THE ROLE OF MACROPHAGES, DENDRITIC AND LANGERHANS CELLS IN THE MOTHER TO CHILD TRANSMISSION OF HIV IN PLACENTAL TISSUE OF NORMOTENSIVE PREGNANCIES.

by

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submitted in partial fulfillment of the requirements for the degree of

MASTER OF MEDICAL SCIENCE

in the
Discipline of Optics and Imaging
College of Health Sciences
University of KwaZulu-Natal
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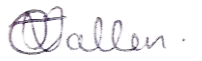
PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Optics & Imaging Centre, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T. Naicker and Mr. V. Dorsamy.



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DECLARATION

I, Camille Josephine Vallen declare that:

(i) The research reported in this dissertation, except where otherwise indicated is my original work.

(ii) This dissertation has not been submitted for any degree or examination at another university.

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Signed:  Date: 10-03-2016

DEDICATION

To my mom and dad,

I would not have been able to do this without your never ending love and support.

Thank you.

I love you.

"*So do not fear, for I am with you; do not be dismayed, for I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand."*

Isaiah 41:10

"'For I know the plans I have for you', declares the Lord. 'Plans to prosper you and not to harm you; plans to give you hope and a future.'"

Jeremiah 29:11

Thank you Jesus

PEER REVIEWED PUBLICATIONS AND CONFERENCE PRESENTATIONS

JOURNAL ARTICLE TO BE SUBMITTED

1. Vallen C, Dorsamy V, Moodley J, Naicker T (2015). The Role of Macrophage, Dendritic and Langerhans Cells in the Mother to Child Transmission of HIV in Placental Tissue of Normotensive Pregnancies. (To be submitted to the European Journal of Obstetrics and Gynaecology).

ABSTRACTS IN PEER REVIEWED INTERNATIONAL JOURNALS

1. Vallen C, Dorsamy V, Moodley J, Naicker T (2015). Immunolocalization of CCR-5 and ICAM-2 expression in the placenta of normotensive and pre-eclamptic placentae. *Placenta*. 36(9):A51.

2. Dorsamy V, Vallen C, Moodley J, Naicker T (2015). Expression of HIV p24 and CD4 receptor on syncytiotrophoblast and fetal circulation in mother to child transmission of HIV. *Placenta*. 36(9):A25.

PEER REVIEWED CONFERENCE PRESENTATIONS

1. V. Dorsamy, C. Vallen J. Moodley and T. Naicker (2014). Immunolocalisation of HIV and CD4 in normotensive and pre-eclamptic pregnancies. *Proceedings of Microscopy Society of Southern Africa* 44, Stellenbosch, South Africa. ISSN 0250-0418: ISBN 0-620-35056-3.

2. V. Dorsamy, C. Vallen J. Moodley and T. Naicker (2014). HIV co-receptor CCR-5 and ICAM-2 in normotensive and pre-eclamptic placentae. *Proceedings of Microscopy Society of Southern Africa* 44, Stellenbosch, South Africa. ISSN 0250-0418: ISBN 0-620-35056-3.

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LIST OF ABBREVIATIONS

°C	degree celcius
µg	microgram
µl	microliter
µm	micrometre
ANOVA	analyses of variance
APC's	Antigen Presenting Cells
ARV's	antiretrovirals
AV	anchoring villi
BM	basement membrane
C	capillary
CI	confidence interval
cm	centimetre
CT	cytotrophoblast
DAB	diamino-benzidine
dH_2O	distilled water
DOH	Department of Health
DNA	deoxyribonucleic acid
DPX	dibutylphthalate
EC	endothelial cell
ER	endoplasmic reticulum
EVT	extravillous trophoblast
g	grams
gag	glycosaminoglycan
H&E	hematoxylin and eosin
HLA	human leukocyte antigen
HIV	human immunodeficiency virus
hrs	hours
IgG	immunoglobulin G
IHC	immunohistochemistry
KZN	KwaZulu-Natal

LU	lumen
L	Langhans cell
MHC	Major Histocompatibility Complex
min	minute
ml	millilitre
mmHg	millimetre mercury
mRNA	messenger ribonucleic acid
mv	microvilli
N	nuclei
nm	nanometer
P	platelet
PBS	phosphate buffered saline
RBC	red blood cell
RNA	ribonucleic acid
RT	room temperature
SB	syncytial bridge
SD	standard deviation
sec	second
SK	syncytial knots
SL	spongy layer
SPSS	statistical package for the social science
ST	syncytiotrophoblast
STD’s	sexually transmitted diseases
tat	trans-activator of transcription
T cells	thymus cells / thymus Lymphocytes
Th2	T helper 2
wks	weeks
yrs	years

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ABSTRACT

Objectives: Mother to child transmission of HIV remains a concern despite the introduction of ARVs during pregnancy. The mechanism by which HIV transmission across the placenta takes place needs to be evaluated.

The placenta is a selective filter and the only barrier between the developing fetus and the mother. Should the implicated proteins of HIV transmission be found in and on placental tissue, it is plausible that these proteins provide the needed pathway for HIV infection of the fetus.

Orthodox mechanisms of transmission involving the CD4-CCR5 interaction have been previously looked at in placenta. This study aims to evaluate expression of these key proteins implicated in the transmission of HIV transmission across placental exchange villi.

Study Design: Following post institutional ethical approval, 60 archived paraffin embedded placental tissue blocks were used for this study. Tissue samples being examined were obtained from normotensive HIV positive and negative mothers who delivered at a rural district hospital in Kwa-Zulu Nata. The tissue samples were immunohistochemically stained with target antibodies (CD68, DC-SIGN and CD1a) and analysed using light microscopy. This was followed by morphometric analysis with subsequent statistical analysis.

Results: Analysis of the demographic data showed a significant difference between the placental weight (p = 0.005), with HIV positive placentae having a greater weight (517.33±81.59) than HIV negative placentae (457.10±77.81).

There was no significant difference in the positive immunoexpression of the CD68, DC-SIGN and CD1a markers noted between the HIV negative and positive groups; nor within the HIV positive subgroups.

DC-SIGN expression was noted on immune cells as well as syncytiotrophoblast layers of conducting and exchange placental villi. CD68 expression on the immune cells showed no difference in expression between HIV positive and HIV negative groups. CD1a expression was not seen in the placental villi nor the immune cells of the different groups.

Conclusion: We have seen that the number of cells which express the CD68 and DC-SIGN markers remain constant regardless of HIV status and tend to increase from placentation to gestation. Further studies need to be conducted to assimilate a firm connection between the two.

Chapter 1

1.1. Introduction

Over three decades has been dedicated to finding a cure and eradicating human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS). Extensive research has been carried out all over the world in an effort to understand the make-up of this resilient virus and the mechanism by which it evades all efforts of neutralization (Kasahara, 2011). The retaliation of the immune system and its constant bombardment with drugs has honed viral evolution by selecting phenotypes that mitigate such onslaught. Antigenic variation and production of immune evasion or sequestering proteins are some ways in which HIV continues to plague humans (Kasahara, 2011).

Mutability of viral RNA is an important key to the success of this pathogen (Iyidogan and Anderson, 2014). In light of this and the global statistics of HIV and its impact on future generations, public health strategies have focused on preventative measures to eliminate current transmission rates. While such strategies are paramount and will eventually reduce mortality rates, understanding the pathophysiology and transmission of HIV is of immediate concern, especially in the most vulnerable groups: pregnant women and their babies (Tiemessen and Kuhn, 2006). This study seeks to better understand the mechanism of transmission of HIV in these groups by examining immune cells targeted by HIV and the placenta, through which *in utero* transmission occurs. Molecules identified on the immune cells include the Cluster of Differentiation 68 (CD68), Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin (DC-SIGN) and Cluster of Differentiation 1a (CD1a). Due to the biological importance as well as their importance in HIV transmission, these molecules have been of interest and have previously documented individually, especially that of DC-SIGN (Boily-Larouche *et al*., 2012; Pillay *et al*., 2014; Tassaneetrithep *et al*., 2003) and CD68 (Kim et al., 2007). However, there has not yet been a study which evaluates the expression of these markers in consecutive sections of placental tissue samples. This study aims to immunolocalize these proteins and compares their expression in HIV positive and HIV negative patients. Such understanding may add valuable insight into mother to child transmission and maternal and fetal mortality.

1.2. Literature Review

1.2.1. Maternal Mortality

Maternal mortality remains a global concern. Defined as death due to complications from pregnancy or childbirth (*Tenth Interim Report on Confidential Enquiries into Maternal Deaths in South Africa*, 2011), it has seen dramatic rise in the last 25 years (*Saving Mothers 2008-2010: Fifth Report on the Confidential Enquiries into Maternal Deaths in South Africa.*, 2010) due to non-pregnancy related infections (NPRI). However, the latest statistics have indicated a slight decrease in the percentage of maternal deaths attributed to NPRI's (*Saving Mothers 2011- 2013: Sixth Report on the Confidential Enquiries into Maternal Deaths in South Africa*, 2015).

The Millennium Development Goals (MDG) were established and agreed upon at the Millennium Summit of the United Nations in 2000 in an attempt to address key health concerns plaguing countries around the world. The MDG's have resulted in the reduction of global maternal mortality by 45 percent. Unfortunately, many developing countries have not achieved these targets, including South Africa. In lieu of this, the World Health Organization has developed the Sustainable Development Goals. This has offered countries the opportunity to work towards the betterment of their circumstances over the next fifteen years by setting goals with multiple targets geared towards various issues of global concern. Latest statistics released by the United Nations states that the maternal mortality ratio in developing countries is still fourteen times higher than developed regions; with only half the women having access to the recommended amount of antenatal care which they need. The Sustainable Development Goals aim to reduce the maternal mortality ratio to less than 70 deaths per 100 000 live births as well as end the AIDS epidemic by the year 2030 (UN, 2015).

According to the World Health Organization, it is approximated that over 800 women die per day due to pregnancy or child related complications (WHO, 2014). The majority of these deaths are concentrated to two main areas; namely Sub-Saharan Africa and Asia (WHO, 2014). A staggering 91% of deaths occur in Sub-Saharan Africa, which has the highest maternal mortality ratio at 510 deaths per 100 000 live births; amounting to approximately 179 000 maternal deaths per year (WHO et al., 2014). Statistics released as part of the Sixth Report on the Confidential Enquiries into Maternal Deaths in South Africa (*Saving Mothers 2011- 2013: Sixth Report on the Confidential Enquiries into Maternal Deaths in South Africa*, 2015) reveal that almost 35% of maternal deaths are due to Non-Pregnancy Related Infections (NPRI's), with HIV being the most prevalent. This can be clearly seen in Figure 1.1. With HIV being so rife in Southern Africa, interventions that reduce these alarming statistics will save many lives.

