasm of target cells, activates caspase-1 and the release of pro-inflammatory cytokines including interleukin (IL)-1b. Of note, the latent replication-competent HIV reservoir infection can trigger immune activation by this mechanism, explaining at least in part, the persistent inflammation seen in PLWH despite excellent virologic control [58]. Second, host-derived allo-antigen may prompt the immune system directly. A key driver of persistent inflammation in PLWH is the translocation of pro-inflammatory bacterial products from the gut lumen into the circulation, as described above [40]. Co-infection with cytomegalovirus is another cause of immune activation and release of pro-inflammatory cytokines.

Systemic inflammation is reflected by elevated circulating levels of pro-inflammatory cytokines and chemokines. A wide range of these molecules have been reported in PLWH. In chapter 3 of this thesis, we assessed systemic inflammation using the classical pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6). These have been shown to have prognostic value for subsequent cardiovascular events [59] . Below, we review selected molecular pathways of inflammation that have IL-6 and TNF.

1.4.5.1 Interleukin 6 (IL-6)

IL-6 plays a key role in the acute phase response and in the transition from acute to chronic inflammation. Dysregulation of IL-6 production contributes to the pathogenesis of chronic inflammation. HIV infection induces expression and secretion of IL-6 by monocytes and macrophages [60].

With regard to the structure of IL-6, it is a glycosylated protein with a four-helix bundle structure [61]. The IL-6 receptor–signaling system is made up of two receptor chains and downstream signaling molecules. Macrophages, neutrophils, $CD4^+$ T-lymphocytes, podocytes, and hepatocytes express IL-6R on their cell surface, and therefore can directly respond to IL-6 [62].

On target cells, IL-6 binds to membrane-bound IL-6R. This complex connects to two molecules of gp130 and initiates signal transduction [62]. JAK (Janus kinase)/STAT3 (signal transducers and activators of transcription) and SHP2/Gab/MAPK (mitogen-activated protein kinase) are the two major pathways involved in gp130 signaling [63]. Besides the membrane-bound, a soluble

form of the IL-6R (sIL-6R) has been identified in body fluids such as blood and urine. sIL-6R binds to IL-6 with similar affinity as the mbIL-6R. Subsequently, the complex of IL-6/sIL-6R can activate gp130. [62, 64].

IL-6 induces the production of acute phase reactants, including C-reactive protein, by hepatocytes [65]. IL-6 is involved in the regulation of T cell differentiation between regulatory T (Treg) cells and T helper 17 (Th17) cells. IL-6 triggers the differentiation of Th17 cells together with TGF-βand dampens the generation of Treg cells via STAT3. Therefore, IL-6 has a clear implication in $CD4^+$ T cell differentiation and expansion and contributes to T-cell-mediated immune response [66].

1.4.5.2 Tumor necrosis factor (TNF)

TNF is a potent pro-inflammatory cytokine which plays a pivotal role in inflammation, cell proliferation, differentiation, and apoptosis. TNF is an abundant early mediator of tissue inflammation. Increased serum and tissue levels are found under inflammatory and infectious conditions. TNF orchestrates the production of a pro-inflammatory cytokine cascade [67].

Macrophages and T-cells are the main sources of TNF. Besides these, other cells can also produce TNF: B cells, NK-cells, neutrophils, mast cells, endothelial cells, smooth muscle cells, cardiomyocytes, fibroblasts, osteoclasts, osteoblasts, astrocytes, dendritic cells, microglial cells, keratinocytes, adipocytes, adrenocortical cells, and glomerular mesangial [68].

TNF is translated as 233 amino acid pro-peptide. It is then processed to a 157 amino acid mature protein by cleavage of a 76 amino acid signal peptide. Proteolytic cleavage of transmembrane TNF (tmTNF) is mediated by the metalloprotease TNF-converting enzyme (TACE), resulting in the release of soluble TNF (sTNF) [69]. Both tmTNF and sTNF are homotrimers with a characteristic cone-shape. Each monomer is made up of two packed β-pleated sheets created by eight antiparallel β-strands arranged in a β-jellyroll topology [67].

The biological response to TNF is mediated by two structurally distinct receptors: tumor necrosis factor receptor type I (TNF-RI) and type 2 (TNF-RII), which are present on the membrane of all cell types except erythrocytes [70]. TNF-RI is responsible for most of the TNF actions. TNF-RII is initially expressed intensively by T cells and endothelial cells. Either it recruits or inhibits specific cell types, such as lymphocytes and clastic lineages, when necessary, and induce a rapid and efficient response. TNF-RI is activated by either sTNF or tmTNF, while TNF-RII is preferentially activated by tmTNF [69].

Upon stimulation, the intracellular domain of TNF-RI binds to the TNF receptor-associated death domain (TRADD) protein. This can further activate either an apoptotic pathway, via the Fas-associated death domain (FADD) protein, or a proinflammatory pathway, via TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein, resulting in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κb) [70]. NF-κb regulates the production of cytokines, adhesive molecules, growth factors, metalloproteinases, and many other proteins that are associated with the synthesis of prostaglandins, leukotrienes, and NO [68].

Unlike TNF-RI, TNF-RII is unable to activate the TRADD/FADD pathway and signals only through the TRAF2-associated pathway [70].

1.4.6 Endothelial activation in HIV-1 infection

Endothelial activation encompasses a constellation of alterations in cell function: release of vasomotor factors, permeability to flux of fluids and solutes, release of cytokines and chemokines, expression and secretion of adhesion molecules, and modulation of local coagulation [71]. Multiple pathways regulate endothelial activation in mature vascular beds, including vascular endothelial growth factor (VEGF), the Angiopoietin (Ang) – Tie2 system, sphingosine-1 phosphate, and Slit2/Robo4 [72].

When endothelial cell injury is induced by vascular inflammation, fragments of activated endothelium, endothelial microparticles, and even entire endothelial cells are shed into the circulation. These circulating levels can easily be measured in the blood which are already been found to be increased in association with coronary endothelial dysfunction, unstable coronary syndromes and vasculitis [73].

Chapter 3 of the present thesis examines several biomarkers of endothelial activation: Ang2, sVEGFR1 and sEng. We review these selected molecules and their regulatory pathways below.

1.4.6.1 Angiopoietin-2 (Ang2)

The Angiopoietin-Tie ligand-receptor pathway plays a critical role in regulating endothelial quiescence, inflammation, and angiogenesis [74]. Angiopoietins are secreted, multimeric ligands. There are three genuine angiopoietins (Ang1, -2, and -4) in humans. Their cellular receptors are tyrosine kinases with Ig and EGF homology domains (Tie). There are two forms, Tie1 and Tie2, which are expressed only by endothelial cells [75].

Angiopoietin-2 (Ang2) is a 496 amino acid protein [76]. The predominant source of Ang2 is endothelial cells and some smooth muscle cells. Ang2 is stored in electron-dense storage granules of endothelial cells called Weibel–Palade bodies [75].

The constitutive stimulation of Tie2 by Ang1 regulates vessel maturation and maintains vascular quiescence. Following binding to Ang1, Tie2 becomes phosphorylated on several cytoplasmic tyrosine residues, which leads to activation of downstream signaling pathways, including the phosphoinositide 3 (PI3)-kinase/AKT and ERK pathways. Downstream effects include the strengthening of tight junctions between endothelial cells. Ang1 also plays a significant role in facilitating interactions between the endothelium and surrounding matrix and mesenchyme [77]. Subsequently, plasma components and leukocytes are unable to pass from the blood vessel to the tissue, thereby strengthening endothelial barrier function [77].

Under normal physiologic conditions, Ang2 expression remains low because of AKT-mediated inhibition of the FOXO1 transcription factor [75]. In conditions associated with low AKT

activity, for example, when Ang1/Tie2 signaling is weak, activation of FOXO1 occurs, which increases Ang2 expression. Ang2 exhibits antagonistic effects to Ang1 at the Tie2 receptor. Ang2 can also be upregulated in areas of injury or inflammation [74]. Ang2 acts through an autocrine mechanism to control endothelial responsiveness to multiple cytokines, including vascular endothelial growth factor (VEGF), permeability-inducing molecules such as histamine and bradykinin, and pro-inflammatory cytokines such as TNF. As a result, the endothelium is destabilized [78].

These findings were validated in murine models. Ang2 deficient mice exhibited a reduced ability to express adhesion molecules on cell surfaces with inflammatory stimuli [79]. Engineered mice lacking Ang1 expression show deficits in vascular development [77].

1.4.6.2 Soluble vascular endothelial growth factor receptor 1 (sVEGFR1)

The family of ligands known as vascular endothelial growth factors (VEGFs) mediate angiogenesis during human development and in pathological conditions [80]. VEGF exerts potent mitogenic effects on the vascular endothelium through the inhibition of apoptosis, chemotaxis, and induction of blood vessel permeabilization [81]. In mature vascular beds, VEGF induces endothelial permeability.

There are several VEGF isoforms, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. These are homodimeric polypeptides, produced by mast cells. VEGFs binds to transmembrane tyrosine-kinase receptors, called VEGF receptors (VEGFRs) [82]. There

are three VEGF receptors, VEGFR-1 (also known as *flt-1*), VEGFR2 (also known as *KDR/flk-1*), and VEGFR-3 (also known as flt-4). The binding-affinity of VEGFs isoforms differs between VEGFRs. VEGF-A binds to VEGFR-1 with one order of magnitude higher affinity than VEGFR-2. VEGFRs also differ in their downstream signaling potential. The kinase activity of VEGFR-1 is about 10-fold weaker than that of VEGFR-2. Thus, by trapping VEGF ligands, VEGFR-1 downregulates angiogenesis [83].

VEGF binding to extracellular domains of VEGFR induces receptor dimerization, autophosphorylation of the tyrosine residues in the intracellular domains, and activation of a cell signaling cascade. This results in several cellular responses, including proliferation, migration, vasculogenesis, and endothelial permeability [84].

Soluble VEGF receptor-1 (sVEGFR1) is a truncated 110 kDa splice variant of the 180 kDa membrane-spanning VEGFR1, which is derived from alternative splicing of messenger RNA or by the proteolytic cleavage of full-length VEGFR1. The sVEGFR1 protein contains the first six N-terminal immunoglobulin-like (Ig)-like extracellular motifs of VEGFR1 but lacks the seventh Ig-like domain, the membrane-anchoring region, the regulatory juxtamembrane domain, and intracellular tyrosine kinase domains [85]. Endogenous sources of sVEGFR1 include endothelial cells, vascular smooth muscle cells, activated peripheral blood mononuclear monocytes, placental trophoblasts, corneal epithelial cells, and proximal tubular cells of the kidney [82].

The molecular interactions of sVEGFR1 with VEGF family ligands are likely similar to those of VEGFR1, given their structural similarities. [82]. The physiologic or pathologic effects of sVEGFR1 occur by blocking the action of VEGF-A. Two mechanisms have been proposed: (1) direct ligand trapping, thereby lowering the effective concentrations of free VEGF; and (2) heterodimerization with surface VEGFRs to build dominant-negative complexes, thereby reducing the effective density of VEGFR available for activation [86, 87].

1.4.6.3 Soluble endoglin (sEng)

Endoglin is a transmembrane glycoprotein, known also as transforming growth factor-β receptor III or CD105. There are two forms of endoglin with several physiological and pathological roles: a membrane-bound form expressed in various tissues and a soluble form (sEng) found in plasma. Endoglin is a homodimer composed of two 95 kDa subunits. Each subunit consists of three domains: a large extracellular domain, a transmembrane domain, and a short intracellular domain [88]. Although Endoglin is predominantly expressed in endothelial cells, it can also be detected in various other cells, including smooth muscle cells, mesenchymal and hematopoietic stem cells, monocytes/macrophages, placental syncytiotrophoblasts and fibroblasts. sEng is generated by the cleavage of the extracellular domain of endoglin by membrane-type metalloprotease-14 (MMP-14) [89]. Receptor cleavage may serve as a naturally occurring antagonist for TGF-β signaling. MMP-14 is highly expressed in malignant epithelial cells and endothelial cells [90].

