REPUBLIQUE DU CAMEROUN
Paix – Travail – Patrie
****
UNIVERSITE DE YAOUNDE I
FACULTE DES SCIENCES
DEPARTEMENT DE BIOCHIMIE
****
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES, TECHNOLOGIE ET GEOSCIENCES
LABORATOIRE D'IMMUNOLOGIE/LABORATOIRE DE PARASITOLOGIE MOLECULAIRE ET DE RECHERCHE SUR LES MALADIES A TRANSMISSION VECTORIELLE, CENTRE DE BIOTECHNOLOGIE

REPUBLIC OF CAMEROUN
Peace – Work – Fatherland
****
UNIVERSITY OF YAOUNDE I
FACULTY OF SCIENCE
DEPARTMENT OF BIOCHEMISTRY
****
POSTGRADUATE SCHOOL OF SCIENCE, TECHNOLOGY AND GEOSCIENCES
IMMUNOLOGY LABORATORY/ MOLECULAR PARASITOLOGY AND DISEASE VECTOR RESEARCH LABORATORY, THE BIOTECHNOLOGY CENTRE

Insulin growth factor axis and angiogenic factors in the intervillous space and, changes in gene expression and pathways in the placenta in HIV and malaria patients

Thesis presented and defended
in partial fulfillment of the requirements for the award of a Doctorate Degree/Ph.D. in Biochemistry

Par : **ESEMU LIVO FORGU**
Master of Science Degree in Biochemistry

Sous la direction de
LEKE FOMBAN GANA Rose
Professor, University of Yaoundé 1
BIGOGA DAIGA Jude
Professor, University of Yaoundé 1

Année Académique : 2019-2020

REPUBLIQUE DU CAMEROUN
Paix—Travail – Patrie
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DEPARTEMENT DE BIOCHIMIE

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P.O. Box 812 Yaounde
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DEPARTMENT OF BIOCHEMISTRY

ATTESTATION DE CORRECTION DE THESE DE DOCTORAT/PhD.

Nous soussignés, **Professeur MOUNDIPA FEWOU Paul**, Président et Membres du jury de Soutenance de la Thèse de Doctorat/Ph.D de **Monsieur ESEMU Livo FORGU**, étudiant au département de Biochimie, **Matricule 09R1315**, Né le 18 Avril 1989 à Ngaoundéré, autorisée par la correspondance **N° 20-788/UYI/VREPDTIC/DAAC/DEPE/SPD/CB-AP** de Monsieur le Recteur de l'Université de Yaoundé 1 en date du 1 Juin 2020.

Attestons qu'après la soutenance publique de sa Thèse de Doctorat/Ph.D en date du 11 Juin 2020 à l'Université de Yaoundé 1, il a effectué toutes les corrections conformément aux remarques et suggestions des membres du Jury.

En foi de quoi la présente attestation lui est délivrée pour servir et valoir ce que de droit.

Yaoundé le 06 JUIL 2020

Présidente de Jury

Paul F. MOUNDIPA
Professor
Enzymology & Toxicology

Membres de Jury

Apinjoh, T

Chef de Département de Biochimie

Moundipa Fewou Paul D3C,D. Etat
Professor
Enzymology-Toxicology

Pr. Wilfred F. Mbacham
MS, DS, ScD (Harvard), FASI, FCAS
Public Health Biotechnologist

UNIVERSITÉ DE YAOUNDÉ I **Faculté des Sciences** Division de la Programmation et du Suivi des Activités Académiques		THE UNIVERSITY OF YAOUNDE I **Faculty of Science** Division of Programming and Follow-up of Academic Affaires
LISTE DES ENSEIGNANTS PERMANENTS		**LIST OF PERMANENT TEACHING STAFF**

PROTOCOL LIST

ANNÉE ACADEMIQUE 2019/2020
(Par Département et par Grade)
DATE D'ACTUALISATION 19 Février 2020

ADMINISTRATION

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VICE-DOYEN / DPSAA : DONGO Etienne, *Professeur*
VICE-DOYEN / DSSE : AJEAGAH Gideon AGHAINDUM, *Professeur*
VICE-DOYEN / DRC : ABOSSOLO Monique, *Maitre de Conférences*
Chef Division Administrative et Financière : NDOYE FOE Marie C. F., *Maitre de Conférences*
Chef Division des Affaires Académiques, de la Scolarité et de la Recherche DAASR : MBAZE MEVA'A Luc Léonard, *Professeur*

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2	FEKAM BOYOM Fabrice	Professeur	En poste
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16	NANA Louise épouse WAKAM	Maître de Conférences	En poste
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18	NGUEFACK Julienne	Maître de Conférences	En poste
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20	MOFOR née TEUGWA Clotilde	Maître de Conférences	Inspecteur de Service MINESUP
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23	BEBOY EDZENGUELE Sara Nathalie	Chargée de Cours	En poste
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30	KOTUE KAPTUE Charles	Chargé de Cours	En poste
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35	Palmer MASUMBE NETONGO	Chargé de Cours	En poste

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37	OWONA AYISSI Vincent Brice	Assistant	En poste
38	WILFRIED ANGIE Abia	Assistante	En poste

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4	DJIETO LORDON Champlain	Professeur	En Poste
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6	FOMENA Abraham	Professeur	En Poste
7	KAMTCHOUING Pierre	Professeur	En poste
8	NJAMEN Dieudonné	Professeur	En poste
9	NJIOKOU Flobert	Professeur	En Poste
10	NOLA Moïse	Professeur	En poste
11	TAN Paul VERNYUY	Professeur	En poste
12	TCHUEM TCHUENTE Louis Albert	Professeur	*Inspecteur de service* *Coord.Progr./MINSANTE*
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29	ETEME ENAMA Serge	Chargé de Cours	En poste
30	GOUNOUE KAMKUMO Raceline	Chargée de Cours	En poste
31	KANDEDA KAVAYE Antoine	Chargé de Cours	En poste
32	LEKEUFACK FOLEFACK Guy B.	Chargé de Cours	En poste
33	MAHOB Raymond Joseph	Chargé de Cours	En poste
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36	MVEYO NDANKEU Yves Patrick	Chargé de Cours	En poste
37	NGOUATEU KENFACK Omer Bébé	Chargé de Cours	En poste
38	NGUEMBOK	Chargé de Cours	En poste
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40	NOAH EWOTI Olive Vivien	Chargée de Cours	En poste
41	TADU Zephyrin	Chargé de Cours	En poste
42	TAMSA ARFAO Antoine	Chargé de Cours	En poste
43	YEDE	Chargé de Cours	En poste

44	BASSOCK BAYIHA Etienne Didier	Assistant	En poste
45	ESSAMA MBIDA Désirée Sandrine	Assistante	En poste
46	KOGA MANG DOBARA	Assistant	En poste
47	LEME BANOCK Lucie	Assistante	En poste
48	YOUNOUSSA LAME	Assistant	En poste

3- DÉPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE VÉGÉTALES (BPV) (32)

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2	BELL Joseph Martin	Professeur	En poste
3	DJOCGOUE Pierre François	Professeur	En poste
4	MOSSEBO Dominique Claude	Professeur	En poste
5	YOUMBI Emmanuel	Professeur	Chef de Département
6	ZAPFACK Louis	Professeur	En poste

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9	KENGNE NOUMSI Ives Magloire	Maître de Conférences	En poste
10	MALA Armand William	Maître de Conférences	En poste
11	MBARGA BINDZI Marie Alain	Maître de Conférences	CT/ MINESUP
12	MBOLO Marie	Maître de Conférences	En poste
13	NDONGO BEKOLO	Maître de Conférences	*CE / MINRESI*
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20	MAHBOU SOMO TOUKAM. Gabriel	Chargé de Cours	En poste
21	NGALLE Hermine BILLE	Chargée de Cours	En poste
22	NGOUO Lucas Vincent	Chargé de Cours	En poste
23	NNANGA MEBENGA Ruth Laure	Chargé de Cours	En poste
24	NOUKEU KOUAKAM Armelle	Chargé de Cours	En poste
25	ONANA JEAN MICHEL	Chargé de Cours	En poste
26	GODSWILL NTSOMBAH NTSEFONG	Assistant	En poste
27	KABELONG BANAHO Louis-Paul-Roger	Assistant	En poste
28	KONO Léon Dieudonné	Assistant	En poste
29	LIBALAH Moses BAKONCK	Assistant	En poste
30	LIKENG-LI-NGUE Benoit C	Assistant	En poste
31	TAEDOUNG Evariste Hermann	Assistant	En poste
32	TEMEGNE NONO Carine	Assistant	En poste

4- DÉPARTEMENT DE CHIMIE INORGANIQUE (CI) (35)

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4	GHOGOMU Paul MINGO	Professeur	*Ministre Chargé deMiss.PR*
5	NANSEU Njiki Charles Péguy	Professeur	En poste
6	NDIFON Peter TEKE	Professeur	*CT MINRESI/Chef de Departement*
7	NGOMO Horace MANGA	Professeur	*Vice Chancelor/UB*
7	NDIKONTAR Maurice KOR	Professeur	*Vice-Doyen Univ. Bamenda*
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9	NGAMENI Emmanuel	Professeur	*DOYEN FS UDs*