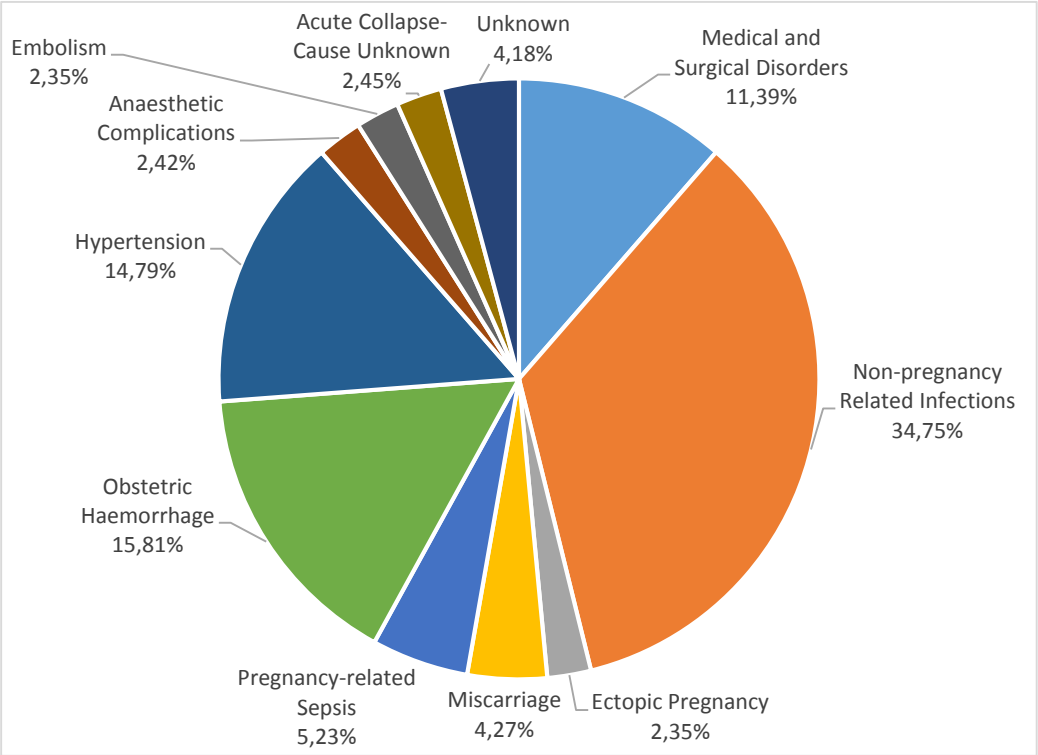

Figure 1.1. Depictions of various causes of global maternal mortality extracted from the Saving Mothers 2011- 2013: Sixth Report on the Confidential Enquiries into Maternal Deaths in South Africa (2015).

1.2.2. HIV

HIV is a lentivirus belonging to a subgroup of viruses known as Retroviridae. Lentiviruses are single-stranded, enveloped RNA viruses which have been said to be gene transfer agents and have the ability to deliver a substantial amount of viral DNA into non-dividing cells (Yamashita and Emerman, 2006). Lentiviruses appear to have a specific tropism for macrophages which essentially then acts as a major reservoir for the virus in an infected individual (Strauss and Strauss, 2002). HIV also infects non-dividing cells such as resting CD4+ T cells (Naldini, 1998; Yamashita and Emerman, 2006). The ability of HIV to invade these cells has brought about the belief that this mechanism of infection is vital to the transmission of the various subtypes of HIV, for example HIV-1 , and the spread of the virus (Fassati, 2006). In this way, Lentiviruses are able to establish life-long, chronic infections that is not able to be cleared by the typical response of the immune system (Strauss and Strauss, 2002).

HIV has various subtypes with varying degrees of severity of disease. Of concern are HIV-1 and HIV-2 subtypes. The HIV-1 subtype is considered to be the main subdivision of HIV due to it accounting for more than 95% of all HIV infections worldwide. The HIV-1 subtype is the general strain when referring to global HIV infection; unlike HIV-2 which is localized to West Africa (Nyamweya et al., 2013). Unlike its counter-subtype HIV-2, HIV-1 has a much higher virulence as well as a faster rate of disease progression (Marlink et al., 1994; Nyamweya et al., 2013).

The primary targets of HIV-1, namely CD4 cells, dendritic cells and macrophages, are involved in the immune response of the body against pathogens such as HIV. By attacking these cells, HIV is able to cause a progressive depletion of these cells at the various mucosal effector sites where they are found (Grossman et al., 2006; Kawamura et al., 2005); effectively resulting in the decreased ability of the immune system to react to life-threatening opportunistic infections due to cellular immunity being lost (Okoye and Picker, 2013).

1.2.2.1. Mechanisms of Transmission

Research has shown an array of theories with regard to the actual mechanism by which HIV enters a host. Broadly, they are divided into two main ideas being CD4-CCR5 dependent HIV transmission (Puigdomenech et al., 2008; Trkola et al., 1996) and CD4-CCR5 independent HIV transmission (Arias et al., 2003b; Boily-Larouche et al., 2012; Bomsel, 1997; Vidricaire et al., 2007a).

1.2.2.1.1. CD4-CCR5 dependent HIV transmission

The orthodox method of HIV transmission in the cell free virus form has been extensively researched. Scientists have determined that the primary binding site for the HIV-1 virus is the CD4+ molecules located on certain white blood cells and is mediated by the viral surface glycoprotein gp120 (Trkola et al., 1996). Subsequent studies looked at a chemokine receptor (CCR5) and its influence on HIV transmission and viral efficacy (Trkola et al., 1996). Trkola *et al* demonstrated that the gp120/CD4+ interaction, although the primary contact, can be substituted by a gp120/CCR-5 interaction; and if both these synapses occur concomitantly, viral efficacy is greatly enhanced (Choe et al., 1996; Trkola et al., 1996). To further support this theory, comparative studies looking at the effect of blocking the biological activity between various leukocyte function-associated antigens (LFA's) and their intracellular adhesion molecule (ICAM) counterparts yielded supporting evidence which excluded such interactions as stand-alone mechanisms of HIV transmission; but rather agents relating to the enhanced transfer of HIV (Trkola et al., 1996). To a lower extent, studies have also looked at other receptors such as C-X-C chemokine receptor 4 (CXCR4) and various CC chemokine ligands (CCL) which have been implicated yet no firm conclusion can be made about their exact effect on HIV transmission (Abbas and Herbein, 2014; Kalinina et al., 2013).

1.2.2.1.2.. CD4-CCR5 independent HIV transmission

The above mentioned orthodox mechanism is the most common known method of HIV infection, but there also exists an immune cell mediated mode of entry that may be relevant when leukocytes laden with HIV infect the trophoblast or facilitates passage of HIV through the barrier (Arias et al., 2003b). Therefore, if such integral contact between infected maternal leukocytes facilitates infection or

transcytosis of HIV, any signalling molecule that recruits and helps attach white cells to the syncytiotrophoblast will be an important factor in transmission.

Another mechanism of HIV transmission across the placental barrier is through endocytosis of the HIV-1 (Parry et al., 2006; Vidricaire et al., 2007a). Although studies have shown that HIV transmission is assisted by placental damage (for example inflammation (Soilleux and Coleman, 2003) or rupture of the fetal membranes (Burton et al., 1996)), the mechanism for HIV transmission in mothers without obstetric complications (for example co-infections with STDs, etc) is poorly understood (Arias et al., 2003a; Parry et al., 2006).

1.2.3. Transmission of HIV

Frequency of transmission is determined by the amount of infectious virus in the body fluid of an infected individual and the amount of contact an individual has with the body fluid of the infected individual (Levy, 2007). The means of transmission from one individual to another are via sexual contact, exposure to contaminated blood and mother to child transmission (Levy, 2007; Strauss and Strauss, 2002).

1.2.3.1. Mother to Child Transmission of HIV

Mother to Child Transmission (MTCT) of HIV, with just under a quarter of a million babies born with HIV annually (Chaib, 2015), is the most common way a child can get infected. HIV is known to be transmitted both during pregnancy, during parturition and post natally during breastfeeding (Mognetti et al., 2000). Significant reduction in transmission rates have been realized though antiretroviral therapy, access to healthcare and changes in breastfeeding advice, but in low and middle income countries access to healthcare is poor, resulting in higher than global transmission rates. In resource poor settings, it is near impossible to achieve sustainable transmission targets (Chaib, 2015; Stats SA, 2013).

In treatment naïve mothers, the overall rate of transmission of HIV to their child is 15-45% compared to the reduced risk of below 5% for those mothers who are on a form of effective intervention to prevent MTCT (PMTCT) of HIV (WHO, 2014). The number of women able to access antiretroviral (ARV) treatment, in an effort to slow the progression of HIV into AIDS, has steadily increased over the years (Barron et al., 2013) but poor healthcare facilities, social stigma as well as a lack of HIV testing still remains a challenge (WHO, 2014).

1.2.3.2. Breastfeeding

Breastfeeding is one of the most intimate and natural interactions between a mother and her newborn child and although the best form of nutrition for the child, it is also a mechanism of HIV transmission from mother to child (Kourtis and Bulterys, 2010; Read and American Academy of Pediatrics Committee on Pediatric AIDS, 2003). Mothers who are not on any form of PMTCT, HIV transmission to their child is almost 40%. For those mothers on PMTCT, that risk is reduced to less than 5%.

In terms of HIV-positive mothers in low- and middle-income countries, such as South Africa, the guidelines in the latest report from the World Health Organization has advised exclusive breastfeeding; especially where there is little access to health services, proper sanitation or clean drinking water (Bland et al., 2008; WHO, 2014). It has been recommended that HIV-positive mothers exclusively breastfeed for the first 6 months followed by weaning for a duration of one month (Kuhn et al., 2008). Any disruption in this system such as the ingestion of tea, water or other forms of nutrition increases the risk of HIV transmission via breastfeeding (Bland et al., 2008).

Although access to ARV's have increased, there is still a significant backlog with regards to ARV's for children. Issues around breast health and providing adequate milk for their infants result in mothers disregarding exclusive breastfeeding, thereby increasing the risk of exposure and transmission of HIV to the infant (Buskens et al., 2007; Engebretsen et al., 2007).

1.2.3.3. In-utero transmission

The placenta functions as a selective filter. However it is not impervious to viruses (Al-husaini, 2009). The main method of infection of the unborn child may occur via the placenta and directly into the embryo's bloodstream (Arias et al., 2003a; Mognetti et al., 2000; Soilleux and Coleman, 2003; Vidricaire et al., 2007b). Studies have shown that the fetus can acquire the HIV infection in the early stages of pregnancy as well as at term. This *in utero* transmission may be caused by direct infection of the trophoblast barrier (Mognetti et al., 2000). Studies have therefore looked at the susceptibility of the trophoblast cells to infection by the HIV-1 virus and also the mechanism of viral infection (Al-husaini, 2009; Arias et al., 2003a; Bomsel, 1997; Derrien et al., 2005; Lagaye et al., 2001b; Vidricaire et al., 2007b, 2004).