Endoglin's physiologic role relates to TGF-β signaling. TGF-β is involved in cell proliferation, differentiation, migration, and survival. The regulatory function TGF-β affects multiple biological processes such as cell development, carcinogenesis, fibrosis, and wound healing, as

well as immune responses. The inhibition of TGF-β1 activity persuades pro-atherogenic changes in the vessel wall of atherosclerotic animal models [88]. sEng is able to bind TGF-β and reduce available ligand for the transmembrane TGF-β receptor III. Thus, sEng is an endogenous antagonist that may play a role in regulating the biological effects TGF- β. sEng interferes with downstream signaling of TGF- β (reduced Smad2/3 activation). This mechanism further inhibits endothelial nitric oxide synthase (eNOS) – mediated vasodilatation [90]. Decreased eNOS activity leads to increased cell adhesion molecules expression, vascular permeability, antiangiogenic effects, alteration of vasodilation, endothelial dysfunction, arterial hypertension, and atherosclerosis [91]. Therefore, eNOS inhibition may play a critical role in mediating the effects of sEng on the vascular endothelium.

1.5 Clinical manifestations of HIV

The symptoms of HIV differ depending on the stage of infection. In the first few weeks following initial infection, people may be asymptomatic or may have an influenza-like illness including fever, headache, rash, or sore throat. Many PLWH are unaware of their status until the later stages.

With progressive immune deficiency caused by HIV infection, PLWH can develop a broad range of signs/symptoms, such as lymphadenopathy, weight loss, fever, diarrhea, and cough. In the absence of treatment, severe illnesses may occur, such as tuberculosis (TB), cryptococcal meningitis, severe bacterial infections, and cancers such as lymphomas and Kaposi's sarcoma [6].

1.6 Diagnosis

The "fourth-generation" HIV test is the standard HIV screening method used in Canada. This assay detects both HIV antibodies and the p24 antigen of HIV-1. This antigen–antibody combination test is highly sensitive. Fifty percent of people will have a positive test within 18 days of infection, 95% within 34 days, and 99% within 90 days, defining a "window period" between infection and HIV-1 detection. If positive, confirmatory HIV testing is performed, with Western blot, or (in Alberta) using a combination HIV-1 and HIV-2 antibody assay (e.g., Geenius™ HIV-1/2 Confirmatory Assay).

Direct detection of HIV nucleic acid is used for viral load (VL) monitoring in patients on cART, and to detect integrated provirus within host cells. These assays are based on the polymerase chain reaction and may be qualitative (positive or negative) or quantitative (copies per volume of blood).

HIV testing and diagnosis is the entry point to HIV treatment for PLWH. As a public health strategy, it is also a method to enhance engagement in HIV prevention HIV seronegative individuals who are at ongoing high risk of transmission. Improved access to HIV diagnostics may be achieved through alternatives to laboratory-based assays such as point-of-care (POC) testing or dried blood spot (DBS) testing.

Rapid POC testing can improve access by circumventing some obstacles associated with

laboratory-based testing. In a traditional testing algorithm, a venipuncture blood sample is collected and sent to a laboratory for HIV screening. The patient must return to the place where they were tested to receive the result in a separate visit days later. In contrast, POC testing from a finger-prick blood sample takes about 20 minutes (including pre-test counselling, receipt of the test result, and post-test counselling) without need for a repeat visit. A reactive (positive) test should be confirmed with a laboratory test. One limitation of POC testing is that the window period can be as long as three months because the assay only looks for HIV antibodies. POC testing is currently only available in some Canadian provinces.

There is growing interest in DBS testing in Canada. This approach uses a sample of blood from a finger prick that is collected as a blot on a card. After the blood spot is dried at room temperature, it is sent to a public health laboratory for screening and confirmatory testing. DBS testing is convenient for use in rural and remote areas because the samples are stable without refrigeration after collection. Even non-medical staff can collect DBS samples [92].

Diagnostic testing plays a key role in the current strategy to prevent vertical transmission. Routine “opt-out” prenatal HIV testing for all pregnant women is recommended during ante-natal clinic visits. Women who are not tested for HIV during pregnancy should undergo rapid HIV antibody testing at the time of delivery. Similarly, if women are at high risk for HIV transmission (e.g., IV drug users, commercial sex workers, or HIV negative women in serodiscordant relationships), repeat testing is encouraged during late pregnancy and at delivery.

Pregnant women living with HIV should be followed by a specialist in the management of HIV and their VL should be monitored regularly. Pediatricians and family physicians who are involved in the post-natal care of infants should ensure that the HIV status of the mother is known and documented prior to discharge [19].

1.7 Treatment

HIV replication can be suppressed with cART, usually consisting of three or more drugs. cART does not cure HIV infection but suppresses viral replication and allows recovery of immune function.

In 2016, the WHO recommended lifelong cART for all PLWH, including children, adolescents, adults, pregnant and breastfeeding women, regardless of their clinical status or $CD4^+$ T-lymphocyte count. By 2019, 182 countries had already implemented this recommendation, which covered 99% of all PLWH worldwide [6].

Over 30 licensed antiretrovirals (ARVs) are in use clinically. These medications can be classified according to their mechanism of action into several categories: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs) and entry inhibitors (e.g. fusion inhibitors and CCR5 antagonists). Combinations of these ARVs provide potent inhibition of viral replication.

Although highly effective, cART regimens differ in their tolerability, long-term safety profile,

and barrier to the development of viral resistance. Some ARV medications induce disturbances in lipid and glucose metabolism which exacerbate the risk of CVD. Older PIs are associated with dyslipidemia, lipodystrophy, and insulin resistance. Prolonged exposure to PIs is associated with an elevated risk of myocardial infarction [94]. Both NRTIs and NNRTIs are associated with lipodystrophy and hyperlipidemia that contribute to the pathogenesis of CVD in HIV patients [95].

Current HIV treatment guidelines recommend new cART options with better tolerability, higher efficacy, and lower rates of treatment discontinuation. Globally, the WHO currently recommends the use of dolutegravir or efavirenz for first-line therapy. Raltegravir and darunavir/ritonavir are recommended for second-line therapy. Switching to dolutegravir has already initiated in 82 low- and middle-income countries which is expected to enhance the durability of treatment and compliance of people living with HIV [6].

Globally, access to HIV treatment remains incomplete. 23.3 million people living with HIV were receiving cART in 2018, representing a global coverage rate of approximately 62%. Efforts are warranted to scale up coverage, particularly for children and adolescents. At the end of 2018,

only 54% of children and adolescents were receiving cART [6]. With expansion of cART coverage, increasingly large numbers of HIV-infected children will start ART over the next decade. Those starting cART may be younger, with less severe immunosuppression, and a longer treatment horizon [55]. This highlights the need to target underlying pathogenic processes of inflammation to improve outcomes in high-risk children, despite universal availability of ART.

1.8 Objectives of the thesis

Chapter 2 of this thesis begins with a case report of vertical HIV transmission due to primary HIV infection in a breastfeeding mother. Next, it documents a retrospective chart review of HIV serodiscordant couples in which the male partner was HIV- positive, and the pregnant female partner was HIV-negative. The objective of this study was to describe pregnancy outcomes and the prevalence of risk factors for HIV transmission in HIV-serodiscordant couples. Risk factors of interest included male VL during the period of pregnancy and breastfeeding. We hypothesized that, despite surveillance programs for VT, residual risk persists, allowing for rare transmission events.

Chapter 3 of this thesis reports a study of CLWH examining biomarkers of MT, systemic inflammation, and endothelial activation. The objective of this study was to examine the correlation between markers of these three pathologic pathways and to investigate possible associations between endothelial activation and clinical, immunological, virologic, and treatment characteristics of the patient cohort. We hypothesized that markers of endothelial activation would be correlated with markers of MT and systemic inflammation. We further hypothesized

that successful early cART treatment would be associated with reduced markers of endothelial activation. We tested these hypotheses using a cross-sectional study design with measurement of selected biomarkers in the Early Pediatric Initiation-Canadian Child Cure Cohort (EPIC[4]), a well-characterized cohort of CLWH across Canada with detailed longitudinal clinical and laboratory data.

CHAPTER 2. Pregnancy among HIV-serodiscordant couples: case report of vertical transmission and retrospective case series

Authors and affiliations:

Bipasha Choudhury[1], Maria Stadnyk[2], Dolores Freire Jijon[1], Luke McLaughlin[3], Wendy Vaudry[1], Stan Houston[3,5], Alena Tse Cheng[1], Michael T. Hawkes[1,4-7]

[1]Department of Pediatrics, University of Alberta, Edmonton, Canada.

[2]STI Clinic, Edmonton General Hospital, Edmonton, Canada.

[3]Department of Medicine, Division of Infectious Diseases, University of Alberta, Edmonton, Canada

[4]Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Canada.

[5]School of Public Health, University of Alberta, Edmonton, Canada.

[6]Distinguished Researcher, Stollery Science Lab

[7]Member, Women and Children's Health Research Institute

Contributions:

Bipasha Choudhury performed the chart reviews, extracted the laboratory results from electronic databases, analyzed the data, wrote the first draft of the manuscript, and revised the manuscript based on feedback from co-authors.

MS collected public health data, followed the serodiscordant couples, and critically reviewed the manuscript.

DFJ wrote the case report and contributed to the literature review, and critically reviewed the manuscript.

LM wrote the case report and critically reviewed the manuscript

WV supervised the analysis and write-up of the first draft of the manuscript and critically reviewed the manuscript.

SH supervised the analysis and write-up of the first draft of the manuscript and critically reviewed the manuscript.

MTH supervised the analysis and write-up of the manuscript and critically reviewed the manuscript.

2.1 Abstract

Background

Monitoring for HIV transmission during pregnancy and breastfeeding among serodiscordant heterosexual couples may yield additional opportunities to identify early HIV infection and prevent vertical transmission. Such a public health strategy could be a valuable tool toward elimination of vertical transmission of HIV-1 infection in Canada.

Objective

To report a case of undetected vertical HIV transmission during breastfeeding and examine the prevalence of risk factors for HIV transmission in the pregnancy and postpartum periods among serodiscordant couples.

Methods

Case report and retrospective chart review of HIV-serodiscordant pregnant couples over an eight-year period in Edmonton, Canada.

Results

We report a case of maternal primary HIV infection during the postpartum period and vertical transmission to a nursing infant that went undetected until the infant presented with AIDS. We also report a series of 47 serodiscordant pregnant couples identified by our public health nurse between 2008 and 2016. No cases of HIV transmission occurred. Among HIV-infected male partners, 15 had detectable viral load (VL) during their partner's pregnancy, with median peak

VL 3,800 copies/mL (IQR 370-14,000), and 16 males had a detectable VL during breastfeeding, with median peak VL 5,000 copies/mL (IQR 210-30,000).

Conclusion

Despite concerted attempts to minimize HIV transmission during pregnancy and breastfeeding in our well-resourced setting, residual transmission risk remains due to non-suppressed viral load within many HIV-serodiscordant pregnant couples.

2.2 Introduction

Human immunodeficiency virus (HIV) continues to be a major public health issue globally. According to the World Health Organization, 37.9 million people were living with HIV in 2018, and of these, 1.8 million were children [3]. Low- and middle-income countries across the world experience the heaviest impact of HIV and acquired immune deficiency syndrome (AIDS); however, HIV continues to be a major health concern in industrialized countries as well [96]. In Canada, ongoing HIV transmission remains an issue of concern with a total of 2,402 new HIV cases were reported in 2017. The incidence appears to be rising, from 5.8 per 100,000 populations in 2014 to 6.5 per 100,000 populations in 2017. Among the provinces, Saskatchewan had the highest HIV infection rate (15.5 per 100,000 population) and accounted for 7.5% of total new HIV cases reported across Canada, while Alberta had the third highest rate (6.6 per 100,000 population) [3]. Over the past two decades, the rate of HIV infection in women has been rising steadily. Towards the end of 2014, while approximately 75,500 Canadians were living with HIV, it was estimated that 16,880 were women, accounting for about 22% of the national total. A further concern is that most of these women with HIV infection are of reproductive age with potential risk of vertical transmission of the virus to their offspring [97].