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13	KEMMEGNE MBOUGUEM Jean C.	Maître de Conférences	En poste
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15	NDI NSAMI Julius	Maître de Conférences	En poste
17	NJIOMOU C. épse DJANGANG	Maître de Conférences	En poste
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24	KENNE DEDZO GUSTAVE	Chargé de Cours	En poste
25	KOUOTOU DAOUDA	Chargé de Cours	En poste
26	MAKON Thomas Beauregard	Chargé de Cours	En poste
27	MBEY Jean Aime	Chargé de Cours	En poste
28	NCHIMI NONO KATIA	Chargé de Cours	En poste
29	NEBA nee NDOSIRI Bridget NDOYE	Chargée de Cours	CT/ MINFEM
30	NYAMEN Linda Dyorisse	Chargée de Cours	En poste
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32	TCHAKOUTE KOUAMO Hervé	Chargé de Cours	En poste
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34	PATOUOSSA ISSOFA	Assistant	En poste
35	SIEWE Jean Mermoz	Assistant	En Poste

5- DÉPARTEMENT DE CHIMIE ORGANIQUE (CO) (35)			
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4	NKENGFACK Augustin Ephrem	Professeur	Chef de Département
5	NYASSE Barthélemy	Professeur	En poste
6	PEGNYEMB Dieudonné Emmanuel	Professeur	*Directeur/ MINESUP*
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11	FOTSO WABO Ghislain	Maître de Conférences	En poste
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14	KOUAM Jacques	Maître de Conférences	En poste
15	MBAZOA née DJAMA Céline	Maître de Conférences	En poste
16	MKOUNGA Pierre	Maître de Conférences	En poste
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22	TCHOUANKEU Jean-Claude	Maître de Conférences	*Doyen /FS/ UYI*
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28	NGNINTEDO Dominique	Chargé de Cours	En poste
29	NGOMO Orléans	Chargée de Cours	En poste
30	OUAHOUO WACHE Blandine M.	Chargée de Cours	En poste
31	SIELINOU TEDJON Valérie	Chargé de Cours	En poste
32	TAGATSING FOTSING Maurice	Chargé de Cours	En poste
33	ZONDENDEGOUMBA Ernestine	Chargée de Cours	En poste

34	MESSI Angélique Nicolas	Assistant	En poste
35	TSEMEUGNE Joseph	Assistant	En poste

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19	DOMGA KOMGUEM Rodrigue	Assistant	En poste
20	EKODECK Stéphane Gaël Raymond	Assistant	En poste
21	HAMZA Adamou	Assistant	En poste
22	JIOMEKONG AZANZI Fidel	Assistant	En poste
23	MAKEMBE. S . Oswald	Assistant	En poste
24	MESSI NGUELE Thomas	Assistant	En poste
25	MEYEMDOU Nadège Sylvianne	Assistante	En poste
26	NKONDOCK. MI. BAHANACK.N.	Assistant	En poste

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5	MBEHOU Mohamed	Maître de Conférences	En poste
6	TCHAPNDA NJABO Sophonie B.	Maître de Conférences	Directeur/AIMS Rwanda

7	AGHOUKENG JIOFACK Jean Gérard	Chargé de Cours	Chef Cellule MINPLAMAT
8	CHENDJOU Gilbert	Chargé de Cours	En poste
9	DJIADEU NGAHA Michel	Chargé de Cours	En poste
10	DOUANLA YONTA Herman	Chargé de Cours	En poste
11	FOMEKONG Christophe	Chargé de Cours	En poste
12	KIANPI Maurice	Chargé de Cours	En poste
13	KIKI Maxime Armand	Chargé de Cours	En poste
14	MBAKOP Guy Merlin	Chargé de Cours	En poste
15	MBANG Joseph	Chargé de Cours	En poste
16	MBELE BIDIMA Martin Ledoux	Chargé de Cours	En poste
17	MENGUE MENGUE David Joe	Chargé de Cours	En poste
18	NGUEFACK Bernard	Chargé de Cours	En poste
19	NIMPA PEFOUNKEU Romain	Chargée de Cours	En poste
20	POLA DOUNDOU Emmanuel	Chargé de Cours	En poste
21	TAKAM SOH Patrice	Chargé de Cours	En poste
22	TCHANGANG Roger Duclos	Chargé de Cours	En poste
23	TCHOUNDJA Edgar Landry	Chargé de Cours	En poste
24	TETSADJIO TCHILEPECK M. E.	Chargée de Cours	En poste
25	TIAYA TSAGUE N. Anne-Marie	Chargée de Cours	En poste
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27	BITYE MVONDO Esther Claudine	Assistante	En poste
28	MBATAKOU Salomon Joseph	Assistant	En poste

29	MEFENZA NOUNTU Thiery	Assistant	En poste
30	TCHEUTIA Daniel Duviol	Assistant	En poste

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9	BOUGNOM Blaise Pascal	Chargé de Cours	En poste
10	ESSONO OBOUGOU Germain G.	Chargé de Cours	En poste
11	NJIKI BIKOÏ Jacky	Chargée de Cours	En poste
12	TCHIKOUA Roger	Chargé de Cours	En poste
13	ESSONO Damien Marie	Assistante	En poste
14	LAMYE Glory MOH	Assistant	En poste
15	MEYIN A EBONG Solange	Assistante	En poste
16	NKOUDOU ZE Nardis	Assistant	En poste
17	SAKE NGANE Carole Stéphanie	Assistante	En poste
18	TOBOLBAÏ Richard	Assistant	En poste

9. DEPARTEMENT DE PYSIQUE(PHY) (42)

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4	NANA ENGO Serge Guy	Professeur	En poste
5	NDJAKA Jean Marie Bienvenu	Professeur	Chef de Département
6	NOUAYOU Robert	Professeur	En poste

7	NJANDJOCK NOUCK Philippe	Professeur	*Sous Directeur/ MINRESI*
8	PEMHA Elkana	Professeur	En poste
9	TABOD Charles TABOD	Professeur	Doyen Univ/Bda
10	TCHAWOUA Clément	Professeur	En poste
11	WOAFO Paul	Professeur	En poste

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13	BODO Bertrand	Maître de Conférences	En poste
14	DJUIDJE KENMOE épouse ALOYEM	Maître de Conférences	En poste
15	EKOBENA FOUDA Henri Paul	Maître de Conférences	*Chef Division. UN*
16	EYEBE FOUDA Jean sire	Maître de Conférences	En poste
17	FEWO Serge Ibraïd	Maître de Conférences	En poste
18	HONA Jacques	Maître de Conférences	En poste
19	MBANE BIOUELE César	Maître de Conférences	En poste
20	NANA NBENDJO Blaise	Maître de Conférences	En poste
21	NDOP Joseph	Maître de Conférences	En poste
22	SAIDOU	Maître de Conférences	MINERESI
23	SIEWE SIEWE Martin	Maître de Conférences	En poste
24	SIMO Elie	Maître de Conférences	En poste
25	VONDOU Derbetini Appolinaire	Maître de Conférences	En poste
26	WAKATA née BEYA Annie	Maître de Conférences	*Sous Directeur/ MINESUP*
27	ZEKENG Serge Sylvain	Maître de Conférences	En poste

28	ABDOURAHIMI	Chargé de Cours	En poste
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NOMBRE D'ENSEIGNANTS					
DÉPARTEMENT	**Professeurs**	**Maîtres de Conférences**	**Chargés de Cours**	**Assistants**	**Total**
BCH	9 (1)	13 (09)	14 (05)	3 (2)	**39 (16)**
BPA	13 (1)	09 (06)	19 (05)	05 (2)	**46 (13)**
BPV	06 (0)	10 (02)	9 (04)	07 (01)	**31 (9)**
CI	10 (1)	09 (02)	13 (02)	02 (0)	**35 (5)**
CO	7 (0)	17 (04)	09 (03)	03 (0)	**35(7)**
IN	2 (0)	1 (0)	14 (01)	10 (02)	**26 (3)**
MAT	1 (0)	5 (0)	19 (01)	05 (01)	**30 (2)**
MIB	1 (0)	5 (02)	06 (01)	06 (02)	**17 (5)**
PHY	11 (0)	16 (01)	10 (03)	03 (0)	**40 (4)**
ST	8 (1)	14 (01)	19 (04)	02 (0)	**43(6)**
Total	**68 (4)**	**99 (27)**	**132 (29)**	**45 (10)**	**344 (70)**

Soit un total de **344 (**70**)** dont :

- Professeurs **68 (4)**
- Maîtres de Conférences **99 (**27**)**
- Chargés de Cours **132 (29)**
- Assistants **46** (10**)**

( ) = Nombre de Femmes **45**

DEDICATION

This piece of work is dedicated to:

The LORD ALMIGHTY

and

The ESEMU FAMILY

ACKNOWLEDGMENTS

I thank the Almighty God for the gift of life, for the courage, strength, and favour given me to carry out this piece of work and also for His continuous blessings on me and all those who support me.

I would equally like to acknowledge and extend my heartfelt gratitude to the following persons who made the completion of this work possible:

My supervisors, **Prof. Rose GANA FOMBAN LEKE** and **Prof. Jude DAIGA BIGOGA** who invested time, knowledge, energy and resources despite their tight schedules to thoroughly supervise this project and trained me in the ways of science. They are beyond supervisors; they are respectable scientists and mentors towards who I have profound admiration and respect.

Prof. Jean Claude MBANYA, The Director of the Biotechnology Center, who keeps the Center informed of current fields in research by organizing seminars, that contributed significantly towards the molding of this dissertation.

All lecturers of the Department of Biochemistry, Faculty of Science of the University of Yaoundé I for the academic training I received from them.

Pregnant women who kindly accepted to enroll into our study and contribute towards the progress of science and healthcare.