1.2.4. The Placenta and Its Structure

The chorioallantoic placenta (Soares and Hunt, 2006) is responsible for the redirecting of the immune, endocrine and metabolic functions in favour of the developing embryo (Cross et al., 1994). At term, the human placenta has a disc-like appearance with an average weight of 470g (Kaufmann, 2006). Placentation begins as soon as the fetal membranes of the blastocyst make a close and stable contact

with the uterine mucosa. The placenta is the combination of embryonic and maternal tissues which work together to supply the much-needed nutrients to the growing fetus. The embryonic contribution towards the formation of the placenta is the result of the splitting of the membranous sac; resulting in two separate surfaces: the chorionic plate and the basal plate (Kaufmann, 2006).

Placental villi originating from the chorionic plate are complex tree-like structures which protrude into the intervillous space found between the above mentioned surfaces (Wang and Zhao, 2010). The surface of the villi is known as the trophoblast layer and is in direct contact with the maternal blood supply. This trophoblastic layer is composed of two different layers of cells; the syncytiotrophoblast layer and the cytotrophoblast layer. The cytotrophoblast layer are the proliferating stem cells which give rise to the syncytiotrophoblast, which together with the fetal capillary basement membrane, which runs close to the maternal blood in the intervillous space, form the maternofetal transport barrier (Kaufmann, 2006; Wang and Zhao, 2010).

There are three main types of villi found within a term placenta: stem, intermediate and terminal villi (Wang and Zhao, 2010). Stem villi or conducting villi are the largest villi and are characterized as having multiple arteries and veins, accompanied by easily identifiable muscular walls. Intermediate villi on the other hand differ from stem villi in that they appear without fetal vessels or stromal fibrosis. Terminal villi have been described to have a grape-like structure and are the side branches of the intermediate villi. Intermediate villi and terminal villi are mainly involved in exchange between the fetal and maternal circulation due to their slender capillaries and dilated sinusoids respectively (Kaufmann, 2006).

Within the placental villi lie the fetal vessels including a mixture of fixed tissue cells, macrophages and connective tissue fibres (Kaufmann, 2006). These fetal vessels are connected to the fetal blood circulation via the chorionic plate and the umbilical cord (Kaufmann P, 2006). Although the maternal and fetal circulations do run very close, the formation of this maternofetal barrier ensures that the two blood systems never mix (Wang and Zhao, 2010).

1.2.4.1. Function of the Placenta

The main role of the placenta is to supply the growing fetus with adequate nutrition. Passage of oxygen and glucose from the maternal to the fetal circulation and contraflow of CO_2 and waste products (Gude et al., 2004) occurs across the trophoblast layer. In this way, the placenta is the equivalent to a filter whereby harmful substances are kept away from the fetus, with only beneficial substances passing through to the fetus (Garnica and Chan, 1996; Kaufmann, 2006; Wang and Zhao, 2010).

The placenta is responsible for the production of a variety of hormones; for example oestrogen and progesterone. These hormones are very important in pregnancy as they prevent premature uterine contractions (Gilbert, 2014) and prime tissue within the uterus in preparation for the birthing process.

The immunological role of the placenta is yet to be fully understood. IgG antibodies which are involved in the protection against infection are able to freely traverse the fetomaternal barrier in order to offer temporary protection to the fetus *in utero* (Simister and Story, 1997). This is a passive immunity offered from the mother which is an exact replica of the mother's long term humoral immunity. IgM however, which is involved in the initial response against an exposure to a pathogen, is unable to traverse the placenta resulting in certain infectious diseases being able to be transmitted from mother to child (Gude et al., 2004).

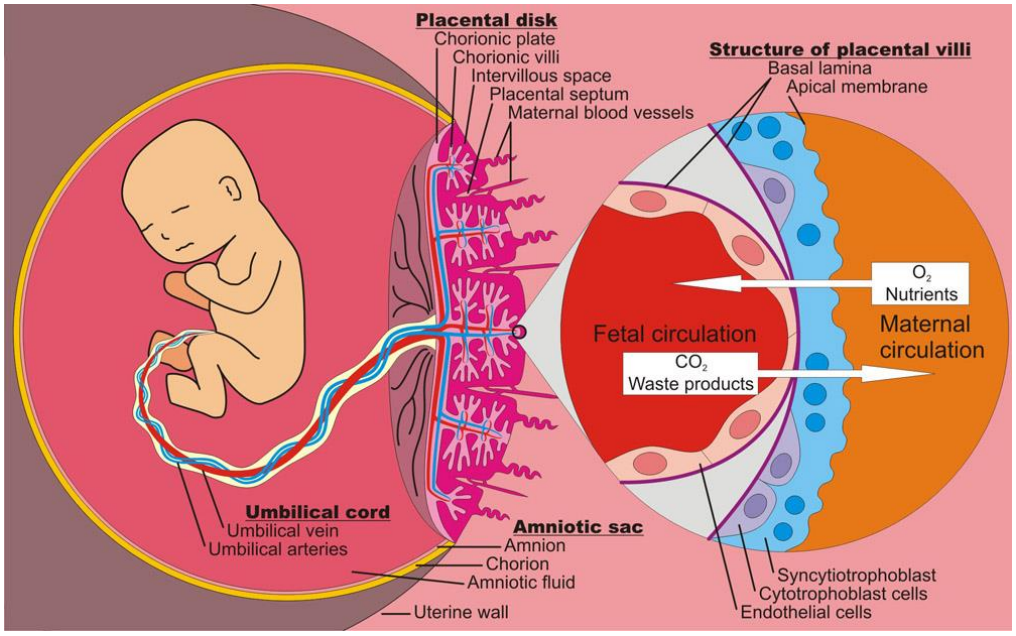

Figure 1.2. Diagram depicting the function of the placenta during gestation. (http://www.ibmm.unibe.ch/unibe/medizin/ntbiomol/content/e537/e666/e1554/e1555/e1564/e1566/PLACENTAbig_eng.jpg).

1.2.5. Natural Immune Response

The development of the immune system is said to be the result of evolutionary pressure of pathogens on a biological system (Kasahara, 2011; Male, 1992). Amongst the many important activities our immune system is responsible for, the main purpose of our immune system is to be able to identify the presence of a pathogen or infectious agent. Subsequent to this, activation of various mechanisms needed to protect an individual against such pathogens is necessary. In essence, an immune response essentially relies on the ability of the immune system to recognise an antigen on a pathogen as a foreign molecule and subsequently mount the appropriate immune response in order to eliminate the source of the antigen (Male, 1992). One of the main activities whereby the immune system is able to carry out its function is known as the recognition phase (Janeway and Medzhitov, 2002).

Recognition of a pathogen by immune cells such as lymphocytes is important but more importantly, immune cells need to have the ability to recognise what is known as "self" molecules. These self-molecules are found on the cells of the individual and these epitopes should not normally be able to mount any sort of immune response.

Immune responses which require lymphocyte recognition of a pathogen are generally referred to as adaptive immune responses (Guidotti and Chisari, 2001; Hsu and Nanan, 2014). Specificity and memory are central to this specific cell immunity as these actions are a second, enhanced reaction compared to the immune response upon the first exposure to said pathogen. First exposure immune reactions in response to a pathogen are normally carried out by the innate immune response (Janeway and Medzhitov, 2002). This immune response is less specific and merely mounts an immune response to any molecule which it identifies as being foreign (Janeway and Medzhitov, 2002).

1.2.5.1. Antigen Presenting Cells

In order for lymphocytes to initiate an immune response against an antigen, recognition must first take place (Janeway and Medzhitov, 2002). The role of APC's is to present an antigen to the lymphocyte is in a recognizable form. When discussing an antigen presenting cell, we are referring to those subset of cells which are capable of MHC Class II antigen presentation. There are multiple cells which fall into this category of APC's, including macrophages, dendritic cells and Langerhans cells (Alberts et al., 2002; Liu, 2001); which will be discussed in more detail further on. It is important to note that although not all cells are able to perform Class II antigenic presentation, cells can be induced to express these molecules. For example, interferon-γ (IFN-γ) is said to be a very strong inducer of MHC Class II molecules on most cell types (Male, 1992).

There are many factors which determine the ability of a cell to be an APC. Most importantly is its ability to internalize antigen and subsequently express the antigenic determinant on its cell surface in association with an MHC Class II molecule (Alberts et al., 2002). It is also important to note the role that cytokines play in combined antigen presentation (Janeway and Medzhitov, 2002). Antigen presentation and the subsequent control of the impending immune response is dependent on the aforementioned attributes.

1.2.5.1.2. Macrophages and the Placenta

In 1908, Elie Metchnikoff was awarded the Nobel Prize on his work regarding phagocytosis and its importance in assisting host defence against infection and injury (Gordon, 2007). In over a century of research into these versatile cells, we have learnt about their distribution within a host, their

heterogeneous phenotypes and multiple complex functions involved in tissue homeostasis in innate as well as adaptive immunity (Gordon, 2007).

Originating in the bone marrow, from hematopoietic stem cells, macrophages are scavenger molecules that appear in a star-like formation (Gordon, 2007). They then travel through the body's blood circulation and take up residence in certain tissues where they co-ordinate development, host defence by cytokine and chemokine secretion and phagocytosis (Gordon, 2007). They move in an amoeboid fashion, engulfing a variety of substances including cellular debris, microbes and foreign substances (Nagamatsu and Schust, 2010). Macrophages are said to be the link between the innate and the adaptive immune system through their functions; namely phagocytosis, digestion and antigen presentation to T-lymphocytes (Abrahams et al., 2004; Gordon, 2007). The great plasticity of macrophages enables them to transform between various phenotypes in an effort to respond to activation agents which they encounter in their micro-environments and based on their expression profiles, macrophages can be classified into two broad groups; namely pro-inflammatory (M1) and anti-inflammatory (M2) macrophages (Olefsky and Glass, 2010; Sica and Mantovani, 2012).

M2 macrophages are said to display immunosuppressive effects and resolve inflammation through the production of anti-inflammatory cytokines and various other molecules as well as being involved in the stimulation of the Th2 immune response (Gordon, 2007). In pregnancy, those macrophages which are found on the fetal side originate from the yolk sac and are known as Hofbauer cells found within chorionic villi while macrophages from the maternal side originate from the aforementioned hematopoietic stem cells and circulate in the blood prior to entering the endometrial decidual stroma and undergoing further differentiation into tissue macrophages (decidual macrophages) (Abrahams et al., 2004; Nagamatsu and Schust, 2010).

CD68 is a transmembrane protein which is expressed by human monocytes and tissue macrophages (NCIB, 2015). This protein has been localized to lysosomes and endosomes with a smaller division circulating to the surface of the cell. This Type 1 integral protein binds to tissue and organ specific lectins or selectins via its heavily glycosylated extracellular domain. As a member of the scavenger receptor family, it is involved in clearing cellular debris, recruiting and the subsequent activation of macrophages as well as the promotion of phagocytosis (NCIB, 2015).

1.2.5.1.3. Dendritic Cells and the Placenta

Similar to macrophages, dendritic cells (DC's) are also derived from hematopoietic bone marrow progenitor cells (Liu, 2001) and are antigen presenting cells of the human immune system. Their main role as messengers between the adaptive and innate immune systems is based on the successful

processing of antigenic material and the subsequent presentation of this material at the cell surface to T cells (Banchereau et al., 2000; Geijtenbeek et al., 2000).