The principal routes of vertical transmission are trans-placental, via the birth canal, and via breastfeeding [98]. Canadian data demonstrated a 16% rate of vertical HIV transmission among women with no antiretroviral treatment during pregnancy, 1.6% with mono- or dual- nucleoside-analogue therapy, and 1.0% with combination antiretroviral therapy [99]. In 2018, there were 259 infants born to HIV-positive mothers, five (1.9%) of whom were vertically infected [3]. Recently, there has been increased investment in programs for the prevention of vertical

transmission of HIV globally. However, most of these programs focus on pregnant women who are already identified as HIV-positive during or before antenatal care. The prevention of vertical transmission still remains challenging for mothers who seroconvert after antenatal screening, later in pregnancy or while breastfeeding [100]. Nevertheless, evidence suggests that pregnant and post-partum women may have heightened susceptibility to HIV infection and higher rates of HIV seroconversion [101]. Furthermore, women who acquire primary HIV-1 infection during pregnancy or breastfeeding are at higher risk of transmitting HIV to their infants than chronically infected women [102]. The mechanism underlying this heightened susceptibility are the high levels of HIV viral load (VL) that occur during acute infection and maternal immune response that may not be sufficiently mature to allow significant transfer of protective immunity to the child during acute infection [103-105]. Considering this challenge, efforts are warranted to monitor pregnancies arising in high-risk heterosexual couples where the male partner is positive, and the pregnant female is seronegative (serodiscordant pregnancies).

This study begins with a case report of HIV seroconversion in a breastfeeding mother which was only discovered when her infant was diagnosed with an AIDS-defining opportunistic infection. We next report a retrospective chart review of a cohort of 47 HIV-serodiscordant pregnant couples, based on a surveillance system in Edmonton. Our objective was to examine the prevalence of risk factors for HIV transmission in the pregnancy and postpartum periods in our well-resourced setting.

2.3 Methods

2.3.1 Setting and participants

The Northern Alberta Program (NAP) is a publicly funded HIV treatment and surveillance program based out of three sites in Edmonton, Canada: the Kaye Clinic, Royal Alexandra Hospital, and the Edmonton Sexually Transmitted Infection (STI) Clinic. PLWH are followed longitudinally for their clinical care, which involves: individualized cART prescription; monitoring for adherence; routine quantification of VL and $CD4^+$ T-lymphocyte count; and assessment of clinical or laboratory signs of opportunistic infections or medication adverse effects. A patient with well-controlled HIV on cART will typically be followed every six months, with VL testing at each visit.

In September 1998, Alberta Health and Wellness introduced the Prenatal HIV Screening Program. Since then, all pregnant women in Alberta have been offered testing for HIV on an "opt-out" basis as part of routine prenatal care. The rate of uptake of HIV testing in women accessing prenatal care in Alberta was 96.3% [106]. Women who test negative in early pregnancy who have characteristics associated with increased risk for acquiring HIV are retested in the late third trimester or during labour. Since 2008, a public health surveillance program has been in place to follow the outcomes of HIV-serodiscordant pregnant couples. The rationale for this surveillance program is that maternal seroconversion in pregnancy represents a high risk for vertical transmission. Early detection of transmission may be an opportunity for intervention prior to delivery. We reviewed the medical records of serodiscordant pregnant couples in the NAP over an eight-year period. Couples were identified when HIV positive male partners followed through the NAP program disclosed to their HIV treatment team that their sexual

partner was pregnant. This disclosure triggered the public health nurse to follow the couple and determine the outcome for mother and baby.

To be eligible for inclusion in this study, the male partners were HIV positive while the female partners were HIV negative and pregnant. Serodiscordant couples were excluded if pregnancy outcome data were missing or if the pregnancy ended in spontaneous or therapeutic abortion or still birth.

2.3.2 Study procedures

A standardized data collection tool was used to collect data by retrospective chart review on risk factors for vertical transmission of HIV. Data gathered on male partners included demographic characteristics, IV drug use, VL, $CD4^+$ T-lymphocyte count, and other STD testing. For pregnant women, the following information was abstracted from the chart record: sociodemographic characteristics, HIV and other STD testing, intravenous drug use. Data on the pregnancy and delivery were also collected, including condom use in pregnancy once the serostatus of male partner known, due date of delivery, mode and date of delivery, and pregnancy outcome. Neonatal characteristics included sex, birthweight, gestational age, congenital anomaly.

Laboratory data was extracted from the medical record for seropositive males (VL, $CD4^+$ T-lymphocyte) and seronegative females (HIV serology screening of VL) during the period nine months prior to delivery (pregnancy period) to 24 months after delivery (presumed breastfeeding period). Sources of laboratory data included the chart record, eClinician (an electronic medical

record which includes laboratory data), and the NAP patient electronic database. In order to ensure complete ascertainment of test results, we plan to search an additional electronic database, the Provincial Laboratory system, which will be the most comprehensive source of laboratory data, pending ethical and operational approvals.

2.3.4 Statistical analysis

Descriptive statistics were expressed as number with percentage for dichotomous variables and median with range or interquartile range (IQR) for continuous variables. Data analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA, 2012), and R. [107]

2.3.5 Ethics statement

This retrospective chart review was approved by the Human Research Ethics Board (HREB) at the University of Alberta (Study identifier Pro00066472).

2.4 Results

2.4.1 Case report

A 5-month old girl was hospitalized with a one-month history of persistent fever and progressive cough. The patient had been admitted to hospital twice in the preceding three months for respiratory and urinary tract infections. In the month preceding her admission, she had visited the emergency department three separate times due to high fevers.

Birth history included a term vaginal delivery to a mother with an uncomplicated pregnancy and adequate prenatal care, including negative first trimester HIV testing. The infant was breastfed. The patient had no known medical conditions or history of immunodeficiency. Her parents had immigrated to Canada from an HIV-endemic country and the patient was born in Canada.

Physical examination on admission revealed an alert, interactive child without signs of lethargy. She was tachypneic and febrile at 40.4ºC. Lung auscultation revealed right-sided bronchial sounds. The rest of the exam was unremarkable. Initial investigations showed thrombocytopenia, elevated LDH, and undetectable immunoglobulins. A chest radiograph showed bilateral upper lobe consolidations consistent with multi-focal pneumonia. Bronchoscopy was performed and examination for pneumocystis organisms was positive. HIV testing was antigen positive, but negative for HIV antibodies. HIV RNA testing showed a VL of 9,322,360 copies/ml (log 7.0).

It was discovered that the patient's father was HIV-infected, had stopped taking his ARVs, and had disengaged from his treatment program. His wife had no knowledge of his HIV positive

status. Following her daughter's HIV diagnosis, the mother was tested and found to be HIV-infected. Retrospective testing of the mother's serum at delivery for HIV RNA showed no detectable virus. The infant was felt to have acquired HIV through breastfeeding.

2.4.2 Retrospective case series of HIV-serodiscordant pregnant couples

In response to the above case of vertically transmitted HIV-1 at our center, we reviewed the public health surveillance records of HIV-seronegative pregnant women who had a known seropositive male sexual partner. Fifty-six HIV-serodiscordant couples were followed by the NAP during the period of 2008-2016. Of these 56 pregnancies, information was missing on birth outcome in 3, therapeutic abortion occurred in 4, and stillbirth in 2. Characteristics of 47 serodiscordant couples who completed the pregnancy with live newborn(s) are outlined in Table 2.1. There were 46 singleton births and one set of live twins delivered.

Table 2.1. Characteristics of 47 serodiscordant pregnancies (seronegative pregnant female and seropositive male sexual partner)

Demographic characteristics	**Female (n=47)**	**Male (n=47)**
Age [years], median (IQR)	35 (22-49)	41 (31-59)
Known Intravenous Drug Use	1 (2%)	7 (15%)
Hepatitis B co-infection, n (%)		
HBsAg positive	1 (2.1%)	7 (15%)
HBsAg negative	40 (85%)	37 (79%)
HBsAg unknown	6 (13%)	3 (6.3%)
Hepatitis C co-infection, n (%)		
HCV serology positive	7 (15%)	14 (30%)
HCV serology negative	34 (72%)	31 (66%)
HCV serology unknown	6 (13%)	2 (4.2%)

With respect to the seropositive male partners, a total of 47 VL measurements during the pregnancy period, and 137 during the period of breastfeeding were available from the paper charts and electronic databases. Median and peak VLs are shown in Table 2.2. Of note, for 19/47 (40%) of males, we did not find a VL measurement during the pregnancy period, based on a search of available electronic medical records. Of those for whom at least one VL was documented, 15/28 (54%) had a detectable VL, with median (IQR) peak VL of 3,800 (IQR 370-14,000) copies/mL. Similarly, during the breastfeeding period, for 14/47 (30%) males, we did not find a VL measurement. Of those for whom at least one VL was documented, 16/33 (48%) had a detectable VL, and the median (IQR) peak VL was 5,000 (210-30,000).

With respect to the seronegative female partners, 98 and 23 serology tests, and 36 and 28 VL tests were performed during pregnancy and breastfeeding, respectively (Table 2.2). All serology results were negative and VLs were undetectable. Consistent condom use was reported by 14 (30%) of couples. None of the women received pre-exposure prophylaxis.

Table 2.2. Virologic and immunologic characteristics of serodiscordant pregnant couples

Characteristic (N=47)	During period of pregnancy	During period of breastfeeding
Male partner		
HIV Viral load		
No viral load found in available databases	19/47 (40%)	14/47 (30%)
Number of viral load measurements documented[1]	2 (1-2)	4 (3-5)
Viral load not suppressed[1]	15/28 (54%)	16/33 (48%)
Highest viral load (copies/mL), median (IQR)[2]	3,800 (370-14,000)	5,000 (210-30,000)
Median viral load (copies/mL), median (IQR)[2]	2,500 (97-8,500)	400 (100-4,400)
Low risk[3]	13/47 (28%)	17/47 (36%)
$CD4^+$ T-lymphocyte count		
No CD4 count found in available databases	19/47 (40%)	14/47 (30%)
Number of CD4 measurements documented[1]	2 (1-2)	4 (2-5)
One or more CD4 count < 500 cells x 10^6/L[1]	18/28 (64%)	23/33 (70%)
Lowest CD4 count (cells x 10^6/L), median (IQR)[1]	440 (320-550)	390 (310-530)
Female partner		
HIV serology		
No serology test found in available databases	13 (28%)	34 (72%)
Number of serology tests documented, median (range)[1]	3 (1-5)	2 (1-5)
HIV Viral load		
No viral load found in available databases	26 (55%)	42 (89%)
Number of viral load tests documented, median (range)[1]	1 (1-4)	1 (1-3)

[1]Among those with at least one measurement documented
[2]Among those with at least one detectable VL
[3]At least one VL was measured and all were undetectable

No cases of HIV transmission were documented between seropositive males and their seronegative pregnant sexual partners during the period of pregnancy and breastfeeding. However, one female participant seroconverted three years after delivery (likely beyond the weaning period and posing no risk to the child). Among the 47 mothers, 41 (87%) had a documented negative HIV serology test beyond the period of weaning, allowing us to confidently exclude HIV transmission to mother and child. Maternal HIV serostatus was unknown for 6 mothers at the end of the breastfeeding period; such that we could not confirm that they remained HIV negative.

Of the 48 newborn infants, 39 (81%) were born at term and 7 (15%) were preterm (gestational age was unknown for one infant). Vaginal delivery occurred for 32 (67%) infants and 14 (29%) infants were delivered by caesarean section (mode of delivery was unknown for one infant). Genetic, congenital, and/or fetal abnormalities were present in 7 (15%) of newborns, including asymmetrical IUGR (n=4), ventriculomegaly with small cerebellum, VSD, and trisomy 21.

2.5 Discussion

Here we describe a case report of vertical HIV transmission during breastfeeding in a high-resource setting which escaped detection by the universal surveillance system for HIV in pregnancy. We also report a case series of 47 serodiscordant couples in which the female partner was pregnant and HIV-1 negative and the male partner was HIV-1 positive. No cases of HIV seroconversion occurred among the pregnant women during pregnancy and breastfeeding in our study; however, high VL in many male partners suggests that a substantial risk persists in our community. In our publicly funded health care system with universal access to HIV screening in pregnancy and freely available antiretroviral treatment, a case of vertical transmission is noteworthy. Vertical transmission in high-income settings has become rare, with only 200 cases annually in the United States, and only 188 in the European Union [108, 109]. Data from the Canadian Perinatal HIV Surveillance Program indicate that the rate of vertical transmission declined from 19.6% between 1990 and 1996 to 2.5% between 1997 and 2012 [97]. Reductions in perinatal transmission may be attributed, at least in part, to the impacts of opt-out prenatal testing and the availability of publically funded antiretroviral drugs. An additional public health strategy implemented at our facility was the real-time prospective follow-up of serodiscordant couples, a strategy not previously described in the literature, to our knowledge. Despite these numerous public health interventions, our case illustrates that vertical transmission continues to occur and may be difficult to prevent entirely with current strategies.