The entire team of co-workers who supported me throughout all the phases of my thesis: **Mr. TAMO John, Dr. FODJO Barriere, Mr. YUOSEMBOME Emile, Dr. DJONTU Jean Claude, Dr. Gabriel EKALI LONI,**

Special thanks go to **Dr. Anna BABAKHANYAN** who provided seeding funds for the initiation of this study

My gratitude is extended towards my mentors **Prof. Diane TAYLOR** and **Prof. Lishomwa NDHLOVU** of the University of Hawaii, as well as the Department of Tropical medicine of the University of Hawaii headed by **Prof. Vivek NERURKAR** who provided all the expertise and the platform for Molecular Biology assays.

I appreciate the effort of our Laboratory technicians, **Mrs. SAMA Grace, Mrs. NYONGLEMA Philomina** and **Miss. Canicia ENOW** for watching over the quality of the experiments we conduct in the laboratory.

My laboratory seniors: **Dr. Gabriel EKALI LONI**, **Dr. FODJO Barriere**, **Mr. SEUMEN Clovis and Dr. TABUE Raymond** who all proposed new ideas and perspectives.

My laboratory mates: **Dr. ZEUKENG Francis, Mr. NJEAMBOSAY Boris, Mr. BEKINDAKA Obase, Mr. AWANAKAM Honore, Miss. Clavella NANTCHO, Mr. YUOSEMBOME Emile, Mrs. NANA Christine** and all members of the laboratory**.** You all contributed towards refining the quality of this piece of work, kindly accept my thanks.

The **ESEMU FAMILY** especially **Mr. ESEMU Ivo, Mrs. ESEMU NKIE, Mr. ESEMU Tasivo, Mrs. ESEMU Ivota** and **Mr. ESEMU Ferivo.** You are God-sent individuals and words will water down the sacrifices and contributions you made not only towards the achievement of this piece of work, but also to what I am. God bless you.

The Household of Faith that kept me spiritually and morally upright for this work.

My dear friends: **Dr. Camille MBA**, **Dr. Yukie LLOYD, Dr. BESONG Michael, Mr. SONELA Nelson, Mr. KAMOGNE Cedric, Mr. CHEBE Anthony, Mrs. MANJIA Jacqueline, Dr. KENJI Obadiah**, **Dr. Abanda NGU**, **Dr. NLENDE MENGALLE** and **Dr. DERMATA Claudia** and those whose names I did not mention, whose friendship and warmth made me surge ahead.

My colleagues at the Institute of Medical Research and Medicinal Plant Studies with special emphasis to those of the "Laboratoire de Biologie Humaine" and "CREMER" who refined the quality of this write up through their critical remarks.

All my near and distant relatives who supported me at critical moments that enabled my work with greater ease during these years.

And finally, to my batch mates and all whose names are not mentioned, I sincerely thank you for your respective contributions towards my training and the realization of this piece of work. May the Almighty God bless you abundantly.

FUNDING

This study was supported by funding obtained from the Northern Pacific Global Health Fellowship issued to Dr Anna Babakhanyan from the University of Hawaii(Grant #R25 TW009345); The HIV research Trust issued to Esemu Livo Forgu of the University of Yaounde I; and Esemu Livo Forgu was also supported by the National Institutes of Health Training Grant #D43TW009074 to train young Cameroonian Scientists.

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ABSTRACT

In sub-Saharan Africa, women disproportionately bear the burden of HIV and malaria endemics. Maternal HIV-1 and malaria infections increase the risk of pre-term birth (<37 weeks of gestation), small-for-gestational-age babies, and fetal intrauterine growth restriction, resulting in low birth weight (LBW) infants (<2500g). However, the mechanisms underlying the dysregulation of fetal growth in these infected pregnant women remain poorly understood. The Insulin-like Growth Factor (IGF) axis and angiogenic factors have been shown to be essential for fetal anchoring and growth. However, their implication in HIV-1 and malaria-associated low birth weights have not been extensively investigated. Moreover, the impact of HIV-1 and malaria on the placenta has not been extensively studied. Here, we sought to investigate the impact of HIV-1 infection and malaria on (i)biomarkers important for fetal growth and (ii) genome-wide expressions and pathways in the placenta of pregnant Cameroonian women at delivery. Overall, 102 mother-neonate pairs were recruited in the study at delivery. A panel of biomarkers including IGF binding proteins (IGFBP1, IGFBP3), angiopoietins (ANG) 1 and 2, matrix metalloproteinases (MMP) 2 and 9, and galectin 13, was measured in plasma of blood collected from the placental intervillous space using the Luminex assay. ELISA was used to measure Insulin-like growth factor (IGF)1 and galectin 13. The impact of HIV-1 infection and malaria on genes expression and pathways in the placenta was assessed using microarray on selected placenta biopsies excised from 9 HIV-1-positive, 5 malaria positive and 8 HIV/malaria negative women. The levels of IGF1, IGFBP1, ANG1, ANG2, MMP2, MMP9, and Gal-13 were not affected by maternal HIV-1, even when adjusted for maternal factors in a linear regression model (all $p>0.05$). A total of 292 genes were mapped as dysregulated by HIV-1. Among these, 166 were upregulated and 126 were downregulated. The dysregulated genes overlapped with 36 canonical pathways amongst which 3 were flagged as activated. These pathways were associated with localized immune activation. Overall, 372 genes were differentially expressed in the placenta of malaria positive women. These genes overlapped with 100 Canonical pathways amongst which 14 were flagged as activated by malaria whereas 4 were identified as inhibited. All the flagged pathways were associated with biological functions important for fetal growth. Our findings portray that HIV-1 and malaria disrupt the homeostasis in the placenta and IVS by affecting a number of molecules and pathways important for pregnancy.

Keywords: HIV-1, Malaria, Placenta, biomarkers, regulated pathway

RÉSUMÉ

En Afrique subsaharienne, les femmes supportent de manière disproportionnée le fardeau des endémies de VIH et de paludisme. Les infections maternelles à VIH-1 et le paludisme favorisent le risque d'accouchement prématuré (<37 semaines) avec une réduction de la taille des bébés pour l'âge gestationnel et une limitation de la croissance fœtale intra-utérine ; ce qui favorise l'obtention des nourrissons de faible poids à la naissance (<2500 g). Cependant, les mécanismes favorisant la dérégulation de la croissance fœtale chez ces femmes enceintes infectées restent peu investigués. Les facteurs de croissance de type insuline (IGF) et les facteurs angiogéniques se sont avérés essentiels pour l'ancrage et la croissance du fœtus. Cependant, leur implication dans l'insuffisance pondérale à la naissance pendant les infections à VIH-1 et le paludisme reste également peu investiguée. De plus, l'impact du VIH-1 et du paludisme sur le placenta n'a pas fait l'objet d'études approfondies. Nous avons ainsi investigué l'impact du VIH-1 et du paludisme sur (i) les biomarqueurs importants pour la croissance du fœtus et (ii) les voies d'expression génique dans le placenta des femmes camerounaises enceintes, lors de l'accouchement. Au total, 102 couples mère-nouveau-nés ont été recrutés pendant l'accouchement. Un ensemble de biomarqueurs dont les protéines de liaison à l'IGF (IGFBP1, IGFBP3), les angiopoïétines (ANG) 1 et 2, les métalloprotéinases matricielles (MMP) 2 et 9 et la galectine 13, a été mesuré dans le plasma sanguin prélevé dans l'espace placentaire par Luminex. La méthode ELISA a été utilisée pour quantifier le facteur de croissance de type insuline (IGF1) et la galectine 13. L'impact du VIH-1 et du paludisme sur l'expression des gènes et leurs voies d'expression dans le placenta a été évalué par mircoarray sur les biopsies de placenta extraites de 9 femmes séropositives à VIH-1, 5 femmes atteintes du paludisme et 8 femmes infectées par le VIH+paludisme. Les taux sanguins des biomarkeurs IGF1, IGFBP1, ANG1, ANG2, MMP2, MMP9 et Gal-13 n'étaient pas affectés par le VIH-1 maternel, même après ajustement avec les facteurs maternels dans un modèle de régression linéaire ($p > 0,05$). Un total de 292 gènes dérégulés par le VIH-1ont été cartographiés dont 166 gènes surexprimés et 126 gènes sousexprimés. Les gènes dysrégulés se chevauchaient avec 36 voies canoniques dont 3 voies activées. Ces voies étaient associées à une activation immunitaire localisée. Au total, 372 gènes étaient différentiellement exprimés dans le placenta des femmes atteintes du paludisme. Ces gènes se chevauchent avec 100 voies canoniques dont 14 voies activées par le paludisme et 4 voies inhibées. Toutes les voies d'expression génique affectées étaient associées à des fonctions biologiques importantes pour la croissance fœtale. Nos résultats démontrent que le VIH-1 et le paludisme perturbent l'homéostasie du placenta et du système IVS avec des conséquences sur les molécules et voies importantes pour la grossesse.