Dendritic cells are found in tissue which is exposed to the external environment; for example the skin, nose and vagina. These dendritic cells have also been found in an immature state in blood where they migrate to the lymph nodes (de Witte et al., 2008) following activation by an antigen. Immature dendritic cells have high endocytic capabilities and a low rate of T-cell activation which aids in the constant sampling of the nearby environment for pathogens present (Banchereau et al., 2000; Steinman, 2015). This sampling is further aided by structures known as dendrites, which are branched projections present during certain stages of dendritic cell development and also the namesake of these antigen presenting cells. Also present are branched projections called pattern recognition receptors (PRR's), which are involved in the identification of a pathogen. The development of different subsets of dendritic cells has resulted in a variation in expression of pattern recognition surface receptors from subset to subset (Banchereau et al., 2000; Liu, 2001).

These transmembrane protein receptors play a crucial role in the innate immune system as it recognises numerous evolutionary divergent pathogens, including multiple parasites as well as a range of viruses which impact on public health. In terms of HIV infection, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), also known as CD209, has been identified as the protein receptor used to bind to dendritic cells. Diseases such as Dengue fever have also been related with DC-SIGN and the proposed mechanism by which the dendritic cell initiates the primary immune response is the same (Tassaneetrithep et al., 2003). It is believed that dendritic cells endocytose the pathogens and transport them into the cell via a lysosomal compartments for degradation (Pantophlet, 2014). This is then followed by the receptor recycling to the surface of the dendritic cell (Steinman, 2015) and by the simultaneous use of MHC Class II molecule, presents the antigenic determinant to T cells thereby initiating an adaptive immune response (Pantophlet, 2014).

However, conflicting reports say that it is possible that the bound HIV within the cell is protected in lysosomal compartments and evades degradation (Geijtenbeek et al., 2000). In this way, the infectious particle which is housed within these aforementioned compartments lie close to the surface of the cell membrane and thus increasing the propensity of HIV spread during the migration of the dendritic cells from the periphery to the lymphoid organs (Geijtenbeek et al., 2000).

In vitro studies have looked at DC-SIGN as a receptor for HIV-1 and the role it plays in its transmission. Studies have shown that HIV-1 may enter either in a *trans* (without dendritic cell infection) or *cis*

(dendritic cell infection) followed by presentation to T cells to induce a robust infection (Ahmed et al., 2015; Lee et al., 2001; Puryear and Gummuluru, 2012).

1.2.5.1.4. Langerhans Cells and the Placenta

Langerhans cells, specifically the epidermal Langerhans cells (LC), have been classified as dendritic cells (Romani et al., 2010) while others have classified Langerhans cells as macrophages (Thorbecke et al., 1980). A distinguishing feature of Langerhans Cells is their expression of CD1, a human thymocyte antigen, which sets this specific subset apart from other dendritic cells (Sutton et al., 1986). CD1a belongs to the family of transmembrane glycoproteins and are structurally related to the MHC proteins and are said to form heterodimers with beta-2-microglobulin, which forms part of the HLA complex and is important in cell-surface structure (Bernier, 1980).

CD1a proteins are said to mediate the presentation of primary lipid and glycolipid antigens of self or those of microbial origin to circulating T cells (Mizumoto and Takashima, 2004; Salamero et al., 2001). The protein localizes to the plasma membrane as well as early recycling vesicles which are part of the early endocytic system (Salamero et al., 2001). Studies have looked at the role of Langerhans cells found in the vaginal mucosa (de Witte et al., 2008, 2007) as well as vaginal epithelium (Kawamura et al., 2005). The epithelial layer in which Langerhans cells have been shown to be present show a positive expression of cytokeratin. Cytokeratin belongs to the intermediate filament (IF) protein family (Barak et al., 2004) and is found in the intracytoplasmic cytoskeleton present in epithelial cells. Cytokeratin is also documented as being a reliable, strong and consistent marker for trophoblast cells throughout pregnancy (Daya and Sabet, 1991). If one had to consider the syncytiotrophoblast layer being analogous to an epithelial layer, it is plausible that Langerhans cells may be present below the syncytiotrophoblast layer in the placenta.

Studies have looked at their presence in placental tissue yet their results have been inconclusive (Sutton et al., 1986). Due to the functioning of these cells and their cell-to-cell communication abilities (Kawamura et al., 2005), Langerhans cells are said to be the initial cellular targets in sexual transmission of HIV (Kawamura et al., 2005). Langerhans cells if present within the placenta may be cellular targets for HIV transmission.

1.2.6. Normal Pregnancy

Pregnancy has been said to be an immunological battle between the mother and the growing semi-allogeneic fetus within her (Veenstra van Nieuwenhoven, 2003). Cells which are genetically distinct from that of the mother are automatically seen as foreign and are killed by the mother's immune system (Veenstra van Nieuwenhoven, 2003). It is therefore imperative that differentiation of self and non-self

molecules is carried out by an individual's immune system. Recognition of "self" is made possible by the presence of histocompatible human leukocyte antigens (HLA's), specifically HLA-A and HLA-B. These membrane bound proteins are 'read' by lymphocytes and if found to be different, damaged or non-existent; the lymphocytes proliferate and kill the abnormal cells (Veenstra van Nieuwenhoven, 2003).

The trophoblastic layer found within the placenta is semi-allogeneic and may be treated as foreign by maternal immune cells. HLA-A and HLA-B proteins in the trophoblast layer are downregulated resulting in no immune response being mounted against the fetus. Rather than express HLA-A and HLA-B, the trophoblastic layer of the placenta produces a membrane protein called HLA-G. HLA-G is immunologically comparable to HLA-A and HLA-B and is therefore able to fool the maternal natural killer cells into believing that the trophoblastic cells are not foreign (Lash et al., 2010). In essence, the downregulation of HLA-A and HLA-B coupled with the production of HLA-G offers protection against the mother's immune system as well as prevents the maternal natural killer cells from attacking the fetus (Veenstra van Nieuwenhoven, 2003).

The placenta produces the female hormone progesterone. Studies have shown that progesterone inhibits lymphocyte proliferation and in this way if there is an immune response which is mounted against the fetus, due to the diminished number of lymphocytes, the immune response cannot be maintained (Lee et al., 2012).

1.2.7. Placenta and HIV Transmission

The conundrum of HIV transmission from mother to child via the placenta is highly debated with multiple theories purported. However, the presence of HIV within the placenta is substantially supported by various studies carried out (Bhoopat et al., 2005; Lairmore et al., 1993; Martin et al., 1992; Vidricaire et al., 2007a).The mechanisms by which HIV traverses the fetal-maternal barrier is yet to be fully understood.

Various studies suggest that the various receptors used by the virus in either the classical transmission pathway or the CD4-CCR5 independent pathways are present on the apical trophoblastic surface subjected to the infected mother's blood. However, there have been studies which dispute the presence of the orthodox CD4 and CCR5 receptors needed by HIV for transmission. Furthermore, studies have shown that differentiation of the cytotrophoblast to syncytiotrophoblast cells causes a decrease in the expression of receptors found on the trophoblast surface (Koi et al., 2001). This further strengthens the possibility of CD4-CCR5 independent transmission pathways.

When the phenotype of the placenta and the infectious activity of HIV are considered, infection of the trophoblast layer or the transcytosis of the virus across the trophoblastic barrier becomes a possibility. Studies to support these theories look at the cell-to-cell interaction (Arias et al., 2003b; Burleigh et al., 2006; Lagaye et al., 2001b) as a form of transcytosis of the virus across the trophoblastic layer (Bomsel, 1997) while other studies have suggested that instead of merely transporting the virus across the barrier to the fetus, the trophoblastic cells are able to be infected (Geijtenbeek et al., 2000).

The association of various immune cells in HIV transmission is an important key in understanding in utero transmission. Their significance in transmission has not been clearly identified and because of this, they continue to be an enigma. By evaluating the expression of cellular receptors specific for placental macrophages, dendritic cells and Langerhans cells which are used for HIV transmission, and comparing their expression in HIV positive and HIV negative patients; we hope to correlate such expression with both orthodox and unorthodox mechanisms of HIV transmission and their mediating factors (Vallen et al., 2015).

Chapter 2

2.1. To be submitted to the European Journal of Obstetrics and Gynaecology.

The Role of Macrophage, Dendritic and Langerhans Cells in the Mother to Child Transmission of HIV in Placental Tissue of Normotensive Pregnancies.

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Condensation

The markers for macrophage, dendritic and Langerhans cells typed the presence or absence of the immune cells in the placental tissue of HIV positive and HIV negative pregnancies, which when combined with their active role in the immune response during pregnancy has resulted in these cells being implicated in alternative mechanisms of HIV transmission.

Abstract

Objectives: Mother to child transmission of HIV remains high despite the use of antiretroviral therapy during pregnancy. The mechanism of HIV transmission across the placenta needs to be evaluated. This study aims to evaluate expression of key molecules implicated in HIV transmission within the placental exchange villi.

Study Design: Immunohistochemical staining of placental tissue from normotensive placentae with target antibodies (CD68, DC-SIGN and CD1a) were analysed using light microscopy followed by morphometric and statistical analysis.

Results: No significant difference was noted in the expression levels of DC-SIGN and CD68 between the groups. DC-SIGN expression was noted on immune cells as well as the syncytiotrophoblast of placental villi. CD68 expression on immune cells showed no difference between HIV positive and HIV negative groups. CD1a expression was not seen in the placental villi or immune cells in either group.

Conclusion: Markers indicating the presence of Langerhans cells were not present in placental tissue. HIV infection does not alter the number of cells and quantity of expression of CD68 and DC-SIGN in normotensive pregnant women. Such expression provides evidence that HIV could use cell-mediated pathways to enter the circulation of the fetus, or at least infect the fetomaternal barrier. Further studies need to be conducted to co-localise these immune cells and markers of HIV infection in these groups.

1. Introduction

According to the World Health Organization, over 800 women die per day due to pregnancy related complications (WHO et al., 2015). Maternal mortality, defined as death due to complications from pregnancy or childbirth (*Tenth Interim Report on Confidential Enquiries into Maternal Deaths in South Africa*, 2011), remains a global concern and has seen a dramatic rise in the last two decades due to non-pregnancy related infections (NPRI's), with 35% of the statistic attributed to HIV infected mothers (*Saving Mothers 2011- 2013: Sixth Report on the Confidential Enquiries into Maternal Deaths in South Africa*, 2015).

Of equal concern is Mother to Child Transmission (MTCT) of HIV which occurs either during pregnancy, during parturition or postnatally during breastfeeding (Mognetti et al., 2000). MTCT has resulted in just under a quarter of a million babies born with HIV annually (Chaib, 2015). Significant reduction in transmission rates have been realized though antiretroviral therapy (ART), access to healthcare and changes in breastfeeding advice; but in low and middle income countries access to healthcare is poor, resulting in higher than global transmission rates. In these resource poor settings, it is near impossible to achieve sustainable transmission targets (Chaib, 2015; Stats SA, 2013).