Transmission of HIV through breastmilk during primary maternal HIV infection, as in our case, was first reported in 1985 in an infant who acquired HIV after being breastfed by his previously healthy mother, who received a postpartum transfusion of HIV-contaminated blood [110].

Several other case reports demonstrated acquisition of HIV infection by infants of breastfeeding mothers, in which the mothers were HIV seronegative until delivery, and acquired primary HIV infection through heterosexual exposure [111-113]. A systematic review of published studies estimated that the risk of transmission from a lactating mother to her infant was 29% during primary infection [114]. The presence of high breast milk VL in primary HIV infection explains this increased risk of transmission [115]. Case series and cohort studies in Australia [116], China [117], and Zimabawe [118] demonstrated a similarly high risk of transmission with the acquisition of HIV-1 infection after delivery.

The late pregnancy and post-partum periods are associated with a 2.82- and 3.97-fold higher transmission risk, relative to the nonpregnancy state, respectively [18]. In our case report, a negative HIV RNA PCR test from serum at the time of delivery strongly supported post-partum acquisition of HIV from her undisclosed seropositive partner. A study of 2,751 HIV-serodiscordant couples in 7 African countries, which included 686 pregnancies, demonstrated an incidence of 5.37 transmission events per 100 person years [18]. Based on this incidence, the expected number of infections in our cohort of 47 Canadian serodiscordant couples would be ~3.2. However, no incident infections were found in the present study, despite numerous risk factors for transmission (e.g., high VL in about one-third of males and condom use in only 30% of couples). Differences in VL, sexual behaviour, condom use, or STI co-infection may explain the lower transmission rate observed in our setting.

A substantial proportion of male partners in our cohort had a high VL during their partners' pregnancy (15 males, median peak VL 3,800) and breastfeeding (16 males, median peak VL

5,000. The probability of HIV transmission increases proportionately with male partner HIV VL [18]. In our case report, the infected male had not attended HIV clinic visits in follow-up, had stopped taking his medications, and likely had an elevated VL. Within the current "90-90-90" [119], "treatment as prevention" [120, 121] and "undetectable equals untransmittable (U=U)" [122] paradigms, the frequency of males with detectable viral load in our study suggests that gaps remain in achieving public health goals for HIV in our context.

HIV non-disclosure in a serodiscordant relationship poses a risk of transmitting the virus to the uninfected partner, as in our case report [123]. It represents a structural barrier to the current surveillance system [124]. HIV transmission in our case report was directly related to non-disclosure in a serodiscordant couple where the male partner was HIV positive without the mother's knowledge. The existing surveillance system failed to identify a high-risk pregnancy in this case. Prior studies showed that HIV disclosure was associated with a reduced risk of HIV transmission by 18% to 41% and increased use of condoms from 4% to 57% in serodiscordant couples [125]. Non-disclosure as a risk factor for transmission would likely be under-represented in our case series since we relied on self-reporting of pregnancy by the infected male partner.

Given the heightened risk and potential for severe consequences of HIV transmission during the pregnancy and postpartum periods, enhanced surveillance and interventions may be contemplated. Our public health nurse follows serodiscordant pregnant couples, yet there may be opportunities for improved surveillance during the post-partum period, since 30% of HIV-infected males had no VL documented and 72% of females had no serology test documented during this time. Of note, testing for seroconversion during lactation is challenging to organize

and appears to be of low interest to patients [108]. Pre-exposure prophylaxis (PrEP) has been used safely, and with high patient acceptability, among Kenyan pregnant and postpartum women, including 193 with an HIV-positive partner [107]. None of the pregnant mothers in our study received PrEP since public funding for PrEP was introduced in Alberta in 2018, after the study period (2008-2016).

This study has several limitations. Our case report and case series were based on a single clinic's experience, which may limit the generalizability of our findings. However, the NAP is the sole provider of HIV care in the geographic area, and therefore our study approaches a population-based survey. Prospective longitudinal studies of discordant couples are the preferred design for the investigation of heterosexual HIV transmission; however, our study was based on retrospective chart review. Serodiscordant pregnant couples in our study were identified through self-reporting by an HIV-positive man; therefore, potentially many more high-risk cases may go undetected by our current strategy. Our case report of HIV transmission in an undisclosed heterosexual partnership is one such example. As a result, our study may underestimate the actual potential for HIV-1 transmission.

Furthermore, the small sample size limited our ability to precisely estimate the transmission risk. Sources for laboratory data included several electronic databases used in clinical practice; however, the completeness of these databases is not known. As a result, there may have been more testing for VL, $CD4^+$ T-lymphocyte count, and maternal serology than we found in our study. A definitive and complete database of laboratory procedures (Provincial Laboratory database) will be examined prior to publication of these results.

Vertical HIV transmission remains a concern, even in well-resourced settings, and raises

important questions about the additional preventive measures that could be implemented. We believe that targeted health educational interventions may prevent this uncommon mode of transmission. These interventions should include parents from high-HIV-prevalence countries. Women who have avoided HIV infection in their countries of origin may not appreciate their ongoing risk of HIV infection after immigration to Canada, even if their male partners are from endemic countries. Education should also focus on health care professionals counseling pregnant women who may not be aware of the increased risk of heterosexual transmission during the postpartum period when the mother may be breastfeeding. Counseling should address condom use and also include education on the high risk of HIV postnatal transmission after heterosexual exposure during breastfeeding. In our experience, having a specific, written, readily available protocol in place that addresses the steps in reducing the rate of transmission in both serodiscordant and seropositive pregnant women is paramount. Although uncommon, HIV infection during the pregnancy or post-partum periods is a devastating event. Our case report and case series highlight residual gaps in public health strategies for prevention of vertical transmission and opportunities for increased vigilance.

CHAPTER 3. Endothelial activation is associated with microbial translocation, systemic inflammation, and treatment regimen in children living with vertically acquired HIV-1 infection

Data from this chapter has been submitted for publication to the journal *HIV Medicine*.

Authors and affiliations:

Bipasha Choudhury[1], Jessica Brown[2], Doris G. Ransy[3], Jason Brophy[4,5], Fatima Kakkar[6], Ari Bitnun[7], Lindy Samson[4], Stanley Read[7], Hugo Soudeyns[3,8], Wendy Vaudry[1], Stan Houston[9,10], Michael T. Hawkes[1,10-13]

[1]Department of Pediatrics, University of Alberta, Edmonton, Canada.

[2]Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Canada

[3]Unité d'immunopathologie virale, Centre de recherche du CHU Sainte-Justine

[4]Division of Infectious Diseases, Children's Hospital of Eastern Ontario

[5]Department of Pediatrics, University of Ottawa

[6]Division of Infectious Diseases, CHU Sainte-Justine, Department of Pediatrics, Université de Montréal

[7]Hospital for Sick Children, Department of Pediatrics, University of Toronto

[8]Department of Microbiology, Infectiology & Immunology, Université de Montréal

[9]Department of Medicine, Division of Infectious Diseases, University of Alberta, Edmonton, Canada

[10]School of Public Health, University of Alberta, Edmonton, Canada.

[11]Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Canada;

[12]Distinguished Researcher, Stollery Science Lab

[13]Member, Women and Children's Health Research Institute

EPIC[4] Study Group. Ariane Alimenti, BC Women's Hospital & Health Centre, Vancouver; Petronela Ancuta, Centre de recherche du Centre hospitalier de l'Université de Montréal (CHUM), Montreal; Ari Bitnun, Hospital for Sick Children, Toronto; Jason Brophy, Children's Hospital of Eastern Ontario (CHEO), Ottawa; Jared Bullard, Children's Hospital of Winnipeg, Winnipeg; Tae-Wook Chun, National Institute of Allergy and Infectious Diseases, Bethesda; Hélène C. F. Côté, University of British Columbia, Vancouver; Joanne Embree, Children's Hospital of Winnipeg; Michael T. Hawkes, Department of Pediatrics, University of Alberta, Edmonton; Fatima Kakkar, Centre hospitalier universitaire (CHU) Sainte-Justine, Montreal; Christos Karatzios, Montreal Children's Hospital, Montreal; Rupert Kaul, University of Toronto, Toronto; John Kim, National HIV and Retrovirology Laboratory (NHRL), Public Health Agency of Canada (PHAC), Winnipeg; Valérie Lamarre, CHU Sainte-Justine, Montreal; Normand Lapointe, CHU Sainte-Justine, Montreal; Pascal Lavoie, BC Women's & Children's Hospital, Vancouver; Terry Lee, Canadian HIV Trials Network (CTN), Vancouver; Deborah M. Money, BC Women's Hospital & Health Centre, Vancouver; Dorothy Moore, Montreal Children's Hospital, Montreal; Stanley Read, Hospital for Sick Children, Toronto; Robert Reinhard, University of Toronto; Lindy Samson, CHEO, Ottawa; Paul Sandstorm, NHRL, PHAC, Winnipeg; Laura Sauve, BC Women's Hospital & Health Centre, Vancouver; Sandra Seigel, McMaster Children's Hospital, Hamilton; Joel Singer, CTN, Vancouver; Hugo Soudeyns, Centre de recherche du CHU Sainte-Justine, Montreal; Ben Tan, Department of Pediatrics, University of Saskatchewan, Saskatoon; Wendy Vaudry, Stollery Children's Hospital, Edmonton.

Contributions:

Bipasha Choudhury performed the ELISA assays, analyzed the data, wrote the first draft of the manuscript, and revised the manuscript based on feedback from co-authors.

JB contributed to the first draft of the manuscript, literature review, and Table 3.2

DGR coordinated the study, data management, and sample storage and shipment

JB, FK, AB, LS, SR, HS were members of the EPIC[4] steering committee which conceived and designed the parent study and obtained funding. They critically reviewed the manuscript

WV recruited study participants, oversaw data and sample collection. She supervised the analysis and write-up of the first draft of the manuscript. She critically reviewed the manuscript.

SH supervised the analysis and write-up of the first draft of the manuscript. He critically reviewed the manuscript.

MTH supervised the analysis and write-up of the manuscript. He critically reviewed the manuscript.

3.1 Abstract

Background

Premature development of CVD in children living with HIV-1 (CLWH) may be associated with microbial translocation (MT), immune activation, systemic inflammation, and endothelial activation. Biomarkers of these pathways may provide insights into pathogenesis of atherosclerotic disease in CLWH.

Methods

This was a cross-sectional study of biomarkers in CLWH enrolled in the multicentre Early Pediatric Initiation-Canadian Child Cure Cohort (EPIC^4) who were on antiretroviral therapy (ART) with undetectable viral load. Plasma biomarkers of MT (intestinal fatty acid binding protein [I-FABP]), systemic inflammation (tumour necrosis factor [TNF] and interleukin-6 [IL-6]), and endothelial activation (angiopoietin-2 [Ang2], soluble vascular endothelial growth factor-1 [sVEGFR1], and soluble endoglin [sEng]) were quantified by ELISA. Correlation and factor analysis of biomarkers were used to examine associations between innate immune pathways.

Results

Among 90 CLWH, 16% of Ang2, 15% of sVEGFR1, and 23% of sEng levels were elevated relative to healthy historic controls. Pairwise rank-correlations between the three markers of endothelial activation were statistically significant ($\rho = 0.69$, $\rho = 0.61$, and $\rho = 0.65$, $p<0.001$ for

all correlations respectively). An endothelial activation index, derived by factor analysis of the three endothelial biomarkers, correlated with TNF ($\rho = 0.47$, $p<0.001$), IL-6 ($\rho = 0.60$, $p<0.001$) and I-FABP ($\rho = 0.67$, $p<0.001$). Current or past treatment with ritonavir-boosted lopinavir (LPV/r) was associated with endothelial activation (OR 5.0 (95%CI 1.7-17), p=0.0020).

Conclusion

Endothelial activation is prevalent in CLWH despite suppression of viral replication with cART and is associated with microbial translocation, systemic inflammation, and treatment with LPV/r.