Mots-clés : VIH-1, paludisme, placenta, biomarkeurs, voie de régulation,

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ANG	Angiopoietin
APC	Antigen Presenting Cells
aRNA	Antisense Ribonucleic Acid
ART	Antiretroviral therapy
AZT	Zidovudine
BM	Basal membrane
BSA	Bovine Serum Albumin
BTC	Biotechnology Center
cART	Combination Antiretroviral therapy
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic acid
CIRCB	Chantal Biya International Reference Centre
CXCL	Chemokine
DEG	Differentially Expressed Genes
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
EDTA	Ethylene-Diamine Tetra-Acetate
ELISA	Enzyme-Linked Immunosorbent Assay
EVT	Extravillous Trophoblast
FGR	Fetal Growth Restriction
Gal 13	Galectin 13
Gal-13	Galectin 13
Hb	Hemoglobin
HIV	Human Immunodeficiency Virus
HRP	Histidine-Rich Protein
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IL	Interleukin
INT	Integrase
IPA	Ingenuity Pathway Analyses
iRBC	Infected Red Blood Cell
IUGR	Intrauterine Growth Restriction
IV	Intravenous
IVS	Intervillous Space
LBW	Low Birth Weight
MAP	Mitogen-activated protein
MFI	Median Fluorescence Intensity

MIP	Malaria in Pregnancy
MMP 2	Matrix Metalloproteinases 2
MMP 9	Matrix Metalloproteinases 2
mRNA	Messenger Ribonucleic Acid
MTCT	Mother-to-child-transmission
MVM	Microvillous Membrane
NIH	National Institutes of Health
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
PfEMP	Plasmodium falciparum Erythrocyte Membrane Protein
pLDH	Plasmodium Lactate Dehydrogenase
PlGF	Placental Growth Factor
PM	Placental Malaria
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
RNA	Ribonucleic Acid
SD	Standard Deviation
SGA	Small for Gestational Age
ST	Syncytiotrophoblast
TE	Tris Ethylene Diamine Tetra Acetate
TIE	Tyrosine kinase with immunoglobin and epidermal growth factor homology domains
TIMP	Tissue Inhibitors of Metalloproteinases
TNF	Tumor necrosis factor
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America
Var2CSA	Var2 Chondroitin Sulphate A
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell
WHO	World Health Organization

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Introduction

In sub-Saharan Africa, women disproportionately bear the burden of the HIV. Each year, 1.4 million HIV-infected women become pregnant, with sub-Saharan African countries bearing the heaviest burden(UNAIDS, 2014). In Cameroon, the national HIV prevalence in 2011 was 5.6% in women and 2.9% in men, but the prevalence of HIV among pregnant women was 7.8%. Maternal HIV-1 infection increases the risk of pre-term birth (<37 weeks of gestation), small-for-gestational age babies, and fetal intrauterine growth restriction, resulting in low birth weight (LBW) infants (<2500g) (Dreyfuss et al., 2001; Lambert et al., 2000; Turner et al., 2000). Although mother-to-child transmission of HIV has dramatically declined, the number of *in utero* HIV-exposed, uninfected infants is on the rise. HIV-exposed infants are at an increased risk of mortality, morbidity and slower early growth than their non-HIV exposed counterparts. Maternal HIV increases the risk of having preterm deliveries, intrauterine growth retardation and low birth weight babies (López et al., 2015; Patel et al., 2010). However, the mechanisms underlying the dysregulation of fetal growth in HIV-infected pregnant women remain poorly understood. The Insulin-like growth factor (IGF) axis and angiogenic factors have been shown to be essential for fetal anchoring and growth. However, their implication in HIV-1- associated low birth weight has not been investigated.

The maternal–fetus interface in HIV-1 infected women may present an altered environment for fetal growth and development. A major consequence of this alteration is an increase in the morbidity and mortality rates in these infants during their postnatal life (Abu-Raya et al., 2016a, 2016b; Adhikari et al., 2016; Bunders et al., 2010). In spite of these consequences, the causes and mechanism(s) underlying these conditions are not well understood. The placenta plays a key role in fetal development, and a healthy placenta increases the chances of the fetus to be healthy later on in childhood(Krishna and Bhalerao, 2011). HIV-1 infection in pregnant women has been associated with inflamed placentas. However, the extent to which HIV affects the placenta is not well understood. Moreover, the advent of Antiretroviral Therapy (ART) has provided a new sphere of unknowns, including a complex interaction between the virus and the drug in the micro-environment of the placenta. We hypothesized that infection with HIV-1 alters the functioning of the placenta, thus impeding the overall development of the fetus.

Akin to HIV infection, an estimated 219 million cases of malaria occurred worldwide in 2017, causing approximately 435 000 deaths of which 93% occurred in sub-Saharan Africa (World Health Organization, 2018). Children under 5 years of age accounted for 38% of malaria associated death(Sakwe et al., 2019)s. Cameroon reported in 2018, 23.9% cases of malaria and 14.6% associated deaths(Ekoyol, 2019.). Pregnancy-associated malaria (PAM) was detected among 22.3% of pregnant women. Also, (PAM) remains amongst the leading causes of adverse pregnancy outcomes in malaria-endemic countries. More specifically, *Plasmodium falciparum* malaria during pregnancy is associated with an increased risk of maternal anemia, low birth weight (LBW) babies, and premature deliveries (Kidima, 2015). The placenta modulates several placenta functions; but, the extent to which PAM affects these functions is not fully understood. Studies on the response of the placenta to malaria have mostly been done *in vitro* and, several response pathways have been reported to be affected by PAM(Kidima, 2015).

Rationale

HIV and malaria infections during pregnancy are associated with devastating consequences to both the mother and newborn. In spite of this, the role of the placenta and its microenvironment in the progress of these diseases has not been extensively studied. Moreover, the changes that occur in the structure and function of the placenta is even less known. Understanding the pathophysiology of these infections during pregnancy and specifically on the placenta would shed more light on the role the placenta in disease progress. This would also enable the identification key targets that would either be used as biomarkers t detect adverse conditions either specific to the placenta or pregnancy as whole, and/or to identify potential drug targets that would remedy consequences of exposure to these diseases.

Hypothesis

Our null hypotheses are:

- HIV-1 does not alter the IGF-1 axis and angiogenic factors important for fetal growth
- Gene expressions and pathways in the placenta are not affected by HIV-1 infection.
- Malaria has no effect on the gene expression and pathway in the placenta

General Objective

To investigate the effect of HIV-1 infection and malaria on (i)biomarkers important for fetal growth and (ii) genome-wide expressions and pathways in the placenta of pregnant Cameroonian women at delivery.

Specific objectives

1. To investigate the effects of maternal HIV-1 on insulin-like growth factor (IGF) axis, some angiogenic factors and related biomarkers implicated in the regulation of fetal growth.
2. To investigate using a genome-wide study, changes in the gene expression and pathways, in the placenta of HIV-1 infected pregnant women, under ART at delivery.
3. To determine the extent to which gene expression and pathways are altered in placentas of women with placental malaria at delivery.

CHAPTER I: LITERATURE REVIEW

CHAPTER I: LITERATURE REVIEW

1.1. Overview of HIV Infection

1.1.1. Definition, Burden and Global Distribution of HIV/AIDS

The Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) is a spectrum of conditions caused by the Human immunodeficiency virus (Blut, 2016). The HIV virus belongs to the family of viruses called retroviruses which are RNA viruses but are capable of converting their genomes into the DNA form. The major existing types of HIV are; HIV-1 which is most common throughout the world and HIV-2 which is mostly confined to Sub-Saharan Africa. There are four subtypes of HIV-1 called groups M, N, O, and P. The most dominant HIV group worldwide is globe is group M which is composed of subtypes A-J. The structure of the virus has been well described by literature. It consists of a bar-shaped electron dense core that harbors the viral genome, two short strands of ribonucleic acid (RNA) of about 9200 nucleotides, along with the enzymes reverse transcriptase, protease, ribonuclease, and integrase, all encased in an outer lipid envelope synthesized from the host cell. This envelope has 72 surface projections, containing an antigen, gp120 that aids in the binding of the virus to the target cells with CD4 receptors. A second glycoprotein, gp41, binds gp120 to the lipid envelope as shown on Figure 1 (Blut, 2016; "HIV strains and types," 2015; Maartens et al., 2014).

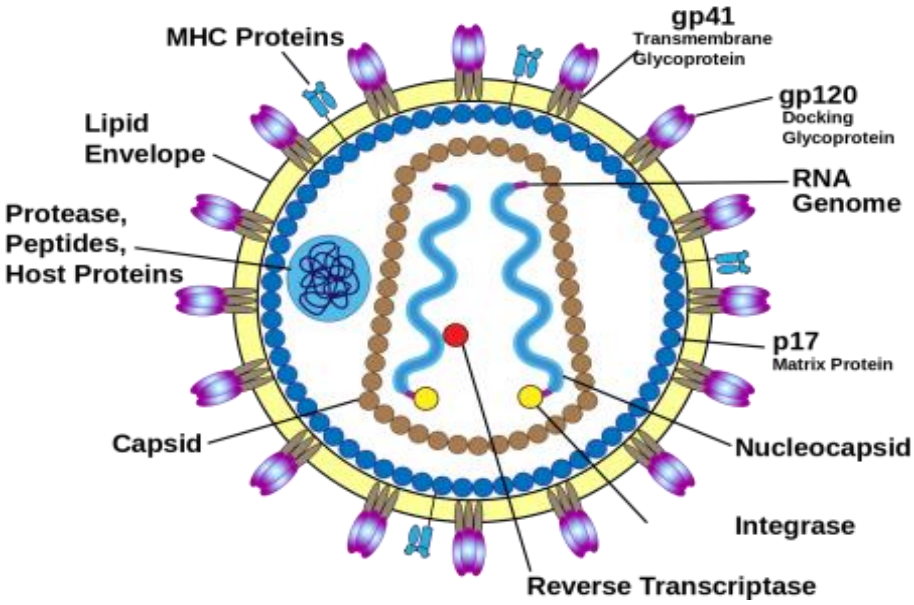

Figure 1: Diagrammatic representation of the HIV

Source:(Toppr, 2019)

Since the start of the epidemic in 1981, about 78 million people have become infected with HIV and 39 million have died of AIDS-related illnesses. In 2018, an estimated 37.9 million people were living with HIV with the vast majority living in low- and middle-income countries, with about 24.5 million people had access to antiretrovirals. HIV was responsible for 770 000 deaths in 2018 (Global HIV & AIDS statistics, 2019). Approximately, 25.5 million sub-Saharan Africans were identified to live with the virus in 2015 (UNAIDS, 2016.). In sub-Saharan Africa, women disproportionately bear the burden of HIV epidemic(*The Gap Report.*, 2014). Each year, 1.4 million HIV-infected women become pregnant(*The Gap Report.*, 2014); in many African countries, up to 15% of pregnant women are HIV positive (World Health Organization, 2009). In Cameroon, national HIV prevalence is 5.6% in women and 2.9% in men; prevalence of HIV among pregnant women is 7.8 % (*Demographic and Health survey and Multiple Indicators Cluster Survey DHS-MICS 2011*, 2012; PEPFAR, 2014).