The main method of infection of the unborn child may occur via the placenta (Arias et al., 2003a; Mognetti et al., 2000; Soilleux and Coleman, 2003; Vidricaire et al., 2007b) and this may occur early or late in pregnancy (Kourtis and Bulterys, 2010; Vidricaire et al., 2004). Such *in utero* transmission may be caused by direct infection of the trophoblast barrier (Mognetti et al., 2000). Several studies report that trophoblast cells are susceptible to HIV infection and attempt to elucidate the mechanism whereby this occurs (Al-husaini, 2009; Arias et al., 2003a; Bomsel, 1997; Derrien et al., 2005; Lagaye et al., 2001b; Vidricaire et al., 2007b, 2004). However, the exact mechanism which HIV utilizes is still unclear. HIV may use a non-receptor mediated pathway to traverse protective barriers such as the placenta in order to spread infection (Arias et al., 2003b; Lagaye et al., 2001a; Mognetti et al., 2000).

The use of these pathways are facilitated by many important immunocompetent cells. These cells are also intricately linked to pregnancy. Successful pregnancy is dependent on a maternal tolerance of the semiallogeneic fetus (Svensson-Arvelund et al., 2015). At the fetomaternal interface the balance between the Th1/Th2 maternal immune response to a foreign body ensures the cell mediated activation of protective processes and recruitment of important immunocompetent cells such as decidual natural killer cells as well as macrophages. HIV present at the maternal fetal interface capitalises on this process by infecting responding cells or killing them, thus increasing the iterative infection of immune cells in the body. This makes macrophages an important subset of immune cells that solicits viral transmission during pregnancy.

Macrophages play key roles in all stages of pregnancy: implantation, placentation and parturition (Kim et al., 2007). Derived from blood monocytes, macrophages are central to successful placentation (Kim et al., 2007) while decidual macrophages found at the fetomaternal interface are needed for maternal tolerance of fetal antigens (Kim et al., 2007). During the gestational period, macrophages are also involved in surface presentation of molecules involved in both the innate and adaptive immune response. One such molecule is Dendritic Cell-Specific Intracellular adhesion molecules-3 (ICAM-3)-grabbing Non-integrin (DC-SIGN) (Pillay et al., 2014). This C-type mannose-binding lectin is said to initiate the interaction between dendritic cells and resting T-cells (Pillay et al., 2014).

DC-SIGN is highly expressed on dendritic cells and decidual macrophages as well as Hofbauer cells (Pillay et al., 2014). It has been proposed that in order for HIV to cause infection, it adheres to the Hofbauer cells and can possibly infect these placental macrophages (Pillay et al., 2014). The expression of DC-SIGN has been seen in the cervix (Trifonova et al., 2014), uterus (Ochiel et al., 2010) as well as the placenta (Geijtenbeek et al., 2001).

Macrophages and dendritic cells, which are targeted by HIV appear to arise from a common lineage (Sutton et al., 1986). Another important cell type that are initial targets in the sexual transmission of HIV are Langerhans cells owing to their specialisation in antigen presentation and their cell-to-cell communication ability (Kawamura et al., 2005). Some epidermal Langerhans cells have been classified as dendritic cells (Romani et al., 2010) while others have classified Langerhans cells as macrophages (Thorbecke et al., 1980).

The association of Langerhans cells with the placenta is uncertain but given the similarity between the types of tissue in which they are found and the placenta, it may be possible that they are found here. Cytokeratin is a common marker for both epithelial cells and trophoblast cells (Barak et al., 2004; Kawamura et al., 2005). This knowledge is suggestive that the presence of Langerhans Cells are not only restricted to the vaginal epithelium but they could possibly be expressed in the placenta. Langerhans cells if present within the placenta may be cellular targets for HIV transmission.

By evaluating the presence and density of expression of proteins specific for placental macrophages and dendritic cells which are used by HIV for its transmission, we will be able to correlate such expression with alternative methods of HIV transmission. In this study, we aim to evaluate the expression of DC-SIGN, CD68 and CD1a in HIV affected pregnancies compared to HIV negative pregnancies.

2. Materials and Methods

2.1. Patient Sampling

Placental samples were obtained from 60 Black South African normotensive primigravid women over the age of 18 yrs from a hospital servicing a catchment of at least 16 clinics in the Durban South region of Kwa-Zulu Natal, South Africa. These patients were stratified into HIV positive (n = 30) and HIV negative groups (n = 30); with the HIV positive group being further stratified according to their CD4+ cell counts; namely CD4+ count below 200 (n = 9) and CD4+ count above 200 (n = 21).

2.2. Sample preparation

This study uses formalin fixed paraffin embedded placental tissue of full thickness sampled from the central area of the placenta avoiding major blood vessels, areas of visible necrosis and/or infection.

2.3. Microtomy

Serial sections with a thickness of 3-4µm were cut on a rotary microtome (Leica 819; Germany) and lifted onto coated glass slides (Xtra™ Adhesive, Leica; Germany) which were baked overnight at 60°C followed by rehydration of the sections in xylene, 100% and a descending series of alcohol. Thereafter slides were incubated in picric acid for 30minutes and then sequentially brought to water.

2.4. Immunohistochemical staining procedure for CD 209, CD1a and CD68.

Immunohistochemical staining was performed using the Envision FlexMini High pH kit (DAKO, Denmark) according to the manufacturer instructions. Briefly, antigen retrieval was performed using the Envision™ FLEX target retrieval solution in the PT link (DAKO, Denmark) for 20 minutes at 95°C. Blocking of endogenous peroxidase present in the tissue was carried out by incubating slides for 5 minutes in enzyme blocking reagent (DAKO, Denmark).

Tissue sections were then incubated with the mouse antihuman CD209 (1:25, BioLegend, Clone 9E9A8), mouse antihuman CD68 (1:50, R&D Systems, Clone #298807) and mouse antihuman CD1a (1:10, DAKO, Clone 010) antibodies diluted in an antibody diluent DAKO REAL™ (DAKO, Denmark). The reaction was visually detected within 10min with diamino-benzidine (DAB) as the chromogen. Nuclei were counterstained with Mayer's haematoxylin (5min). Sections were subsequently rehydrated and mounted in dibutylphthalate xylene (DPX).

During optimization studies of the primary antibodies, both negative and method controls were used substituting the primary antibody with antibody diluent.

2.5. Morphometric image analysis of antibody expression

All immunostained sections were viewed on the Zeiss Axioscope A1 Photomicroscope. Automated image analysis at an initial magnification of 40x was used to quantify immunostaining on slides.

Two images per sample were captured for CD209 and CD68 staining. This was not possible with CD1a due to the complete absence of staining.

2.6. Statistical Analysis

All data was tabulated and analyzed using SPSS 23 (IBM, Armonk USA).

Demographic data was analyzed using Students T-test and Mann-Whitney U tests based on tests for normality. All categorical variables were analysed using Fisher's/$\chi 2$ tests. A p-value of $<.05$ was considered statistically significant. Statistical analysis of the expression of CD209 and CD68 was performed to compare expression between HIV positive and HIV negative patient groups. The percentage of brown stain obtained within the placental villi per field area was captured in two consecutive images. The percentage from each of the two images from each sample was averaged. This average percentage was converted to a percentage out of 100.The value obtained was the value used for the various statistical tests.

3. Results

3.1. Demographic Statistics

Demographic statistics are outlined in Table 1. There was no significant difference of baby weight between the different groups however there was a significant difference between placental weight ($p = 0.005$), with HIV positive placentae having a greater weight (517.33±81.59) than HIV negative placentae (457.10±77.81). No histopathological differences were noted between placentae of the various groups.

Table 1.1. Table showing demographic data of HIV Positive and HIV Negative normotensive groups.

	Normotensive pregnant women		
	HIV negative	**HIV positive**	**P value**
N	**30**	**30**	
Maternal age (years)	19.87±1.93	23.63±3.17	<.001*
Maternal weight (kg)	75.78±12.01	73.96±14.95	.286
BMI (kg/m^2)	31.85±4.83	28.94±4.28	.016*
Systolic pressure (mmHG)	121.36±13.19	119.87±9.47	.812
Diastolic pressure (mmHG)	70.36±10.02	69.50±13.06	.762
Gestational age at birth (weeks)	38.42±1.46	38.90±1.60	.225
Baby weight (kg)	3.32±0.43	3.22±0.39	.344
Placental weight (g)	457.10±77.81	517.33±81.59	.005*

3.2. DC-SIGN expression in HIV positive and HIV negative normotensive placentae.

DC-SIGN expression was immunohistochemically determined. Positive staining was noted on the syncytiotrophoblast layer of placental villi (Figure 1.1.) as well as immune cells (Figure 1.2.) in the fetal and maternal blood circulations in both HIV negative and HIV positive groups.

Staining was noted in conducting villi (Figure 1.1.a, c and e) and exchange villi (Figure 1.1.b, d and f). Independent samples Mann-Whitney U test was performed to determine significance of expression between conducting and exchange villi. There was no significant difference in expression in the conducting villi or the exchange villi [$p = 0.671$]. There was also no significant difference in expression in the syncytiotrophoblast layer between the HIV negative (18.09±11.95) (Figure 1.1.a and b) and HIV positive groups (21.05±16.24) (Figure 1.1.c, d, e and f). There was no difference in expression of DC-SIGN with CD4 counts above 200 (Figure 1.1.c and d) or CD4 counts below 200 (Figure 1.1.e and f).

Immune cells which stained positive for the expression of DC-SIGN were found in the fetal as well as the maternal blood in both HIV negative normotensive placentae (Figure 1.2.a) as well as HIV positive normotensive placentae (Figure 1.2.b).