3.2 Introduction

Every year, there are an estimated 160,000 new cases of pediatric HIV-1, usually as a result of vertical transmission. With the introduction of potent cART, people living with HIV (PLWH) have an increased life-expectancy and reduced frequency of opportunistic infections. However, non-communicable diseases, including CVD, have now surpassed opportunistic infections as the leading cause of death in PLWH [126]. Children living with vertically acquired HIV-1 (CLWH) face lifelong exposure to HIV and may be at risk of early CVD [127]. CVD precursor conditions such as dyslipidemia, carotid artery thickening, arterial stiffness, and coronary arteriopathy are prevalent in CLWH [128]. Accelerated progression of atherosclerosis in CLWH is evidenced by early complications such as myocardial infarction (MI) [32, 128].

Chronic systemic inflammation is well-recognized in PLWH, even among patients with excellent virologic control on cART, and may contribute to elevated risk of early cardiovascular complications [129]. Systemic inflammation is reflected by elevated circulating levels of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-6 (IL-6), which have been shown to predict subsequent cardiovascular events [130]. A key driver of persistent inflammation in PLWH is thought to be the translocation of pro-inflammatory bacterial products from the gut lumen into the circulation [131]. In this model, HIV infection of gut-associated lymphatic tissue causes reduced barrier function, and abnormal flux of bacterial products into the blood stream. This, in turn, may stimulate innate immune mechanisms, leading to the release of inflammatory cytokines [40, 132]. Intestinal epithelial damage is reflected by increased circulating levels of intestinal fatty acid binding protein (I-FABP), which has been widely used as a marker of MT in PLWH [54, 133, 134].

Endothelial activation, a hyperpermeable and pro-coagulable state of the endothelium, is observed in acute systemic infections [135, 136], as well as chronic HIV infection [130, 137]. Ang2 plays a critical role in potentiating endothelial activation through its interaction with the tyrosine kinase with immunoglobulin-like and EGF-like domains-2 (Tie2) receptor. Endothelial activation is also associated with shedding of endothelial cell surface receptors such as vascular endothelial growth factor receptor-1 (VEGFR-1) and endoglin (Eng), a component of the tissue growth factor-β receptor complex. Elevated circulating soluble VEGFR-1 (sVEGFR1) and soluble endoglin (sEng) are therefore markers of endothelial activation [33].

Chronic inflammation is implicated in the pathogenesis of CVD in PLWH [133]; however, linkages between pathways of MT, systemic inflammation, and endothelial activation have not previously been explored in CLWH. We hypothesized that markers of MT, inflammation, and endothelial activation would be correlated in CLWH. We tested this hypothesis in a cross-sectional multicentre study of CLWH engaged in care and receiving effective cART.

3.3 Methods

3.3.1 Study design

This was a cross-sectional study of CLWH on cART with undetectable VL. Children were recruited from eight pediatric HIV care centres across Canada as part of the Early Pediatric Initiation-Canadian Child Cure Cohort (EPIC[4]) study. For the current analysis, inclusion criteria were: perinatally acquired HIV-1; receiving cART; and undetectable VL at the time of the visit. Ethics approval was granted by all participating institutions. Adolescents consented to participate if they were deemed capable of providing consent. Parents of children provided informed consent, and assent was sought from children, as developmentally appropriate.

3.3.2 Clinical definitions

Undetectable viral load was defined as HIV RNA measurement below the limit of quantification by the clinical virology laboratory at each center (target not detected, <20, or <40 copies/mL). The proportion of life on effective cART was defined as the sum of all time periods during which the participant received effective cART associated with undetectable VL in days (numerator) divided by participant age in days (denominator). The proportion of life with undetectable VL was defined as the sum of all time periods during which SVS was achieved in days (numerator) divided by participant age in days (denominator). Data on all treatment regimens (lifetime antiretroviral exposure) was abstracted from the clinical chart record.

3.3.3 Measurement of Biomarker levels

Whole blood was collected in EDTA tubes at each clinical site and shipped within 24 hours for processing to a central laboratory. After centrifugation and isolation of plasma, samples were

stored at -80°C until the time of analysis. Biomarker levels in plasma were quantified by commercially available ELISA kits according to manufacturer's instructions (R&D Duoset, Minneapolis, MN). Laboratory biomarker assays were performed blinded to all clinical data. Background signal was determined from blank wells on each plate and subtracted from all samples and standards prior to analysis. A four-parameter logistic regression curve was fitted to the optical densities of standards of known concentration on each plate. This standard curve was used to determine biomarker concentrations from the ELISA optical density.

A reference range for all biomarkers was obtained from a literature search of studies which measured the biomarkers of interest and included healthy control subjects. The range of values observed in historic healthy controls, including adults and children from any geographic area, was used as the "normal" reference range. The proportion of participants in our cohort with biomarker levels above the upper limit of the reference range was determined.

3.3.4 Statistical Analysis

Non-parametric statistical methods were used where possible since the distributions of biomarker levels were not Gaussian. Descriptive statistics used the median and interquartile range (IQR). Correlations were assessed using Spearman's rank correlation coefficient (ρ). For multivariable linear regression analyses examining the association between different biomarkers, we used log-transformation of biomarker levels. We confirmed normal distribution of log-transformed biomarker levels using the Kolmogorov-Smirnov test. Analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA), and R (R Core Team, version 3.3.1).

3.4 Results

Ninety children were included, with samples collected between April 2015 and September 2016. The median age was 13 years (range 8 to 16) and 49% were female. Table 3.1 shows the characteristics of the cohort, disaggregated by endothelial activation status (described below). Twenty-four (27%) children were initiated on cART before six months of age and 46 (51%) had achieved sustained viral suppression for more than 5 years. Plasma concentrations of inflammatory, endothelial, and MT biomarkers are presented in Table 3.2, together with a reference range derived from past studies which measured the same biomarkers in healthy individuals, in other disease states, and in PLWH.

Table 3.1. Characteristics of 90 children living with vertically acquired HIV with undetectable viral load on cART.

Characteristic	**All participants (N=90)**	**Quiescent endothelium (n=40)[1]**	**Activated endothelium (n=40)[1]**	**P-value**
Demographics				
Age (yr), median (IQR)	13 (8.7-16)	14 (10-18)	12 (8.4-15)	0.10
Female sex, n (%)	49 (54)	20 (50)	23 (57)	0.65
HIV Clade, n (%)				0.51
Clade A	29 (32)	13 (32)	13 (32)	
Clade B	17 (19)	7 (18)	9 (22)	
Clade C	1 (1.1)	1 (2.5)	0 (0)	
Clade G	2 (2.2)	2 (5)	0 (0)	
Other	19 (21)	11 (28)	5 (12)	
Unknown	22 (24)	6 (15)	13 (32)	
History of HIV control, median (IQR)				
Age at initiation of any ARVs (yr)	2.0 (0.36-5.4)	2.4 (0.48-5.6)	1.7 (0.29-4.2)	0.28
Age at initiation of effective cART (yr)	2.2 (0.41-6.1)	4.0 (0.87-6.8)	2.0 (0.36-4.2)	0.18
Duration of viral suppression (yr)	5.1 (2.5-8.5)	5.1 (2.8-7)	6.1 (2.7-9.6)	0.45
Immunologic variables, median (IQR)				
CD4+ T-cell count (x10^6/mL)	800 (570-1100)	780 (550-1000)	750 (540-1100)	0.85
CD4+ T-cell percent	38 (32-42)	38 (31-43)	37 (32-40)	0.29
CD4+ T-cell lifetime nadir (x10^6/mL)	430 (250-570)	400 (240-560)	460 (320-590)	0.30
CD8+ T-cell count (x10^6/mL)	670 (490-920)	620 (470-890)	630 (480-860)	0.89
CD8+ T-cell percent	32 (27-37)	32 (27-36)	31 (27-35)	0.70

[1]In ten participants, missing data for Ang2, sVEGFR1, or sEng prevented the calculation of endothelial activation index; these participants were excluded from the comparative analysis between quiescent and activated endothelium. ARV antiretroviral.

The majority (67%, 60/90) of subjects had TNF levels above the range of healthy historical controls, while 36% (33/90) and 10% (9/90) had elevated IL-6 and I-FABP levels (Table 3.2). Inflammatory cytokine concentrations were positively correlated with each other and with I-FABP (Figure 3.1A-C).

For the endothelial biomarkers, 16% of Ang2, 15% of sVEGFR1 and 23% of sEng concentrations were higher than the reference range (Table 3.2). Pairwise rank-correlations between the markers of endothelial activation were statistically significant (Figure 3.1D-F, $\rho = 0.68$, $\rho = 0.43$, and $\rho = 0.54$, $p<0.001$ for all correlations). Given the correlation between the biomarkers of endothelial activation, we used factor analysis to derive an index of endothelial activation (latent construct). A single factor (mean 0 and unit variance), representing a linear combination of Ang2, sVEGFR1, and sEng concentrations (factor loadings 0.88, 0.87, and 0.66, respectively) explained 65% of the variance and was used as an index of "endothelial activation." We found significant correlations between the endothelial activation index (EAI) and IL-6 (Figure 3.1G-I, $\rho = 0.60$, $p<0.001$), TNF ($\rho = 0.47$, $p<0.001$), and I-FABP ($\rho = 0.67$, $p<0.001$).

Table 3.2. Plasma concentrations of biomarkers of endothelial activation, inflammation, and microbial translocation in 90 children living with HIV, together with historical controls (reference range).

Biomarker	Population	Range	Comment	References
TNF (pg/mL)	**Current study**	**31.2 (3.4-160)**	**66% patients above ref. range**	
	Healthy controls	0.76 - 5.4	Serum level in children	[138, 139]
	Disease state	280 – 390	Children with sepsis	[130, 140]
	HIV	350 – 830	Children with HIV	[141]
IL-6 (pg/mL)	**Current study**	**19 (4.7-65)**	**36% patients above ref. range**	
	Healthy controls	0.8-32	Serum level in children and adults	[142-145]
	Disease state	97-220,000	Children and adults with sepsis	[49, 139]
	HIV	0.50-2.1	Perinatally infected children	[141]
I-FABP (pg/mL)	**Current study**	**1,200 (590-2,900)**	**10% patients above ref. range**	
	Healthy control	100 – 5500	Serum level in adults	[49, 146]
	Disease state	900 – 6,100	Children with severe sepsis	[146]
	HIV	540 – 4,700	Chronic HIV with SVS	[49, 147]
Ang2 (pg/mL)	**Current study**	**1,000 (510-2,900)**	**16% patients above ref. range**	
	Healthy controls	1,400 – 4,900	Serum level in adults	[135]
	Disease state	5,000 – 46,000	Severe pediatric malaria, placental malaria, sepsis	[135, 136, 140]
	HIV	30 – 16,000	Serum level in adults, after 1 year of cART	[130]
sEng (pg/mL)	**Current study**	**28,000 (19,000 – 51,000)**	**23% patients above ref. range**	
	Healthy controls	730 – 47,000	Plasma and serum levels in adults and children	[135, 136, 143]
	Disease state	820 – 120,000	Severe pediatric malaria, placental malaria, sepsis	[136, 143, 148]
	HIV	7,300 – 16,000	Pregnant Women with HIV	[130]
sVEGFR1 (pg/mL)	**Current study**	**1,400 (560-4,000)**	**15% patients above ref. range**	
	Healthy control	0 – 5,900	Children	[139, 144]
	Disease state	38 – 6,600	Children with HUS, placental malaria, sepsis	[145, 148]
	HIV	830 – 2,500	Pregnant Women with HIV	[130]