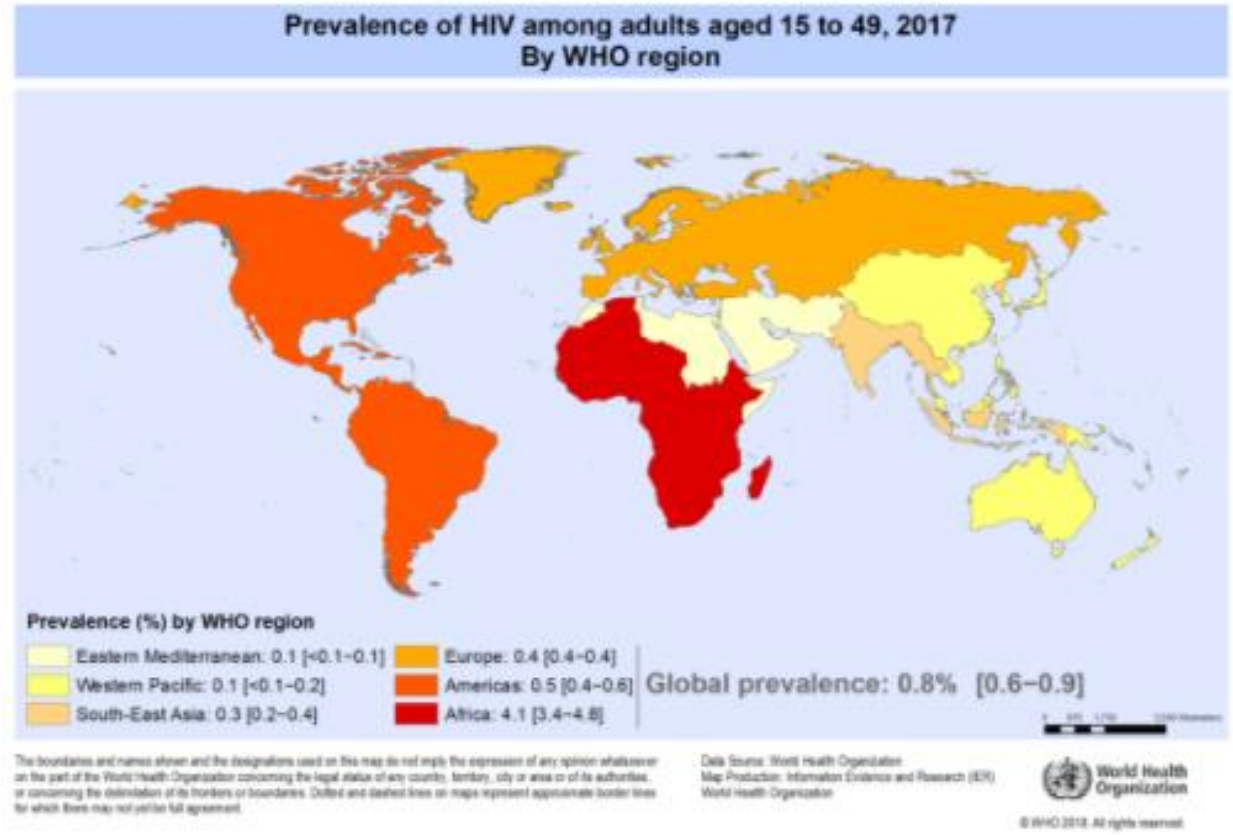

Figure 2: Prevalence of HIV among adults aged 15 to 49, 2017 by WHO Region
Source: (UNAIDS, 2017)

Although, mother-to-child transmission of HIV has dramatically declined, the number of in *utero* HIV-exposed uninfected infants is on the rise (Abu-Raya et al., 2016a). Figure 2 depicts the global distribution of HIV by region.

1.1.2. The Replication Cycle of HIV

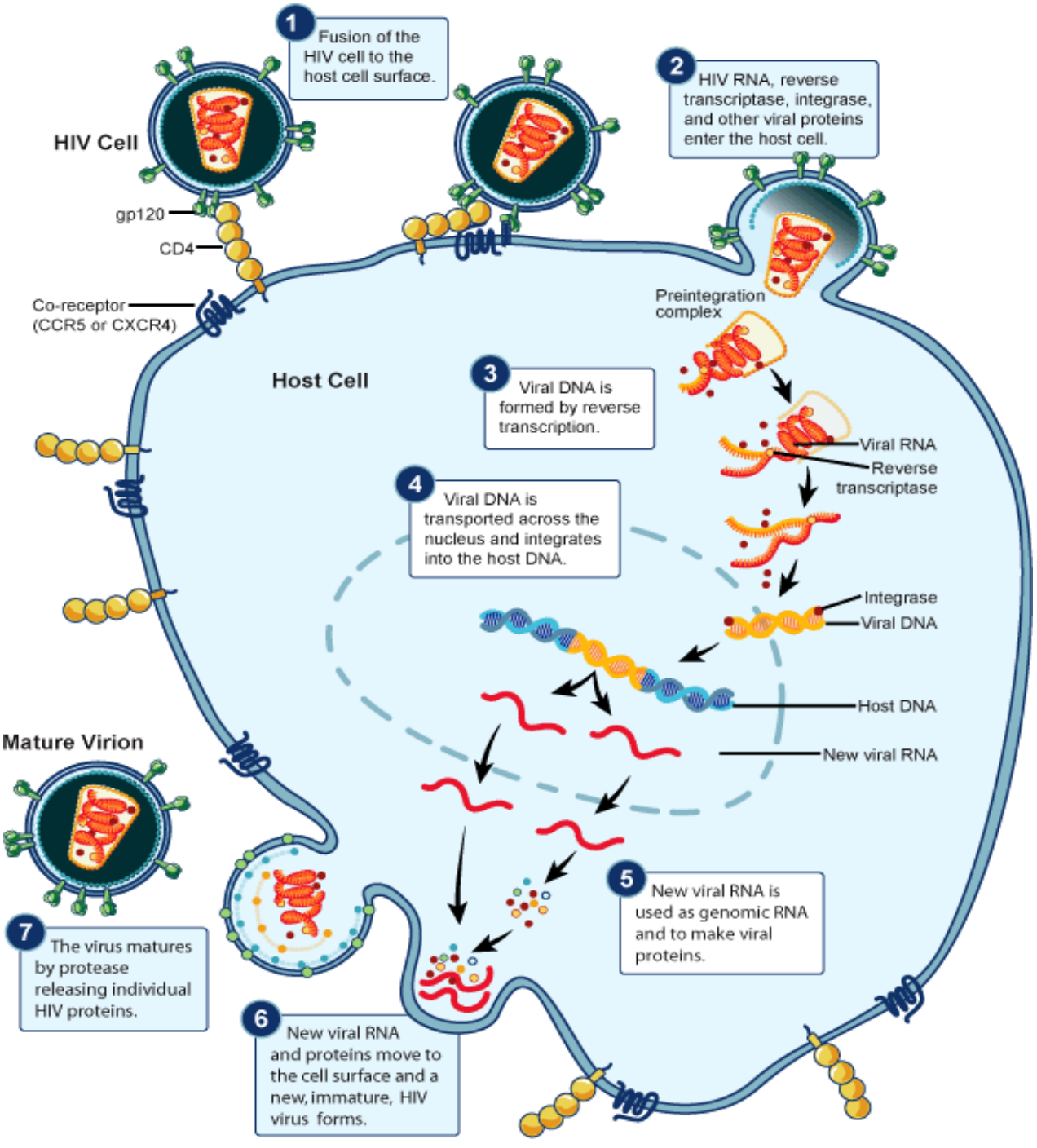

Figure 3: The Life Cycle of HIV

Source:(NIAID, 2018)

As depicted on Figure 3 above, HIV begins its life cycle when it binds to a CD4 receptor and one of two co-receptors on the surface of a CD4+ T- lymphocyte. The virus then fuses with the host cell. After fusion, the virus releases RNA. its genetic material, into the host cell. An enzyme of the virus called reverse transcriptase converts the single-stranded HIV RNA to double-stranded DNA and the newly formed DNA enters the host cell's nucleus, where an enzyme called integrase "hides" the viral DNA within the host cell's own DNA. The integrated viral DNA is called provirus. When the host cell receives a signal to become active, the provirus uses a host enzyme called RNA polymerase to create copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of viral proteins. In fact, an enzyme of the virus called protease cuts the long chains of viral proteins into smaller individual proteins. As the smaller HIV proteins come together with copies of RNA genetic material, a new virus particle is assembled. The newly assembled virus pushes out from the host cell. During budding, the new virus takes up part of the cell's outer envelope. This envelope, which acts as a covering, is studded with protein/sugar combinations called HIV glycoproteins. These viral glycoproteins are necessary for the virus to bind CD4 and co- receptors. The new copies of HIV can now move on to infect other cells (Blut, 2016).