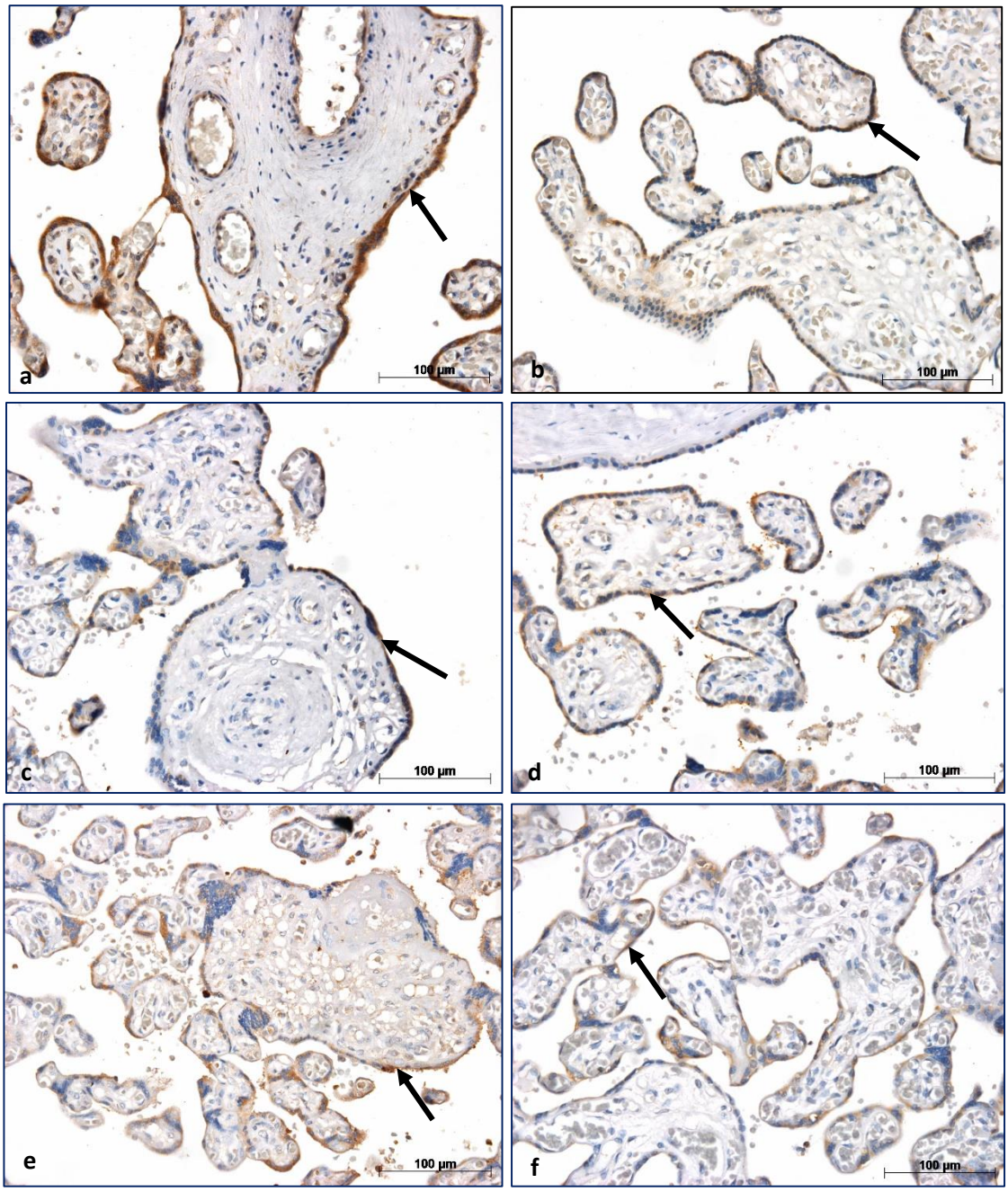

Figure 1.1. Immunostaining for DC-SIGN (indicated by the black arrows) of both HIV positive and negative placentae with (a),(c) and (e) showing conducting villi sections and (b), (d) and (f) depicting exchange villi sections. Both (a) and (b) show DC-SIGN positive immunoreactivity in normotensive HIV negative conducting villi (a) and HIV negative exchange villi (b). Micrograph (c) depicts staining of conducting villi in an HIV positive sample with a CD4+ cell count greater than 200 while positive staining of the exchange villi can be seen in micrograph (d). In micrograph (e), we can see the presence of DC-SIGN in the conducting villi in the placenta while micrograph (f) demonstrates the similar result in exchange villi of placenta from patients with a CD4+ count less than 200.

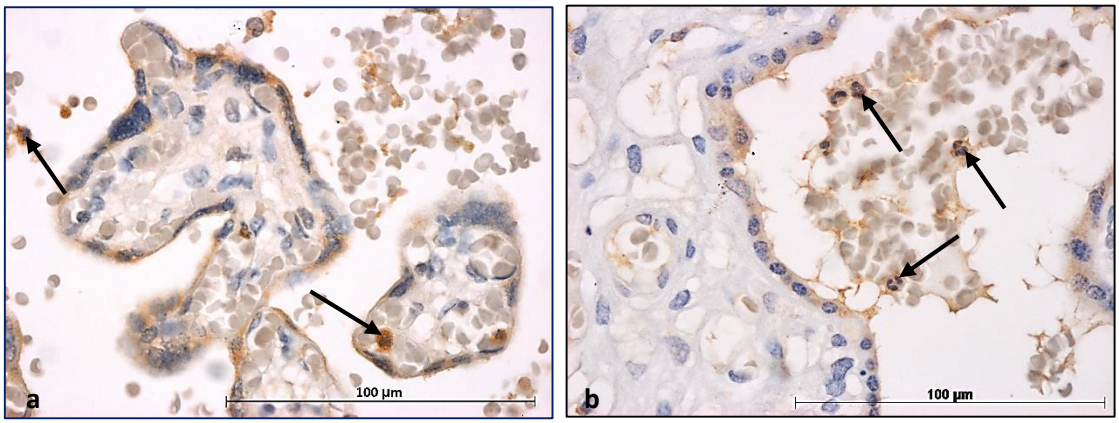

Figure 1.2. Immunostaining for DC-SIGN on immune cells (indicated by the black arrows) of HIV negative and positive placentae with (a) showing HIV negative normotensive sections and (b) depicting HIV positive sections. Both (a) and (b) show immunoreactivity on immune cells in both the fetal as well as maternal circulation. Positive expression can also be noted on the syncytiotrophoblast layer in micrograph (a) and less intensely in micrograph (b).

3.3. CD68 expression in HIV positive and HIV negative normotensive placentae.

Expression of CD68 was noted on circulating immune cells in both fetal and maternal blood circulations of the HIV negative group (Figure 1.3.a and b) as well as the HIV positive group (Figure 1.3.c, d, e and f) as well as Hofbauer cells in placental villi (Figure 1.3.b). Independent Samples Whitney U test was performed to determine significance of expression between conducting and exchange villi. No significant difference was noted between the conducting villi or the exchange villi [p = .355]. There was also no significant difference in expression between HIV negative placenta (13.33±7.49) (Figure 1.3.a and b) and HIV positive placenta (10.58±4.33) (Figure 1.3.c, d, e and f). Further statistical analysis was carried out on the expression found in the subgroups of the HIV positive placenta and no difference was noted between those placentae with a CD4+ count above 200 (Figure 1.3.c and d) and placentae with a CD4+ count below 200 (Figure 1.3.e and f).

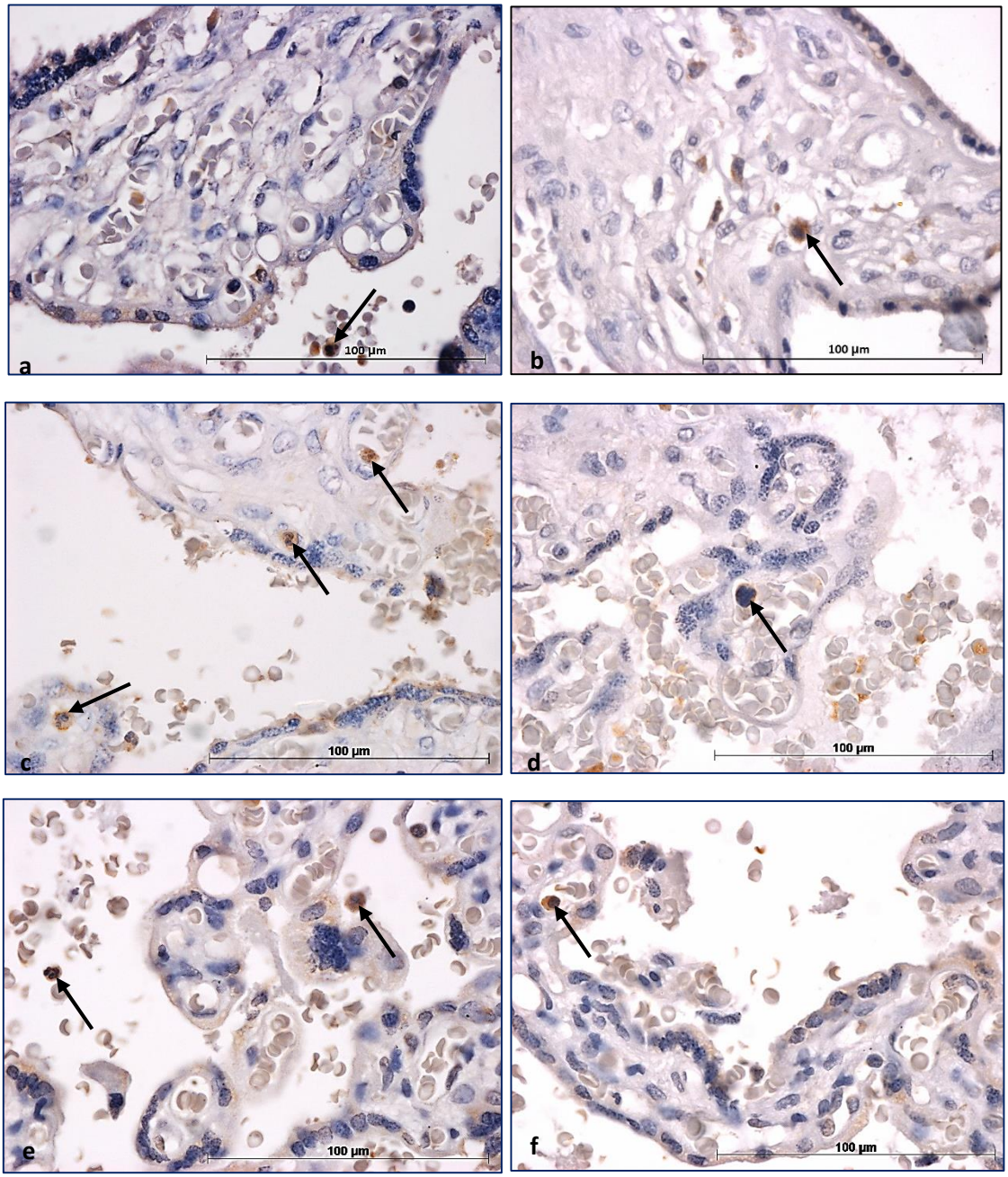

Figure 1.3. Immunostaining for CD68 of both HIV positive and negative placentae with (a), (c) and (e) showing expression on immune cells present in the maternal circulation and (b), (d) and (f) depicting positive expression of CD68 on immune cells present in placental villi tissue. Both (a) and (b) show CD68 positive immunoreactivity in normotensive HIV negative maternal circulation (a) and HIV negative placental villi (b). Micrograph (c) depicts staining of immune cells in the maternal as well as fetal circulation in an HIV positive sample with a CD4+ cell count greater than 200 while positive staining of an immune cell within the placental villi can be seen in micrograph (d). In micrograph (e), we can see the presence of CD68 in both the maternal and fetal circulation within the placenta while micrograph (f) demonstrates the similar result in the fetal circulation within placental tissue from patients with a CD4+ count less than 200.

3.4. CD1a expression in HIV positive and HIV negative Normotensive placentae.

Optimization of the CD1a antibody utilized skin tissue where Figure 1.4a depicts the negative control and Figure 1.4.b depicts the positive control, where expression can be seen on the Langerhans cells found embedded in the upper layers of skin. Immunohistochemistry of CD1a was carried out on all samples. No expression of CD1a was noted on HIV positive samples (Figure 1.4.c and d) or HIV negative samples (Figure 1.4.e and f). Images were captured at a magnification of 40x (Figure 1.4.d and f) and 20x (Figure 1.4.c and e) which shows no CD1a expression in the placental tissue samples in the control group or the test group for this study.