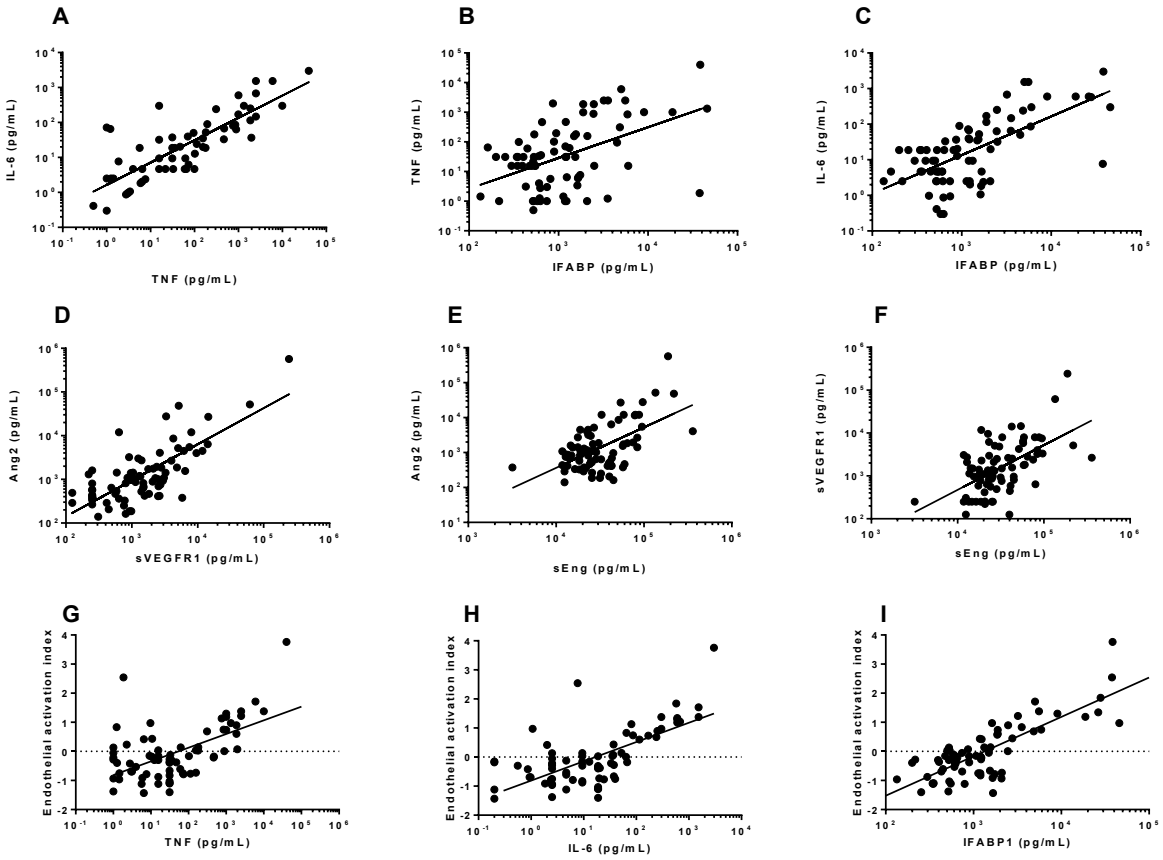

Figure 3.1. Correlation between biomarkers of systemic inflammation, endothelial activation, and microbial translocation. A. Correlation between inflammatory markers: TNF and IL-6 (ρ=0.82, p<0.001). **B-C.** Correlation between microbial translocation marker (I-FABP) and inflammatory markers: **B.** I-FABP and TNF (ρ=0.48, p<0.001); and **C.** I-FABP and IL-6 (ρ=0.82, p<0.001). **D-F.** Correlation between biomarkers of endothelial activation: **D.** Ang2 and sVEGFR1 (ρ=0.68, p<0.001); **E.** Ang2 and sEng (ρ=0.43, p<0.001); and **F.** sVEGFR1 and sEng (ρ=0.54, p<0.001). **G-I.** Correlation between endothelial activation index (EAI) and inflammatory and microbial translocation biomarkers: **G.** EIA and TNF (ρ = 0.47, p<0.001); **H.** EAI and IL-6 (ρ = 0.60, p<0.001) **I.** EAI and I-FABP (ρ = 0.67, p<0.001). Concentrations of the biomarkers were measured using ELISA. Non-parametric Spearman's rank correlation coefficient (ρ) and associated p-value are shown.

We used the EAI to dichotomize the cohort into “high” and “low” levels of endothelial activation (Table 3.1). No statistically significant differences in clinical characteristics, lifetime virologic control, or immunological characteristics were noted between participants with high and low levels of endothelial activation (Table 3.1). In a subset of 13 children who had initated cART before six months of age and who had achieved sustained viral suppression for more than five years, the EAI was not statistically significantly different from children who has started cART later or had not achieved SVS (p=0.28).

We next compared current and past cART regimens in patients with high and low levels of endothelial activation (Table 3.2). Ritonavir-boosted lopinavir (LPV/r) was associated with endothelial activation, relative to patients without prior LPV/r exposure (OR 5.0 (95%CI 1.7-17), p=0.0020). The EAI in patients treated with LPV/r was higher than those with no prior protease inhibitor (PI) exposure, and those who had received PI regimens other than LPV/r (Figure 3.2). In a multi-variable linear regression model adjusting for age, sex, TNF, IL6, and I-FABP, LPV/r exposure remained a statistically significant independent determinant of higher EAI (p=0.010).

Table 3.3. Association between cART treatment and endothelial activation

Characteristic	All patients (N=90)	Quiescent endothelium (n=40)	Activated endothelium (n=40)	P-value
Current cART regimen, n (%)				0.18
NNRTI-based	43 (48)	24 (60)	17 (42)	
Protease-inhibitor-based	19 (21)	5 (12)	10 (25)	
Integrase inhibitor-based	18 (20)	5 (12)	10 (25)	
Other/complex	9 (10)	6 (15)	2 (5)	
Lifetime exposure to ARVs[1]				
NRTIs				
Lamivudine	84 (93)	38 (95)	37 (92)	>0.99
Zidovudine	69 (77)	27 (68)	33 (82)	0.20
Abacavir	45 (50)	21 (52)	19 (48)	0.82
Tenofovir	31 (34)	19 (48)	12 (30)	0.17
Emtricitabine	28 (31)	18 (45)	10 (25)	0.10
Stavudine	17 (19)	8 (20)	7 (18)	>0.99
NNRTIs				
Efavirenz	36 (40)	19 (48)	15 (38)	0.50
Nevirapine	26 (29)	12 (30)	11 (28)	>0.99
Protease inhibitors				
Ritonavir[2]	48 (53)	16 (40)	26 (65)	**0.044**
Lopinavir	31 (34)	7 (18)	21 (52)	**0.0023**
Nelfinavir	19 (21)	10 (25)	6 (15)	0.40
Atazanavir	13 (14)	6 (15)	4 (10)	0.74
Integrase inhibitor				
Raltegravir	17 (19)	7 (18)	7 (18)	>0.99

[1]Antiretrovirals (ARVs) shown were administered to at least 10 children. Additional ARVs administered to fewer than 10 children included: didanosine (8), zalcitabine (1), etravirine (3), rilpivirine (7), darunavir (7), saquinavir (2),
indinavir (1), elvitegravir (5), dolutegravir (5), and maraviroc (1).
[2]Ritonavir exposure included full dose and boosting dose, in combination with other protease inhibitors.

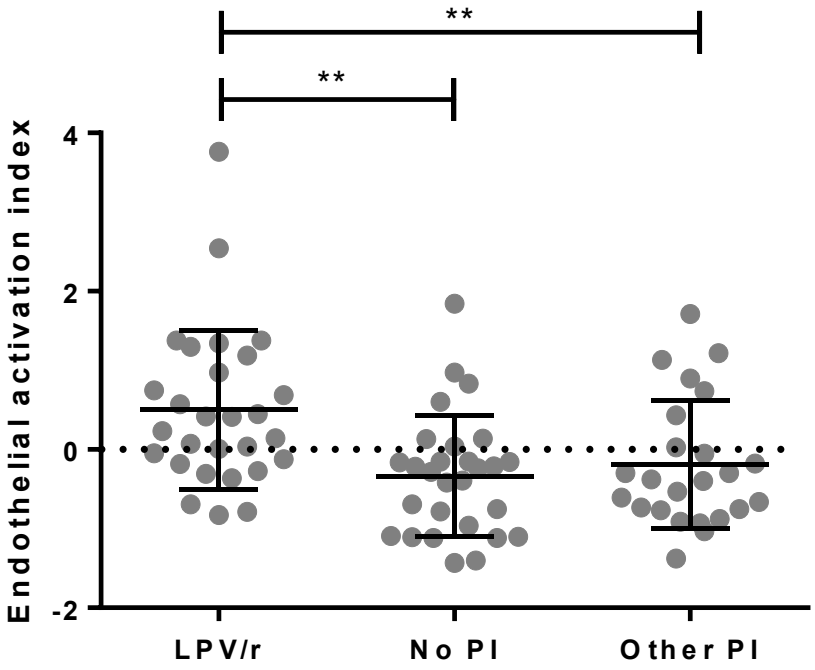

Figure 3.2. Past or current treatment with ritonavir-boosted lopinavir (LPV/r) was associated with endothelial activation. The endothelial activation index (derived from plasma levels of Ang2, sEng, and sVEGFR1) was higher in patients with past or current treatment with LPV/r compared to patients who had never received a regimen containing a protease inhibitor (PI, p=0.0004), and compared to patients who had exposure to PIs, but not LPV/r (p=0.0037).

3.5 Discussion

This study adds to the limited data on endothelial activation in CLWH, linking it to systemic inflammation, MT, and treatment with LPV/r. Collectively, these data are consistent with a model of endothelial activation in which: (a) gut barrier dysfunction due to the effects of HIV-1 infection on GALT leads to translocation of bacterial products into the circulation; (b) MT leads to systemic inflammation through activation of innate immune mechanisms [127]; and (c) microbial products and/or pro-inflammatory cytokines activate the endothelium [127, 129]. Iatrogenic endothelial activation appears to be associated with LPV/r treatment. All children included in the study had a suppressed viral load on cART at the time of testing, yet had varying degrees of endothelial activation, MT, and systemic inflammation. These findings suggest that innate immune activation persists in some CLWH despite virologic control and may be exacerbated by some treatment regimens.

In our study, 66% and 36% of participants had TNF and IL-6 levels above the range reported in healthy historical controls, respectively [40, 131, 132]. Similarly, in another case-control study, CLWH had higher circulating levels of IL-6 when compared to healthy children [33]. Levels of TNF and IL-6 in our study were not as high as in children with severe acute infections such as sepsis or severe malaria [142, 146], consistent with chronic low-level systemic inflammation in CLWH. In our study, 10% of CLWH had evidence of elevated I-FABP, relative to normal values previously reported (Table 3.2). I-FABP has been used as a surrogate marker of intestinal injury, impairment of gut barrier function, and MT [132, 135]. Previous reports noted correlations between markers of MT and inflammatory cytokines in PLWH [127, 149]. Our results in CLWH

are consistent with these findings: I-FABP levels were significantly positively correlated with IL-6 and TNF levels.

While it is established that inflammatory markers remain elevated in PLWH despite virologic control [33], less research has been done on endothelial activation to date. We found significant correlations between Ang2, sVEGFR1, and sEng, and used factor analysis as a dimensionality reduction technique to derive an endothelial activation index (EAI). Our findings suggest that a gradient of endothelial activation exists in CLWH. Sources of elevated levels of endothelial biomarkers may include chronic release of endothelium-specific Weibel-Palade body contents (Ang2) and shedding of cell surface molecules (sVEGFR1 and sEng) into the circulation. Peripheral blood Ang2 levels are often below the limit of assay detection in healthy persons [145]; however, one previous study done in adults living with HIV showed Ang2 levels of 700 (30-15900) in plasma one year after initiating cART [135]. Consistent with this finding, the majority of participants in our study had detectable plasma Ang2 levels despite achieving viral suppression. Similarly, sVEGFR1 and sEng were also found to be high in CLWH compared to healthy controls both in our study and previous studies [49, 136]. Correlations between the EAI, pro-inflammatory cytokines, and a MT marker in CLWH (Figure 3.1G-I) suggest interaction between these pathologic pathways.

Initiation of cART was initiated after six months of age in 65 (73%) of patients in our cohort. Early initiation of cART is associated with reduced latent replication-competent viral reservoir [150] and might also be expected to be associated with endothelial quiescence.

However, the EAI was not associated with the age at initiation of cART (median 2.0 years) or the duration of viral suppression (median 5.1 years). In a subset of patients (n=13, 14%) who initiated cART before six months of age and had achieved SVS for more than five years, the EAI was not statistically different from children with later cART initiation or without SVS. Although we expected that early and prolonged viral suppression would be associated with endothelial quiescence, other factors (e.g., MT, effects of antiretroviral medications) may be more important drivers of endothelial activation. The lack of association of EAI with clinical, virologic, and immunologic characteristics in our cohort may indicate that processes underlying accelerated atherosclerosis in CLWH may be relatively silent and sub-clinical. Biomarkers measurable in peripheral blood may therefore be of clinical utility in monitoring CVD risk.