1.1.3. Natural Course HIV Infection and AIDS

The pathogenesis of HIV infection is a function of the virus life cycle, host cellular environment, and quantity of the virus in the infected individual. After entering the body, the viral particle is attracted to a cell with the appropriate CD4 receptor molecules where it attaches by fusion to a susceptible cell membrane or by endocytosis and then enters the cell. The probability of infection is a function of both the number of infective HIV virions in the body fluid which contacts the host as well as the number of cells available at the site of contact that have appropriate CD4 receptors (Maartens et al., 2014).

The typical course of HIV infection can be described in three phases: primary infection, clinical latency and AIDs as shown on Figure 4.

1. Primary infection (1 to 3 months): After infection, there is in general a first peak in HIV RNA copies and a steep decline in CD4 cells in the blood. These changes can be explained by the fact that during the early days, HIV can replicate without being controlled by the immune system. When the body's anti-HIV immune response begins (antibody responses begin to develop 4 to 8 weeks after infection), symptoms of seroconversion may develop and viral load falls.

2. Clinical latency (on average 8-10 years): After the acute infection phase, CD4 cell concentration in the peripheral blood increases again, although not as high as before infection. HIV RNA copy number in the plasma declines again, and the stabilized plasma concentration after the peak of the primary infection is called the viral set-point.

3. AIDS (on average 2-3 years after clinical latency): The third phase is characterized by a rapid increase in HIV RNA copies and a decline in CD4 cell counts in peripheral blood (Maartens et al., 2014).

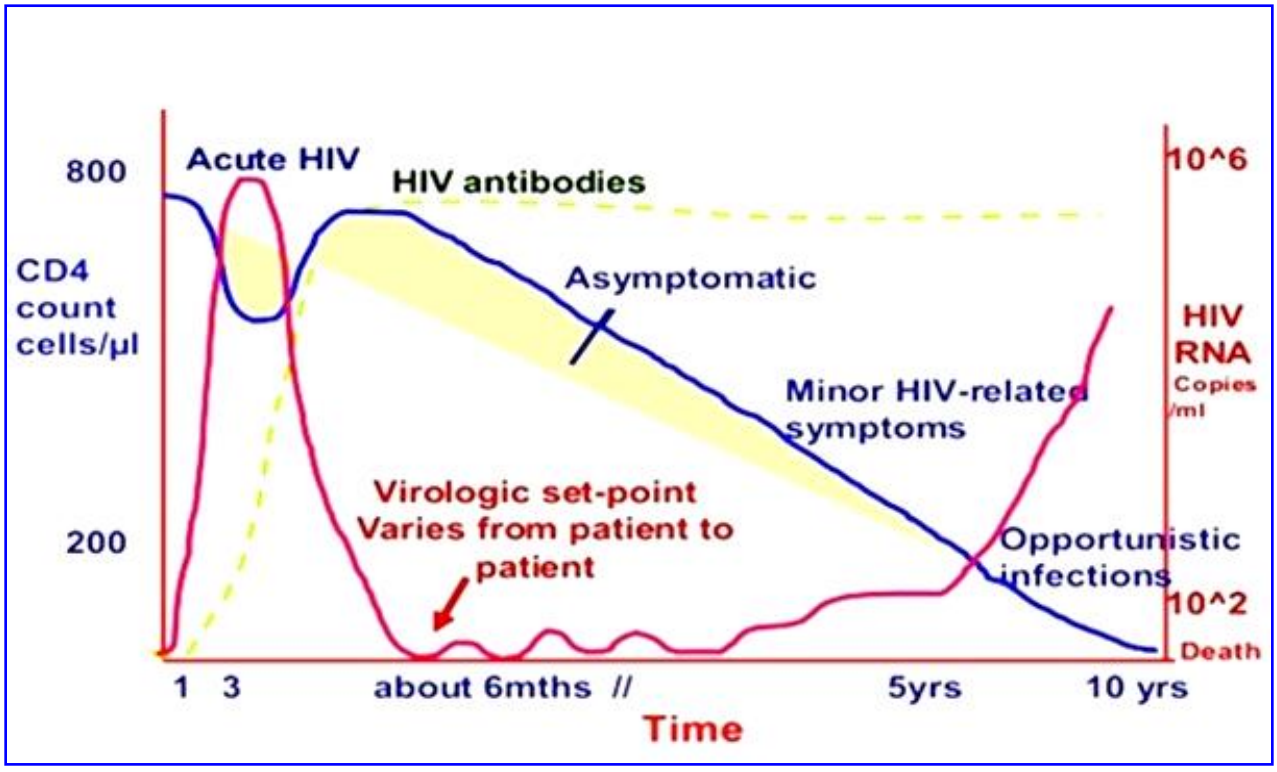

Figure 4: The Natural History of HIV infection

Source:(*Shruthi, 2013)*

1.1.4. Diagnosis, Treatment and Prevention of HIV/AIDS

There are multiple HIV testing methods available. The most commonly used HIV tests are serologic methods based upon detection of antibody to HIV in blood or body fluids, while the p24 assay detects HIV antigen. The PCR (polymerase chain reaction) and *in situ* hybridization techniques are used primarily with fresh and fixed tissue samples but can also be applied to blood samples. HIV viral culture can be performed on both fluids and tissues. Immunologic alterations detected through lymphocyte subset quantification in blood are used clinically to detect and follow the effect of HIV infection on the immune system. Table I below depicts a list of tests routinely used in the diagnosis of HIV. Some tests are for diagnostic purposes, while others are supplemental in monitoring disease progression, such as CD4 and viral load (Fearon, 2005).

Table I: Tests used in the diagnosis and monitoring of HIV treatment

	PURPOSE OF TEST	TYPE OF TEST
1.	HIV diagnosis (Antibody/Antigen testing)	Enzyme Immunoassays
		Rapid tests
		Western blot
2.	Early diagnosis in infants	p24
		DNA/RNA PCR
3.	Initiation and monitoring of ART	Viral Load
		CD4 Count

A variety of therapies have been developed since 1984 for persons infected with HIV. Antiretroviral therapies are aimed at diminishing HIV replication and eventually preventing subsequent destruction of the immune system with progression to AIDS. To date there is no cure for HIV/AIDs and no vaccine against HIV has been developed. A variety of pharmacologic agents have been developed to treat HIV infection. None of these agents can completely eliminate HIV from infected persons. For pregnant women, anti-HIV medications are used at the following times to reduce the risk of mother-to-child transmission of HIV (Maartens et al., 2014).

– During pregnancy, pregnant women infected with HIV receive a regimen (combination) of at least three different anti-HIV medications.

– During labor and delivery, pregnant women infected with HIV receive intravenous (IV) AZT and continue to take the medications in their regimens by mouth.

– After birth, babies born to women infected with HIV receive liquid AZT for 6 weeks. Babies of mothers who did not receive anti-HIV medications during pregnancy may be given other anti-HIV medications in addition to AZT (Siemieniuk et al., 2017).

Prevention of HIV is based on reducing the risks of transmitting the virus from one infected person to an unaffected person and the reduction of mother-child transmission of HIV. Methods to reduce rates of HIV include;

- Reduce levels of poverty in society that lead to increased risks through drug abuse and promiscuity

- Provide HIV testing and counseling to identify infected persons who can reduce their risk to others, provide educational programs for children and adults which describe how to avoid sexually transmitted diseases

- Promote sexual barrier precautions among high-risk commercial sex workers and clients
- Create health care programs with ongoing support to provide antiretroviral therapy to extend their lives and reduce HIV transmission rates
- Give HIV-infected pregnant women antiretroviral therapy to reduce prenatal HIV transmission.

1.1.5. The immune-pathophysiology of HIV-1 infection during pregnancy

During pregnancy immune homeostasis is strictly maintained for the proper development of the fetus. This delicate line is maintained such that, the fetus that is foreign should not be rejected by an overreactive maternal immunity and the maternal immunity is still sufficiently effective to fight potential infections (Arck and Hecher, 2013; Pfeifer and Bunders, 2016). This feat is possible through the action of a highly compartmentalized immune system with a predominance of T regulatory and T helper 2 cell produce by maternal, placental and fetal immune cells (Pfeifer and Bunders, 2016). In the presence of pathogen like during HIV infection, there is an upset to this equilibrium by an increase in the number pro-inflammatory cytokine T helper 1 cells which favors the rejection of "non-self" and a decrease in the level of cytokines produced by T regulatory cells which favors immune quiescence.