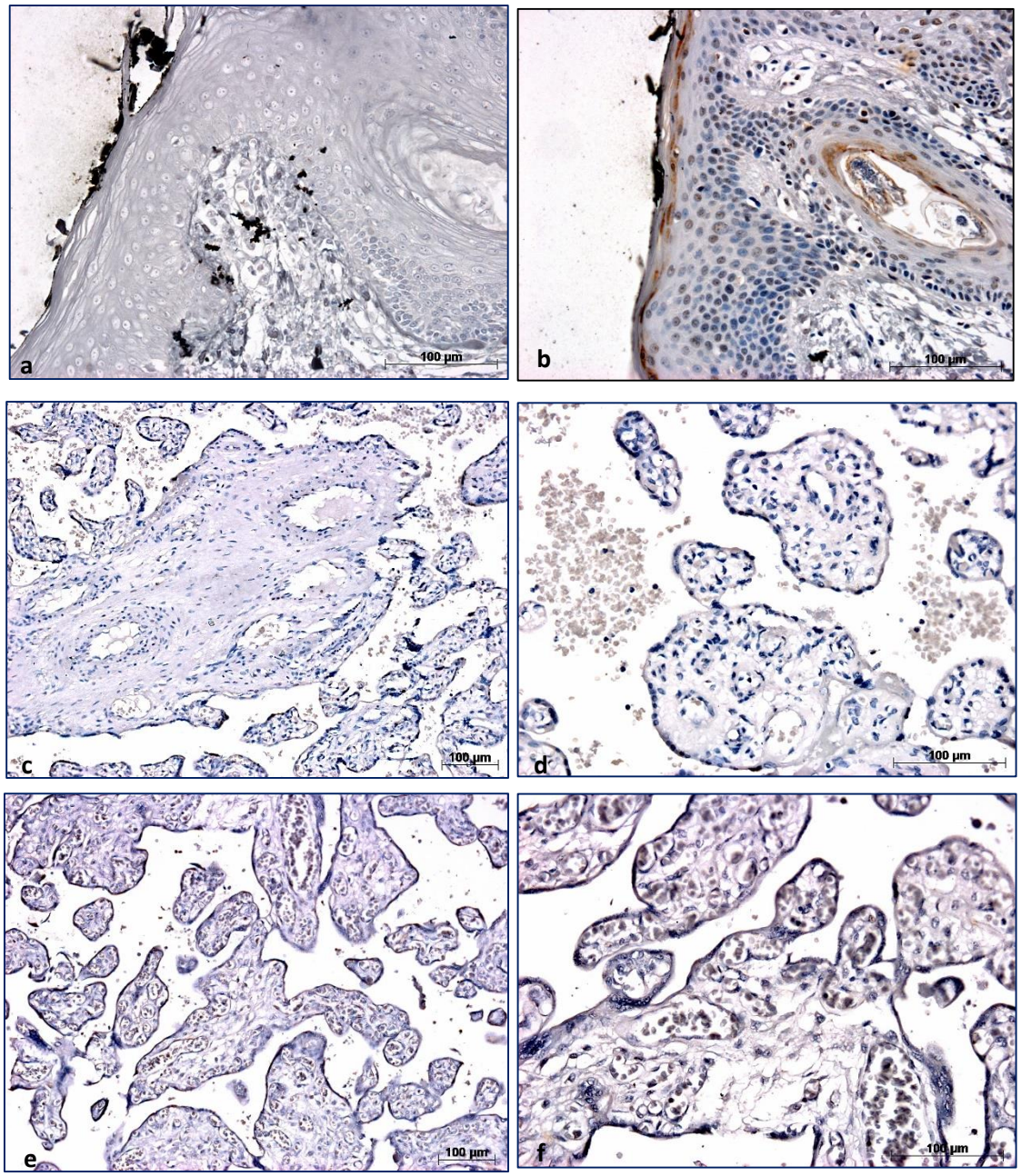

Figure 1.4. Immunostaining for CD1a of HIV positive and negative placentae with (a) showing the negative control using skin tissue and (b) showing the positive control using skin tissue. Micrograph (c) and (d) depicts HIV negative normotensive sections while (e) and (f) depict HIV positive sections. Micrograph (a) captured at 20x and micrograph (b) captured at 40x show no immunoreactivity in normotensive HIV negative conducting villi (c) and exchange villi (d). Similar results can be seen in micrograph (c) captured at 20x depicting no staining of stem villi as well as no immunoreactivity noted in exchange villi (d), captured at 40x, within HIV positive villi of the placenta.

4. Comment

The aim of this study was to localize proteins such as DC-SIGN, CD68 and CD1a which have been implicated in HIV transmission from mother to child. When comparing the results obtained from the HIV positive and HIV negative placentae, we found that the expression of DC-SIGN was the same in both HIV negative as well as HIV positive samples. A similar observation was seen for CD68. The Langerhans cells on which CD1a is expressed was not found in the HIV positive or the HIV negative placentae in this study.

CD1a which is a specific marker of Langerhans cells is associated with the presentation of antigens to T cells (Mizumoto and Takashima, 2004). CD1a is important as it mediates antigen presentation by an HLA-independent pathway (Schulke et al., 2008). Studies have looked at cells similar to those on which CD1a are found and have reported a decline in their presence as the pregnancy progressed. As this study used term placenta, this could possibly explain the lack of CD1a positive cells (Qian et al., 2015). Further studies looking at pre-term placenta would be beneficial as it is possible that CD1a positive cells could be involved in the initial infection by HIV.

In a normal pregnancy, of the number of immune cells found in the placental tissue, approximately 70% of them are natural killer cells, 20-25% are macrophages and 1.7% are dendritic cells (Mor, 2007). The importance of dendritic cells and macrophages in pregnancy has been shown through various studies where a decrease in dendritic cell populations have resulted in the lack of trophoblast invasion as well as decidual formation (Abrahams et al., 2004) and where macrophages play a pivotal role in the vascular remodelling within the uterine wall to ensure the appropriate utero-placental circulation (Nagamatsu and Schust, 2010), amongst other functions.

In this study, we have seen that CD68 is present on immune cells in the maternal circulation (Figure 1.3a, c and e) as well as immune cells found within the fetal circulation (Figure 1.3b, d and f). Specialized macrophages of the placenta known as Hofbauer cells have also been seen in HIV negative (Figure 1.3b) as well as HIV positive placental tissue. The lack of difference in the number of CD68 positive cells in HIV infection and their involvement in HIV infection, suggests that these cells provide a reservoir for HIV infection, even where there are depleted T cell subsets in progressed disease. Unlike the orthodox CD4+ T cells which are the primary targets of HIV which decrease as the severity of the disease increases, macrophages are active from the initial placentation process and remain constant throughout the pregnancy till parturition (Abrahams et al., 2004).

As important as macrophages are in various gestational processes, studies have shown that macrophages are able to be infected in vivo with HIV-1, resulting in both viral antigens as well as infectious virus in the culture supernatants (Kesson et al., 1993; McGann et al., 1994). Macrophages have been shown to express various C-type lectins on their cell surface which have the ability to recognize both foreign ligands originating from microbes as well as those originating from endogenous microbes. Once such lectin is the dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin (DC-SIGN); which was first isolated and identified on immature dendritic cells (Nagamatsu and Schust, 2010).

The exploitation of the dendritic cells, on which DC-SIGN was first isolated, by HIV is still unclear but it is said to increase efficiency of T cell infection (Geijtenbeek et al., 2001; Geijtenbeek et al., 2000; Pöhlmann et al., 2001). Studies have stated that DC-SIGN does not function as a receptor for the virus but rather promotes the efficient *in trans* infection of T cells which express CD4 and chemokine receptors by increasing concentration of virus at the dendritic cell surface (Geijtenbeek et al., 2000), while others have reported that the function of DC-SIGN is independent of the co-expression of CD4 and the associated appropriate receptors (Geijtenbeek et al., 2001). This is supported by studies which have shown that the viral gp120 which is used by HIV to enter T cells has a higher affinity for DC-SIGN compared to CD4 (Curtis et al., 1992; Geijtenbeek et al., 2000). This is further confirmed by studies which have shown that blockage of DC-SIGN has resulted in a slightly decreased transfer of HIV to T-cells and ultimately confirming the independent mechanisms of HIV transmission (Granelli-Piperno et al., 2005). Together with this, DC-SIGN is able to capture HIV for more than four days which not only preserves its effectiveness but also provides the time needed to enter lymph node, as transport by dendritic cells only requires two days (Geijtenbeek et al., 2000).

In the light of this, the importance of DC-SIGN cannot be overlooked. In this study, the expression of DC-SIGN was seen on immune cells in both the fetal and maternal circulation and in contrast to other studies (Pillay et al., 2014), also on the syncytiotrophoblast layer of both conducting as well as exchange placental villi.

The evidence provided by various studies regarding DC-SIGN support the theory whereby infection of the placenta takes place prior to the infection of the fetus. Given the greater affinity of gp120 for the DC-SIGN membrane protein (Geijtenbeek et al., 2000), it is plausible to believe that as a result of a greater expression of DC-SIGN on placental villi, a greater concentration of virus and viral particles may be found at these exchange barriers; providing a possible point of entry into the fetal circulation.

The implication of DC-SIGN and CD68 as possible mechanisms of HIV transmission is supported by the results which indicate a continual presence of cells presenting these proteins throughout gestation (Abrahams et al., 2004; Soilleux et al., 2001a). We have seen that the number of cells which express these markers remain unchanged regardless of HIV status. As studies have proven the ease in which these proteins can be exploited, in the absence of orthodox receptors where HIV transmission to the baby has been reported, it is important to assess these proteins to determine the manner in which they successfully perform their immunological role and how this immunological role can be altered by which transmission could possibly result. In this study we have seen that the HIV status of a mother does not alter the number of cells and quantity of expression of CD68 and DC-SIGN in normotensive pregnant women. With DC-SIGN positive Hofbauer cells easily being able to travel from the mother via the umbilical vein to the fetus (Soilleux et al., 2001a), an increase in number of these cells expressing these proteins from placentation through to gestation is of great significance. Such expression provides evidence that HIV could use a cell-mediated pathway to enter the fetal circulation, or at least infect the fetomaternal barrier.

As the participants of this study were on a form of ARV treatment, the effect of the treatment on the results obtained needed to be determined. Although ARV's have greatly reduced HIV transmission, according to previous studies, it is possible that the implementation of ARV treatments such as HAART tend to direct HIV infection towards a cell-associated mechanism as there appears to be a higher efficiency of the vertical transmission of HIV in cases where viral loads remain low. If the function of DC-SIGN is minimal when the viral load is reported low, it is plausible to assume that their efficiency in promoting the vertical transmission of HIV is greatly increased.

CD68 and DC-SIGN play a role in HIV transmission yet their connection to one another is unclear. It is possible that the mechanism by which HIV-1 infects trophoblast cells incorporates the conventional as well as the non-conventional receptors mediated route to ensure a successful infection. Confirmatory tests to isolate markers of HIV infection within these cells would provide valuable insight into their role in transmission and we propose this for further study. Identifying the association of these cells with placentae at varying stages of gestation and the immunological phenotype will elucidate their role not only in transmission of HIV but also their activity in pregnancy.