Treatment with LPV/r was associated with endothelial activation in our cohort. LPV/r was the most commonly used PI in our cohort, with 31 (34%) of children having received LPV/r as part of a current or past cART regimen. Increased risk of coronary artery disease has been previously observed in adults treated with a PI-containing cART regimen [151, 152]. CVD precursor conditions such as dyslipidemia, lipodystrophy, and insulin resistance are recognized adverse effects of PIs [94]. However, some newer PIs such as ritonavir-boosted darunavir (DRV/r) and atazanavir (ATV/r) have less metabolic toxicity and were not associated with increased risk of MI in one study [153]. This is consistent with our finding that LPV/r, but not ATZ/r, was associated with endothelial activation (Table 3.3). Alternatively, statistical power may not have been adequate, given the small number of patients receiving ATZ/r (13 patients). Among nucleoside reverse transcriptase inhibitors (NRTIs), zidovudine, stavudine, and lamivudine did not increase risk of cardiac event, whereas abacavir (ABC) and didanosine had a 90% and 49%

higher rate of MI, respectively, in one adult study [154]. The association between ABC and CVD is inconsistent across studies and could not be confirmed in a meta-analysis [155]. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and integrase strand-transfer inhibitors (INSTIs) do not appear to contribute to CVD risk [94]. In patients switching from boosted PI regimens to a raltegravir-containing regimen, dyslipidemia improved, and IL-6 levels decreased [156]. These observations are consistent with our findings, which implicated LPV/r but not NRTIs, NNRTIs, or INSTIs in endothelial activation, as measured by circulating biomarker levels.

Our study has several limitations. The study design was cross-sectional, which limits our ability to draw causal relationships between immune activation pathways. We did not include a group of healthy historical controls, which would have assisted in the interpretation of biomarker levels. Instead, we compared the levels measured in our study to a reference range based on historical published healthy controls in the medical literature. This may have led to over- or under-estimation of the proportion of CLWH with elevated levels of endothelial activation markers due to differences in study populations and/or analytical methods. We measured selected biomarkers to assess endothelial activation (Ang2, sVEGFR1, and sEng), MT (I-FABP) and inflammation (IL-6, TNF). The high correlation between groups of biomarkers suggests that they provided a representative measurement of the pathways interrogated; however, additional biomarkers could also be measured to more strongly support the association between those pathological processes. Additional drivers of inflammation (e.g., chronic cytomegalovirus infection and/or latent replication-competent viral reservoir) as well as additional independent CVD risk factors (e.g., dyslipidemia and hypertension) could be measured for a more comprehensive description of

CVD risk. With respect to clinical outcomes of interest (e.g., CVD events), a long-term prospective cohort study would be required to determine the association between endothelial activation and subsequent cardiovascular complications.

In summary, our findings suggest that many CLWH who have achieved sustained viral suppression show signs of ongoing endothelial activation, which may be driven by MT from the gut, systemic inflammation, or antiretrovirals themselves. Based on the importance of endothelial activation in non-infectious complications of HIV such as atherosclerosis and CVD [33], the biomarkers used in our study may be clinically informative predictors of future CVD risk in CLWH.

CHAPTER 4. CONCLUSIONS

4.1 Summary of research

Reported in this thesis are two studies addressing the prevention of HIV in serodiscordant couples and endothelial activation in CLWH.

The global reduction in the vertical transmission of HIV among women living with HIV during pregnancy has been among the great success stories of the HIV pandemic. However, risk of vertical transmission still remains for mothers who seroconvert after the antenatal screening, later in pregnancy, or while breastfeeding [22]. Despite public health interventions, vertical transmission continues to occur in these groups and may be difficult to prevent entirely with current strategies. Considering this challenge, efforts are warranted to monitor pregnancies arising in high-risk heterosexual couples where the male partner is seropositive, and the pregnant female is seronegative (serodiscordant pregnancies). Such a public health strategy could be a valuable tool toward the elimination of vertical transmission of HIV-1 infection in Canada.

In chapter 2 of the present thesis, we reported a case of maternal primary HIV infection during the postpartum period and vertical transmission to a nursing infant despite an advanced and publicly funded program for the prevention of vertical transmission. We also reported a series of 47 serodiscordant pregnant couples identified through our nurse-led public health program to follow serodiscordant couples. Although no cases of HIV seroconversion occurred, high viral load (VL) among some seropositive males was observed during the period of pregnancy and breastfeeding. This suggests that there are additional opportunities for surveillance and optimization of cART therapy and adherence during this period of heightened risk.

In chapter 3, we studied selected biomarkers of pathological processes that put the CLWH at risk of premature development of CVD. Atherosclerotic CVD has become a leading cause of mortality for patients with HIV infection in the era of effective cART [157]. The pathogenesis of atherosclerotic vascular disease, culminating in cardiovascular events such as myocardial infarction and stroke, commences with several mechanisms that may include microbial translocation, systemic inflammation, and endothelial activation [94, 130]. Specific biomarkers may be of use to study how HIV infection and antiretroviral exposure influence CVD pathogenesis in CLWH [130, 158, 159].

We hypothesized that markers of MT, inflammation, and endothelial activation would be correlated in CLWH. We tested this hypothesis in a cross-sectional multicenter study of CLWH with suppressed VL on cART. Plasma biomarkers of MT (I-FABP), systemic inflammation (TNF and IL-6), and endothelial activation (Ang-2, sVEGFR1, and sEng) were quantified by ELISA. Correlation and factor analysis of biomarkers were used to examine associations between innate immune pathways. Among 90 CLWH, 16% of Ang2, 15% of sVEGFR1, and 23% of sEng levels were elevated relative to healthy historic controls. Pairwise rank-correlations between the three markers of endothelial activation were statistically significant. An endothelial activation index, derived by factor analysis of the three endothelial biomarkers, correlated with TNF, IL-6, and I-FABP, suggesting interaction between these pathways. Current or past treatment with ritonavir-boosted lopinavir (LPV/r) was associated with endothelial activation (OR 5.0 (95%CI 1.7-17), p=0.0020).

In summary, in chapter 3 we showed that endothelial activation was prevalent in CLWH, despite suppression of viral replication with cART, and was associated with microbial translocation, systemic inflammation, and treatment with LPV/r.

4.2 Recommendations arising from this research

Our results in chapter 2 have clinical implications and may be actionable. Vertical HIV transmission remains a rare but concerning occurrence, even in well-resourced settings with established prevention programs. Our case report and case series highlight residual gaps in public health strategies for the prevention of vertical transmission. Our findings logically lead to the following recommendations: (1) increase the frequency of VL testing for HIV seropositive males during period of pregnancy and breastfeeding of an HIV-negative female sexual partner; (2) increase the frequency and duration of surveillance (serology and direct viral detection) HIV seronegative pregnant and breastfeeding women with an HIV seropositive sexual partner. These recommendations could be piloted and tested or directly implemented in clinical practice, given their imminent feasibility. A knowledge translation plan would be needed to reach key stakeholders in order to increase the likelihood that these recommendations would be implemented in clinical practice.

4.2.1 ***Recommendation 1:*** *Increase the frequency of VL testing for HIV seropositive males during period of pregnancy and breastfeeding of an HIV-negative female sexual partner.*

Our first recommendation is to increase the frequency of VL testing when HIV-infected males disclose that they have a pregnant partner who is HIV seronegative. Our results indicate that males are tested median of 2 (IQR 1-2) times during pregnancy and 4 (IQR 3-5) times during the

breastfeeding period in current practice in our setting. These results are subject to some limitations because we extracted data from two electronic databases which may not have included all test results. We plan to access a third database, the Provincial Laboratory database, which should be comprehensive and definitive and may identify additional test results. Nonetheless, it appears that there may be an opportunity to increase VL testing in this higher risk situation. VL testing of male partners could serve a dual purpose of surveillance and education. Detectable VL might trigger optimization of the cART regimen or emphasis on adherence, as indicated. Secondly, communicating results of a detectable VL may provide an opportunity to emphasize the potential consequence of vertical transmission, and promote safer sexual practices.

Increased VL testing frequency should ideally be implemented during the entire period of pregnancy and breastfeeding. The optimal frequency of VL testing is unknown; however, given that the period of elevated risk is finite and well-defined, increased testing frequency need not be excessively costly or burdensome from a public health perspective. The impact of this strategy warrants further study. Once the benefit (reduction in vertical transmission) is quantified, the cost-effectiveness of this strategy could be formally evaluated. Limitations of this strategy include incomplete ascertainment of serodiscordant couples since this strategy relies on HIV-positive males to report when their partner is pregnant.

4.2.2 ***Recommendation 2****: Increase the frequency and duration of surveillance (serology and direct viral detection) for HIV seronegative pregnant and breastfeeding women with an HIV seropositive sexual partner.*

Our second recommendation is to increase testing of pregnant and breastfeeding women with an HIV-positive male sexual partner. Universal screening for HIV during antenatal care is already routine in our setting. Furthermore, when HIV serodiscordant couples are identified in the NAP program, increased frequency of testing is implemented for the pregnant woman. In chapter 2, we found that the frequency of testing during pregnancy was median 2 (IQR 1-2) times. This could be increased to promote early detection of seroconversion, especially if the male partner has a VL greater than 40 copies/mL. As described above, communication of test results could serve to reassure but also to heighten the awareness of risk and promote sexual behaviors to avoid transmission. We propose that testing should be extended until infant weaning, since seroconversion during breastfeeding is associated with a high risk of vertical transmission, as in our case report in chapter 2. Follow-up serology testing for HIV after weaning for mother (or infant, if maternal serology is not available) would be another rational consideration to definitively rule out transmission prior to discharging the infant from further follow-up.

As for Recommendation 2 (section 4.2.1 above), the effectiveness and cost-effectiveness of increased maternal screening are unknown but could be studied. Limitations of this strategy include the possibility that pregnant women in serodiscordant couples may be reluctant to undergo repeated testing. This problem may be compounded where access to testing is limited (e.g., remote communities). HIV POC tests or use of DBS testing may be considered to improve access and offset the increased patient burden of repeated laboratory testing.

4.3 Significance and impact of findings

In addition to the actionable findings from chapter 2 which lead to the recommendations above (Section 4.2), this thesis provides several noteworthy contributions to the understanding of HIV care and pathogenesis.

4.3.1 Highlighting an innovative public health surveillance program for HIV-serodiscordant couples

In chapter 2, we reported a case series of HIV serodiscordant couples identified through a public health surveillance system in Edmonton which offers real-time prospective follow-up of serodiscordant pregnant couples. A designated public health nurse maintains an ongoing list of HIV positive male partners who self-report the pregnancy of their HIV uninfected partners, and information is communicated at regular multidisciplinary team rounds. To our knowledge, this surveillance system is unique in Canada, and our literature search did not reveal previous publications reporting such a system. Thus, this is noteworthy as a public health strategy implemented under the Northern Alberta Program (NAP). It builds on more established vertical HIV prevention strategies such as universal HIV screening in pregnancy, which is widely implemented across Canada. This program could be implemented at other HIV care centers, but requires integration of adult HIV services, antenatal health services, and pediatric HIV care. Such an integrated approach is ideally managed through public health, as is our program.

4.3.2 Awareness of ongoing vertical transmission of HIV in Canada

Despite universal screening for HIV in pregnancy, our case of post-natal HIV transmission illustrates that vertical transmission continues to occur in Canada and may be difficult to prevent entirely. Missed opportunities for prevention might be present in other jurisdictions, as in ours. This case highlights the importance of continued vigilance among frontline physicians across Canada for rare but disastrous cases of post-natal transmission through breastfeeding in Canada [22].

4.3.3 Insights into pathogenesis of endothelial activation in children living with HIV

In chapter 3, we investigated endothelial activation in CLWH, which may be a risk factor for long-term cardiovascular complications. While it is established that systemic inflammation persists in PLWH, less research has been done on endothelial activation so far [71]. Our findings provide insight into the pathophysiology of CVD in CLWH, demonstrating a link between the processes of microbial translocation, systemic inflammation, and endothelial activation.

4.3.4 Recognition of endothelial toxicity of ritonavir-boosted lopinavir

Secondly, this study contributes toward increased recognition of a potential long-term toxicity of LPV/r, through activation of the endothelium. Lifelong cART is required to maintain HIV suppression, improve immune function, and minimize morbidity and mortality associated with HIV disease progression [158]. However, side-effects of some antiretrovirals may include metabolic abnormalities with long-term consequences for CVD risk [94, 159]. Given that newer PIs or different antiretroviral classes (e.g., INSTIs) with fewer metabolic side-effects are

available, documenting the adverse effect of LPV/r on the endothelium should prompt increased adoption of these safer choices when initiating or changing a cART regimen.