Chronic inflammation that results from the activation of T helper 1 cells which then secrete cytokines is a hallmark of HIV infection. This phenomenon has also been reported to be similar in pregnant women(Pfeifer and Bunders, 2016). The skewing of immune responses toward inflammation is not restricted to the circulation, innate and adaptive cells in the placenta have been reported to produce more proinflammatory cytokines and growth factors in HIV-infected women, including increased production of granulocyte–macrophage colony stimulating factor (GM-CSF) (Ackerman and Kwiek, 2013; Kumar et al., 2012)

Activating immune cells in the materno-fetal interface has been associated with MTCT of HIV and can be further enhanced by coinfections. In healthy pregnancies, placental CD14þ cells and CD4þ T cells are relatively resistant to infection by HIV R5 strains compared with blood-derived monocytes and CD4þ T cells. However, once activated they have increased susceptibility viral infection and replication. In coinfections with other infections such as placental malaria, there is an upregulation of chemokine receptor 5 (CCR5) on placenta macrophages, the coreceptor for HIV on placental cells facilitating HIV infection(Tkachuk et al., 2001). Other maternal coinfections such as herpes simplex virus-2 (HSV-2), chlamydia and gonorrhoea are also associated with MTCT of HIV(Adachi et al., 2015). Interestingly, MTCT of HIV is not correlated to an increase of HIV shedding in these HSV-2 positive women, which suggests that other mechanisms than mere

increased exposure of the fetus and infant to HIV could play a role in the MTCT process (Drake et al., 2007).

The advent of combination Antiretroviral therapy (cART) during pregnancy has greatly decreased the deleterious effects of the maternal HIV infection on pregnancy outcome, suggesting that decrease in viral load induced by cART and the subsequent reduction of inflammation restores the homeostasis within the placenta (Figure 5). A study in Africa reported that when under cART, pregnancy and infant outcome did not differ between HIV infected mothers and healthy mothers however, there was an increase in the number of spontaneous abortions and stillbirth in the HIV/cART group. It is thought that cART during pregnancy is associated with an increased risk of preterm birth (Townsend et al., 2010) although, the underlying mechanisms are not fully understood. In severely immune compromised HIV-infected patients, initiation of cART can lead to an immune reconstitution syndrome (Pfeifer and Bunders, 2016) and in pregnant HIV-infected women the changes in the maternal immune system upon initiation of cART might disturb the maternal/fetal equilibrium causing deleterious consequences to the fetus. The above comments are best summarized on Figure 5 below.

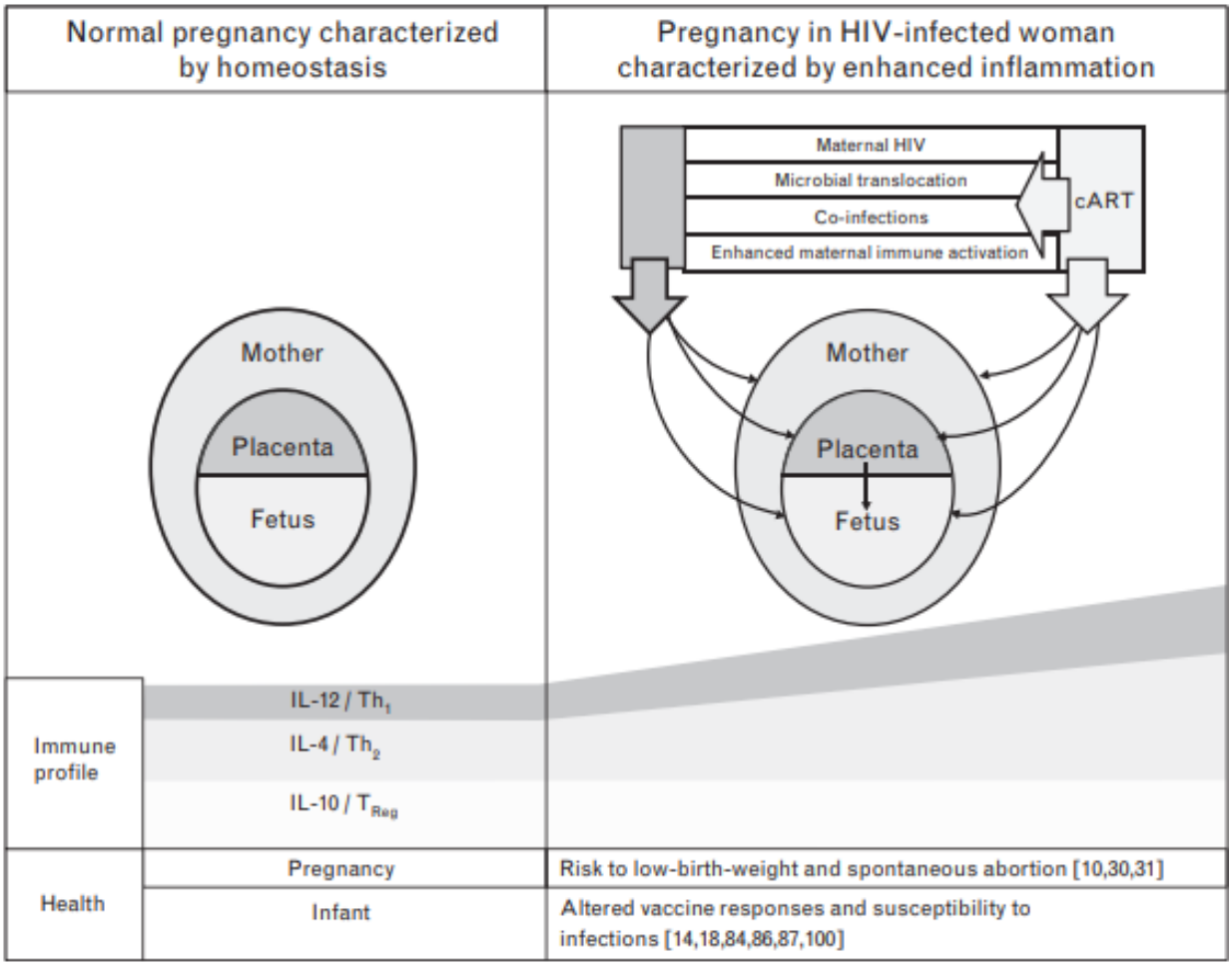

Figure 5: Model for HIV-induced inflammation in pregnant HIV-infected women and their offspring

Source: (Pfeifer and Bunders, 2016)

1.2. Overview of Malaria

1.2.1. Definition, Burden and Global Distribution of Malaria

Malaria is the most important parasitic disease of human beings. The etiologic agent of malaria is the protozoa *Plasmodium sp*., which is transmitted by infected female Anopheles mosquitoes (White et al., 2014). Malaria continues to be endemic in 87 countries in the world (mostly developing countries), with half of the world's population being at risk. According to WHO, 228 million cases of malaria occurred globally in 2018 with an estimated 93% in Africa(World Health Organization, 2019). In 2018, there were an estimated 405 000 deaths due to malaria globally with 94% of these deaths occurring in Africa (World Health Organization, 2019). The WHO reported more than one million cases of malaria in Cameroon in 2014. Children and pregnant women are

particularly susceptible to infection(World Health Organization et al., 2015, 2014). Figure 6 depicts the global distribution of the malaria by region in 2017.

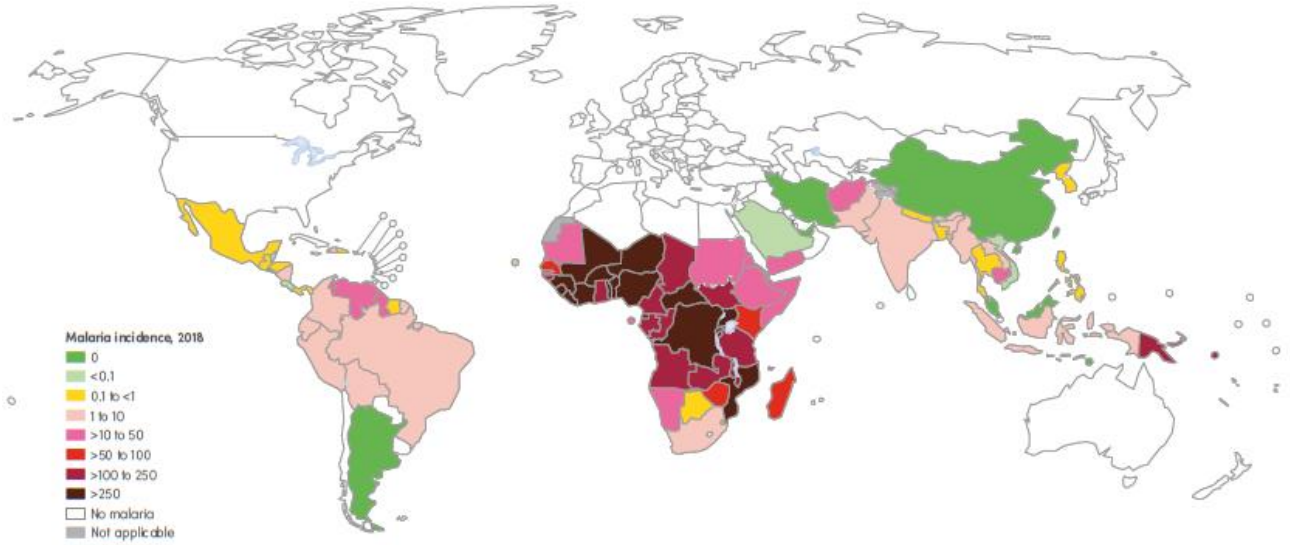

Figure 6: Global Malaria distribution

Source:(WHO, 2019)

In Cameroon, 23.9% of consultations was attributed to malaria and malaria was responsible for 14.6% of deaths in health structures across the nation in 2018(Ewane Ekoyol, 2019). Furthermore, in children under 5 years, 38% of deaths are due to malaria-related causes. All ten regions of Cameroon are affected by malaria (Sakwe et al., 2019). Pregnant women are of particular interest when strategizing in malaria control. In 2018, 22.3% of Cameroonian women were reported to have placental malaria (the sequestration of *Pf* infected erythrocytes in the placental intervillous space)(Leke et al., 2010), and this condition has been associated with pre-term deliveries and low birth weight (Leke et al., 2010; Rogerson et al., 2007; Tako et al., 2005).