5. Conflict of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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CHAPTER 3

3.1. Synthesis

The aim of this study was to localize proteins such as DC-SIGN, CD68 and CD1a which have been implicated in HIV transmission from mother to child. Upon comparison, the results obtained from the HIV positive and HIV negative placentae showed that the expression of DC-SIGN was the same in both HIV negative as well as HIV positive samples. A similar observation was seen with regards to CD68 in the placental samples. The Langerhans cells on which CD1a give the idea that they are not present as no expression was noted in the HIV positive or the HIV negative placentae in this study.

CD1a which is a specific marker of Langerhans cells is associated with the presentation of antigens to T cells (Mizumoto and Takashima, 2004). This CD1a protein is of biological importance as it mediates antigen presentation by an HLA-independent pathway (Schulke et al., 2008). Earlier studies have evaluated cells similar to those on which CD1a are found and have reported a decline in their presence as the pregnancy progressed. This study used term placenta which could possibly explain the lack of CD1a positive cells (Qian et al., 2015). Further studies looking at pre-term placenta would be beneficial as it is possible that CD1a positive cells could be involved in the initial infection by HIV after which other cell mediated mechanisms of HIV transmission may come into play.

To support the theory of placental infection, it is important to note that during pregnancy, the apical aspect of the syncytiotrophoblast is in direct contact with infected maternal blood and is therefore exposed to HIV infected cells and cell free virus (Lagaye et al., 2001b). Expression of viral antigen in the chorionic membranes of the placenta has previously been reported in HIV-1 infected pregnant women (Sheikh et al., 2000) but immunolocalisation of HIV in the tissue was not clear (Arias et al., 2003a). As stated by Mognetti et al (2000), infection of the placental cells requires the crossing of HIV-1 via the trophoblast layer into the underlying cells and tissue for further infection. Although there has been *in vitro* studies to determine the ability of cell-free HIV to infect trophoblastic cells, results showed that the trophoblastic cells showed a resistance to the infection (David et al., 1992; Lagaye et al., 2001b). Extrapolating these results to model the in vivo situation compared to the *in vitro* conditions; for example, the cells used in these studies were unpolarised (David et al., 1992; Lagaye et al., 2001b) , could be a contributing factor to the supposed resistance to infection. Studies later on have proved that HIV transmission does enter trophoblast cells via endocytosis, as these cells have the ability to sustain virus production (Vidricaire et al., 2004, 2003).

In the case of a normal pregnancy, of the immune cells found in placental tissue, approximately 70% of them are natural killer cells, 20-25% are macrophages and 1.7% are dendritic cells (Mor, 2007). Immune

cells such as dendritic cells and macrophages which are derived from a common lymphocyte lineage are said to infiltrate the uterine decidua and amass themselves around the invading trophoblast cells (Ashkar et al., 2000). The importance of these cells in pregnancy has been made known through various studies which have shown that a decrease in dendritic cell populations have resulted in the lack of trophoblast invasion as well as decidual formation (Abrahams et al., 2004). With regards to the role of macrophages, amongst their other functions, they have been shown to play a pivotal role in the vascular remodelling within the uterine wall to ensure the appropriate utero-placental circulation (Nagamatsu and Schust, 2010).

This study has presented evidence that CD68 is present on immune cells in the circulation of the mother as well as immune cells found within the fetal circulation. Specialized macrophages of the placenta known as Hofbauer cells have also been seen in HIV negative as well as HIV positive placental tissue. The absence of a significant difference in the number of CD68 positive cells in HIV infection compared to that of HIV negative samples as well as their involvement in HIV infection is suggestive of the fact that these cells could possibly provide a reservoir for HIV infection even where they are depleted T cell subsets in the progressed disease. Unlike the orthodox CD4+ T cells which are the primary targets of HIV, which decrease as the severity of the disease increases, macrophages are active from the initial placentation process and remain continuous throughout the pregnancy till parturition (Abrahams et al., 2004). This seems to remain this way regardless of whether the mother is HIV positive or not.

As important as macrophages are in various gestational processes, studies have shown that macrophages are able to be infected *in vivo* with HIV-1, resulting in both viral antigens as well as infectious virus in the culture supernatants (Kesson et al., 1993; McGann et al., 1994).

Macrophages located in the placental decidua, which have been categorized according to the expression of CD68 and CD14, have been shown to express various C-type lectins on their cell surface. This provides them with the ability to distinguish between foreign ligands originating from microbes as well as those originating from endogenous microbes. Once such lectin is the dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin (DC-SIGN); which was said to be first isolated and identified on immature dendritic cells (Nagamatsu and Schust, 2010).

The DC-SIGN protein is said to be involved in the capture of microorganisms that enter and are found in mucosal tissue and migrate to secondary lymphoid organs where they present antigens of the microorganism to resting T cells, thereby initiating the adaptive immune response (Geijtenbeek et al., 2000). The mechanism exploitation of the dendritic cells, on which DC-SIGN was first isolated, by HIV is still unclear but it has been found to be involved in a greater efficiency of infection of T cells (Geijtenbeek et al., 2001; Geijtenbeek et al., 2000; Pöhlmann et al., 2001).

The mechanism by which dendritic cells, on which DC-SIGN was first isolated, are said to be exploited by HIV is still unclear but it is said to increase efficiency of T cell infection. Previous studies have stated that DC-SIGN does not function as a receptor for the virus but rather supports the efficient *in trans* infection of T cells which express CD4 and chemokine receptors by increasing the concentration of virus at the dendritic cell surface (Geijtenbeek et al., 2000); while others have reported that the function of DC-SIGN is independent of the co-expression of CD4 and the associated appropriate receptors (Geijtenbeek et al., 2001). This theory is supported by studies which have shown that the viral gp120 protein which is used by HIV to enter T cells has a higher affinity for DC-SIGN compared to CD4 (Curtis et al., 1992; Geijtenbeek et al., 2000). This is further confirmed by studies which have shown that by obstructing the access to DC-SIGN, there has been a slight decrease in the transfer of HIV to T-cells and ultimately confirming the independent mechanisms of HIV transmission (Granelli-Piperno et al., 2005). Together with this, DC-SIGN is able to capture HIV for more than four days which not only preserves its effectiveness but also provides the time needed to enter lymph node, as transport of the virus via dendritic cell movement only requires two days (Geijtenbeek et al., 2000).

Given that all participants of this study were on some form of ARV treatment, it is important to establish the possible relationship between the two. Studies have further looked at the correlation between DC-SIGN and its possible relationship with ARV treatment. It has been proposed that a cell-associated transmission mechanism has a higher efficiency in pregnancies where the viral load remains low due to ARV treatments such as HAART, as *in utero* transmission still occurs (Dorenbaum, 2001). Studies have reported DC-SIGN's ability to greatly enhance infection in this instance which is of great importance in vertical transmission (Dorenbaum, 2001). As DC-SIGN has been localized on Hofbauer cells within the placenta, one of the proposed mechanisms by which DC-SIGN achieves this greater efficiency is said to be via the movement of infected Hofbauer cells into the umbilical cord vein which migrates directly into the fetus (Soilleux et al., 2001b). In the light of this, the importance of DC-SIGN cannot be overlooked. In this study, the expression of DC-SIGN was seen on immune cells in both the fetal and maternal circulation and in contrast to other studies (Pillay et al., 2014), also on the syncytiotrophoblast layer of both conducting as well as exchange placental villi.

Various studies have provided evidence regarding DC-SIGN which support the theory whereby infection of the placenta takes place prior to the infection of the fetus. Due to the greater affinity of the gp120 protein for the DC-SIGN membrane protein (Geijtenbeek et al., 2000), it is plausible to believe that as a result of a greater expression of DC-SIGN on placental villi, a greater concentration of virus and viral particles may be found at these exchange barriers; allowing for a possible point of entry into the circulation of the fetus.

3.2. Conclusions

In conclusion, this study demonstrates a similar DC-SIGN and CD68 immunolocalization profile as well as morphometry across HIV negative as well as HIV positive normotensive placentae. Moreover, we report an absence of antigen presenting Langerhans cells in the placenta. This study suggests that there is a possibility of an alternative mechanism of infection due to the correlation between normal immune function of these proteins, specifically DC-SIGN, and the ability of HIV to utilize and exploit these processes to its own advantage. It has been established that DC-SIGN has a higher efficiency attachment of HIV-1 when compared to the orthodox gp120 protein and as both DC-SIGN and CD68 have been positively identified on decidual macrophages and Hofbauer cells, their presence could possibly provide a mechanism by which transmission may take place in the absence of the orthodox CD4+ receptor.

The implication of DC-SIGN and CD68 as possible mechanisms of HIV transmission is supported by the results which indicate a continual presence of cells presenting these proteins throughout gestation (Abrahams et al., 2004; Soilleux et al., 2001b). We have seen that the number of cells which express these markers remain unchanged regardless of HIV status. As studies have proven the ease in which these proteins can be exploited, in the absence of orthodox receptors where HIV transmission to the baby has been reported, it is important to assess these proteins to determine the manner in which they successfully perform their immunological role and how this immunological role can be altered by which transmission could possibly result. In this study we have seen that the HIV status of a mother does not alter the number of cells and quantity of expression of CD68 and DC-SIGN in normotensive pregnant women. With DC-SIGN positive Hofbauer cells possibly being able to travel from the mother via the umbilical vein to the fetus (Soilleux et al., 2001b), an increase in number of these cells expressing these proteins from placentation through to gestation is of great significance. Such expression provides evidence that HIV could use a cell-mediated pathway to enter the fetal circulation, or at least infect the fetomaternal barrier.

As the participants of this study were on a form of ARV treatment, the effect of the treatment on the results obtained needed to be determined. Although ARV's have greatly reduced HIV transmission, according to previous studies, it is possible that the implementation of ARV treatments such as HAART tend to direct HIV infection towards a cell-associated mechanism as there appears to be a higher efficiency of the vertical transmission of HIV in cases where viral loads remain low.
If the function of DC-SIGN is minimal when the viral load is reported low, it is plausible to assume that their efficacy in promoting the vertical transmission of HIV is greatly increased.

In conclusion, we found that the expression of the DC-SIGN and CD68 proteins as markers on immune cells appear to be unaffected by the HIV status of a patient or the administration of ARV treatment.

3.3. Recommendations

The CD68 and DC-SIGN proteins investigated in this study play a role in HIV transmission yet their connection to one another is not clearly understood. It is a possibility that the mechanism by which HIV-1 infects trophoblast cells incorporates both the conventional as well as the non-conventional receptor routes in order for transmission to occur by effectively exploiting these proteins to mediate successful entry. For further studies, confirmatory tests to isolate markers of HIV infection within these cells would provide valuable insight into their role in transmission. By being able to identify the association of these proteins with placentae at varying stages of gestation as well as the immunological phenotype, we will be able to elucidate their role not only in transmission of HIV but also their activity in pregnancy.

APPENDICES

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