4.3.5 New therapeutic targets to prevent cardiovascular complications of HIV

As the leading cause of death in PLWH, increasing attention has turned to cardiovascular complications for potential therapeutic interventions. Lifestyle modification with diet or exercise have been shown to reduce inflammatory biomarkers in PLWH [160-163]. Optimization of cART regimen, including switch to agents with less atherogenic potential, early cART initiation in infants [137], and intensification of cART regimens [164-168] may reduce long-term inflammation and endothelial activation. Statins, antiplatelet drugs (aspirin, clopidogrel), and blood pressure medications significantly lower the risk of CVD in PLWH [169]. In addition to controlling traditional co-existing CVD risk factors, our study and others point to new pathophysiologic pathways that may be amenable to pharmacologic modulation. For example, in relation to MT, probiotics may improve mucosal immune function, maintain intestinal barrier integrity, and decrease systemic inflammation, without significant adverse side effects [170]. Even potent anti-inflammatory agents such as corticosteroids [171] or cyclosporine [172] have been tested in PLWH to reduce systemic inflammation. Toxicity limits the long-term use of these agents.

Identification of endothelial activation in our study provides another promising avenue of investigation. It is tempting to speculate that endothelial activation pathways represent potential molecular targets for novel therapies to reduce CVD risk in PLWH. Below, we explore potential molecular targets in endothelial activation pathways in a focused literature review.

4.3.5.1 Therapeutics targeting VEGF signaling

Given the importance of VEGF signaling in endothelial activation, licensed pharmacologic agents targeting the VEGF/VEGFR1 pathway could have utility as adjunctive therapies for reducing endothelial activation in PLWH. The binding of VEGF to VEGFR initiates the activity of the tyrosine kinase domain of the receptor [84]; therefore, small-molecule tyrosine kinase inhibitors (TKIs) might be considered for PLWH. Several TKIs with activity against all VEGF receptors have been approved for clinical use for different indications (e.g., malignancy). These include apatinib, axitinib, cabozantinib, lenvatinib, nintedanib, pazopanib, regorafinib, sorafenib, sunitinib, and vandetanib, with several others in development [173]. Anti-VEGF therapy has mainly been utilized in the treatment of cancer and sepsis [174, 175]. Anti-VEGF studies completed in HIV-infected subjects have focused on treatment of Kaposi's Sarcoma and have been well-tolerated [176-178]. No studies of VEGF antagonists have assessed generalized endothelial activation and CVD risk in PLWH, to our knowledge. On the other hand, several studies have identified an increased risk of cardiovascular complications in association with TKIs sorafenib and sunitinib [179-182], with the exception of one study which concluded no difference between placebo and treatment groups [183]. Cardiovascular side effects of sorafenib and sunitinib include hypertension, left ventricular dysfunction, arterial thromboembolism [184] and accelerated atherosclerosis [185]. The mechanism involves inhibition of other cellular tyrosine kinases in cardiomyocytes, independent of effects on VEGFR [186]. Thus, given their broad action against critical cellular enzymes, TKIs may have too many off-target effects to be clinically useful.

Rapamycin, an immunosuppressive drug which inhibits mammalian target of rapamycin (mTOR). mTOR is a regulator of multiple cellular pathways, integrating input from upstream growth factors including VEGF. Rapamycin inhibits microvascular permeability induced by VEGF [187]. Inhibition of mTOR with rapamycin has shown promise in the prevention of atherosclerosis [188] and should be further explored in PLWH.

4.3.5.2 Therapeutics targeting Ang-Tie2 signaling

Chapter 3 implicated Ang2 as a key marker of endothelial activation in CLWH. Ang1 is a Tie2 agonist that promotes endothelial quiescence, while Ang2 is a Tie2 antagonist that causes endothelial destabilization and resulting inflammatory effects [74]. Therefore, Tie2 agonists/Ang1 mimetics and Ang2 antagonists may be potential targeted therapies of endothelial activation in chronic HIV infection.

Potential therapeutic application of the Ang-Tie pathway has been mainly studied in the context of sepsis, cancer and malaria [189-193]. Vasculotide, a Tie2 agonist peptide, was shown to reduce endothelial barrier dysfunction, vascular inflammation, and endothelial adhesion molecule expression in cancer [194] and sepsis [189, 195] animal models. Studies using murine models with acute lung injury have similarly noted reductions in proinflammatory cytokine expression associated with vasculotide or Ang1 gene therapy [196-198]. Peptide-Fc fusion protein L1-7(N), an Ang2 inhibitor, was shown to inhibit endothelial destabilization, and promote Ang1 effects, in nude mice models with colon carcinoma [199]. Finally, a recombinant human Ang1 protein, termed BowAng1, has been developed for as a novel therapeutic in sepsis and malaria [200].

BowAng1 contains the C-terminal fibrinogen-like domain of the angiopoietin protein, fused to human immunoglobulin G1 Fc fragments engineered into a tetramer conformation for optimal Tie2 binding [201]. BowAng1 induces phosphorylation of Tie2 and may promote endothelial quiescence [201]. BowAng1 has not yet been studied in PLWH, to our knowledge. The effects of Tie2 agonists/Ang1 mimetics and Ang2 antagonists on CVD risk and chronic HIV infection have not been explored and warrant further investigation. However, the novelty of these agents also carries risk (little is known about their clinical safety profile). Moreover, the cost of these biologics for long-term use in PLWH to prevent CVD may be prohibitive.

4.4 Future Directions

4.4.1 Evaluation and implementation of recommendations for increased diagnostic testing in serodiscordant couples during pregnancy and breastfeeding

In section 4.2, we proposed increased VL testing of both males (VL quantification) and females (serology or direct HIV detection) as recommendations arising from chapter 2 of this thesis. A future direction of this work could be to evaluate and implement these recommendations in clinical practice.

The effectiveness of increased VL testing in serodiscordant couples could be evaluated using a quality improvement methodology or quasi-experimental (before-after) design. This would entail assessing the outcome (vertical transmission event) before and after the implementation of the intervention, using the present data as a baseline. Because vertical transmission is rare, a large sample size would be needed to demonstrate a change with the intervention. Surrogate outcomes such as proportion of males with suppressed VL during the period of risk could be used as an

alternative outcome. It should be noted that a before-after quality-improvement study would be limited by certain factors, such as lack of randomization of the intervention, leading to potential bias, and difficulty ascribing changes over time to the intervention itself.

If the utility of increased testing can be defined (e.g., number needed to treat to prevent one transmission event to mother or to infant), then a cost-effectiveness analysis could be performed. The costs of the increased testing for all serodiscordant couples could be compared to the probability and costs associated with a transmission event (rare event but carrying substantial cost) using the incremental cost-effectiveness ratio (ICER), for example [202].

In addition to these recommendations, the use of Pre-Exposure Prophylaxis (PrEP) during the pregnancy and breastfeeding periods has been a considerable risk-reduction strategy for women with known HIV-infected partners [203]. Future studies could assess delivery models for PrEP within routine maternal and child health services.

A knowledge translation (KT) plan would be needed to implement these recommendations. Key stakeholders for KT include infectious disease physicians managing adult men with HIV, obstetricians or family doctors managing pregnant women in serodiscordant relationships, and pediatric infectious disease physicians managing infants at risk. Key messages for KT include: (1) ongoing residual risk of VT in serodiscordant couples as highlighted by our case report; (2) prevalence of HIV-positive males without suppressed VL; and (3) awareness of the risk period, which extends through to the end of breastfeeding. Methods for KT could include presentation at national and international scientific conferences where clinicians will be in attendance,

publication in peer reviewed journals, and at the local level, presentation at regularly scheduled educational rounds (e.g., disciplines of infectious diseases, obstetrics, and public health).

4.4.2 Further examination of endothelial activation in CLWH

Our study in chapter 3 examined a limited set of biomarkers to assess MT, systemic inflammation, and endothelial activation. Additional biomarkers could be measured to provide a more comprehensive description of these processes. With respect to MT, we used circulating levels of I-FABP as a marker of intestinal epithelial damage. I-FABP alone has limitations as a biomarker of MT since levels may be affected by metabolic lipid changes due to the disease process or medications [43, 47]. Additional biomarkers of interest include LPS [42], bacterial 16SrDNA [43], sCD14 [44], LBP, anti-flagellin antibodies [43], antibodies to LPS [45]. Finally, another marker of enterocyte damage, zonulin has previously been used [47, 48]. These could readily be measured in samples within the EPIC^4 biorepository. With respect to systemic inflammation, numerous cytokines and chemokines besides TNF and IL-6 could be measured , including soluble TNF receptor-1 and -2, IFN-γ, IL-1, -8, -10, -17, and -23, CXCL10 (IP-10), monocyte chemotactic protein-1, macrophage inflammatory proteins −1α and −1β, C-reactive protein, and procalcitonin [204]. Finally, with respect to endothelial activation, several molecules could be measured from the VEGF pathway (VEGF-A, VEGF-B, soluble VEGFR2 receptor), the Ang-Tie2 pathway (Ang1, soluble Tie2), and other endothelial cell surface molecules shed into the circulation upon endothelial activation (e.g., soluble intercellular adhesion molecule-1, soluble P-selectin). Methods exist for simultaneous quantification of multiple analytes from a small volume of plasma such as the magnetic microsphere-based Luminex® platform [205-207] and the microfluidics-based platform, Ella^{TM} [208, 209].

A relatively recently described pathway of endothelial regulation, Slit2/Robo4, also warrants further study in the context of HIV infection. Slit/Robo is a cell signaling pathway that mediates a broad range of functions including neuronal axon guidance and angiogenesis [210]. Slit refers to a secreted protein and Robo refers to its transmembrane receptor. There are three different slit ligands in vertebrates (Slit1, Slit2, and Slit3) that bind to Robo family receptors (Robo1, Robo2, Robo3/Rig-1, and Robo4) [211]. The Slit2/Robo4 signaling pathway is recently been identified as a regulatory pathway of endothelial permeability [212, 213]. Slit2/Robo4 signaling blocks the Src family kinase activation to inhibit VEGF- mediated vascular leak [214]. The ligand Slit2 can be readily measured in patient plasma by ELISA. Investigation of this recently described pathway in CLWH would be novel and would build on our findings implicating endothelial activation in the pathobiology of chronic HIV infection.

Our study implicated MT as a potential driver of systemic inflammation and endothelial activation by showing a correlation between I-FABP and inflammatory cytokines and an endothelial activation index. However, other drivers of systemic inflammation have been recognized in PLWH, especially chronic cytomegalovirus infection and latent replication-competent HIV reservoir. The EPIC^4 parent study has collected a wealth of clinical and laboratory data on a large cohort of CLWH, including CMV serostatus and sophisticated measurements of viral reservoir [150]. Future analyses should examine if associations exist between endothelial biomarkers, CMV co-infection, and the latent replication-competent HIV viral reservoir.

4.4.3 Predictive value of endothelial activation biomarkers

An important goal of future research should be to elucidate whether CVD biomarkers improve risk stratification beyond HIV-related and traditional risk factors. This question could ideally be addressed with a long-term prospective cohort study, with serial measurement of biomarkers in CLWH and assessment of clinically important outcomes (e.g., myocardial infarction or stroke). Such a study could determine a temporal relationship between endothelial activation and subsequent cardiovascular complications. However, considering that it would require decades of observation to document such complications, this would be a costly and challenging study. Surrogate outcomes such as dyslipidemia, carotid artery intimal medial thickness, or hypertension might be more feasibly assessed in a prospective cohort study. Biorepositories, such as the EPIC[4] banked samples, if properly maintained, could also be used in a retrospective case-control study, once a sufficient number of clinical events have occurred in the EPIC[4] cohort.

4.5 Concluding remarks

In summary, this thesis draws attention to the rare but serious occurrence of VT of HIV in serodiscordant couples during pregnancy or breastfeeding. Second, our novel findings on endothelial activation in CLWH open new avenues for research into predictive biomarkers and molecular targets for CVD prevention. These findings may contribute to ongoing efforts to eliminate VT of HIV in Canada and optimally support CLWH to avoid long-term cardiovascular complications.

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