There exist five major species of Plasmodium that cause malaria in humans, namely; *P.falciparum, P. vivax, P. ovalae, P. malarae*, and *P. knowlesi*. Four of these – *P. falciparum, P. vivax, P. malariae* and *P. ovale* – are human malaria species that are spread from one person to another via the bite of female mosquitoes of the genus Anopheles (World Health Organization, 2014).

1.2.2. Life Cycle of the Malaria Parasite

Malaria is transmitted through the bite of an infected female Anopheles mosquito. Of the over 400 *Anopheles* species, only 30–40 can transmit malaria. The infection starts, when a female mosquito injects (in its saliva) "sporozoites" (one form of *P. falciparum*) into human skin while taking a blood meal. A sporozoite travels (in the bloodstream) into the liver where it invades a liver cell. It matures into a "schizont" (mother cell) which produces 30,000–40,000 "merozoites" (daughter cells) within six days. The merozoites burst out and invade red blood cells. Within two days one merozoite transforms into a trophozoite, then into a schizont and finally 8–24 new merozoites burst out from the schizont and the red cell as it ruptures. Then the merozoites invade new red cells. *P. falciparum* can prevent an infected red cell from going to the spleen (the organ where old and damaged red cells are destroyed) by sending adhesive proteins to the cell membrane of the red cell. The proteins make the red cell to stick to small blood vessel walls. This poses a threat for the human host since the clustered red cells might create a blockage in the circulatory system(CDC-Centers for Disease Control, 2019).

A merozoite can also develop into a "gametocyte" which is the stage that can infect a mosquito as summarized on Figure 7 below. There are two kinds of gametocytes: males (microgametes) and females (macrogametes). They get ingested by a mosquito, when it feeds on infected blood. Inside the mosquito's midgut, male and female gametocytes merge into "zygotes" which then develop into "ookinetes." The motile ookinetes penetrate the midgut wall and develop into "oocysts." The cysts eventually release sporozoites, which migrate into the salivary glands where they get injected into humans during a blood meal. The development inside a mosquito takes about two weeks and only after that time can the mosquito transmit the disease. *P. falciparum* cannot complete its life cycle at temperatures below 20 °C (CDC-Centers for Disease Control, 2019).

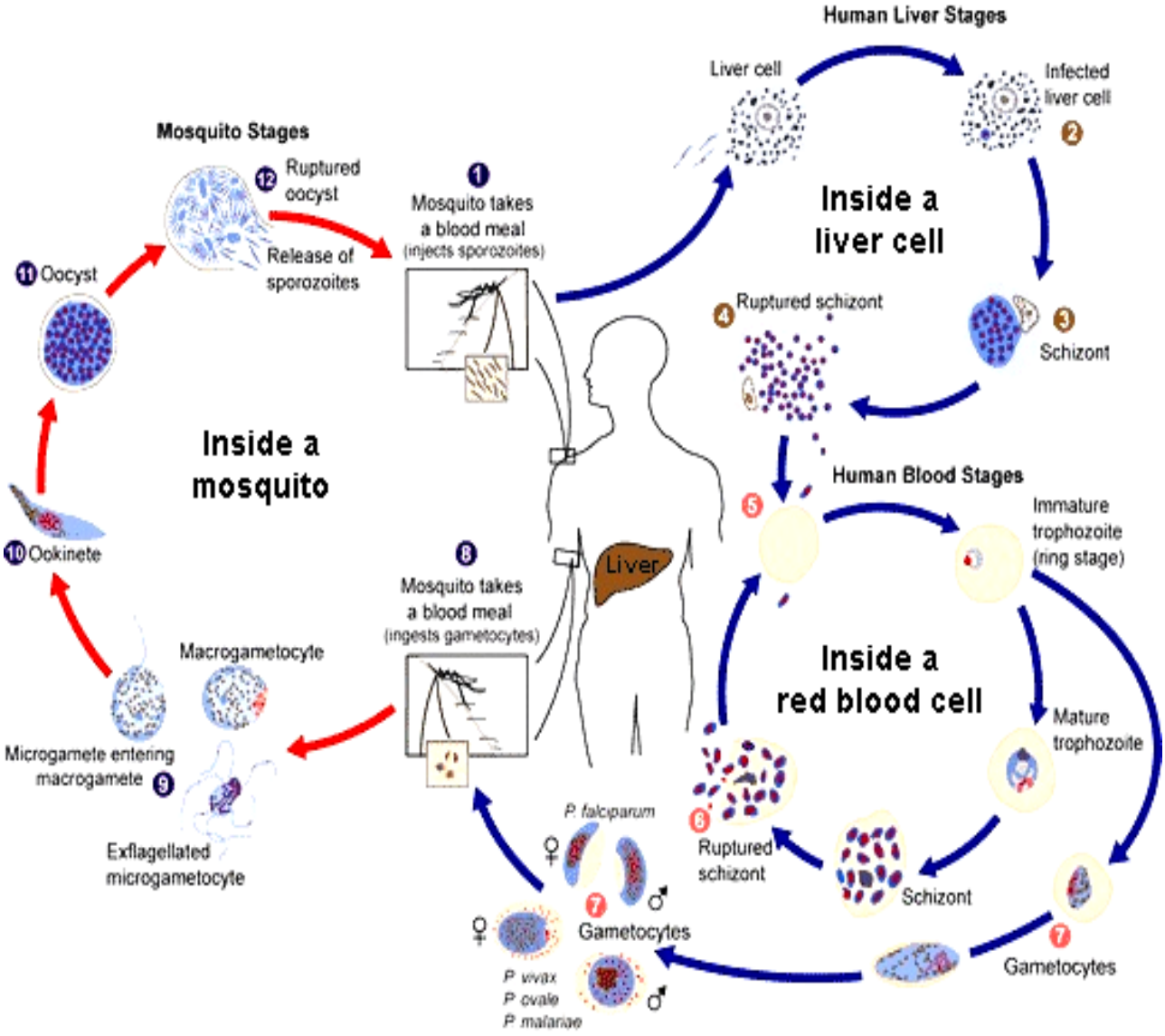

Figure 7: Life Cycle of the *falciparum spp*

Source :(CDC-Centers for Disease Control, 2019)

Legend: *Anopheles mosquito inoculates sporozoites into the human host* 1. *Sporozoites infect liver cells* 2 *and mature into schizonts* 3, *which rupture and release merozoites* 4. *After this initial replication in the liver (exo-erythrocytic schizogony* A*), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony* B*). Merozoites infect red blood cells* 5. *Trophozoites mature into schizonts, which rupture releasing merozoites* 6. *Some differentiate into sexual stages (gametocytes)* 7. *Gametocytes ingested by Anopheles mosquito during a blood meal* 8. *Parasites' multiplication in the mosquito is the sporogonic cycle* C. *In the mosquito's stomach, microgametes penetrate the macrogametes generating zygotes* 9. *Zygotes become motile and elongated (ookinetes)* 10 *which invade the midgut of the mosquito and develop into oocysts* 11. *Oocysts grow, rupture, and release sporozoites* 12, *which migrate to the mosquito's salivary glands. Inoculation of the sporozoites* 1 *into a new human host perpetuates the malaria life cycle.*

1.2.3. Clinical Aspects and Diagnosis of Malaria

After being bitten by an infected mosquito, symptoms usually begin within 10–30 days. Malaria can be uncomplicated or severe. Symptoms of uncomplicated malaria might include chills, diarrhea, fever, headaches, muscle pain, nausea, sweating, vomiting, weakness. Some less noticeable manifestations: enlargement of the spleen or liver, increased breathing frequency, mild anemia, mild jaundice (yellowish eye whites and skin) (Bartoloni and Zammarchi, 2012).

The disease can turn into severe malaria if there are serious organ failures or abnormalities in the bloodstream or metabolism. Symptoms of severe malaria might include breathing difficulties, coma, confusion, death, focal neurologic signs, seizures, severe anemia. Some less noticeable manifestations: abnormalities in blood coagulation, hemoglobin in the urine, high acidity of the blood, hypoglycemia (low blood glucose), low blood pressure and kidney failure(Bartoloni and Zammarchi, 2012).

During pregnancy, malaria can lead to premature baby delivery or delivery of a low-birth-weight baby. The infant can get the parasite from the mother and develop the disease. Central nervous system involvement (cerebral malaria) can cause (especially in small children) blindness, deafness, speech difficulty, paralyses and trouble with movements (Bartoloni and Zammarchi, 2012).

Malaria is usually diagnosed by examining a drop of blood stained on a slide under a microscope. There are also test kits that detect antigens of *P. falciparum* in the patient's blood. These immunologic tests are known as rapid diagnostic tests (RDTs). RDTs can detect two different malaria antigens, one for *P. falciparum* and the other is found in all four human malaria species. RDTs usually show results in about 20 minutes. It is a good alternative to microscopy when a reliable microscopic diagnosis cannot be done. RDT might not detect some infections if there are not enough malaria parasites in the patient’s blood. A negative RDT result can be followed up by microscopy. If a patient with a positive RDT result is not responding to treatment, another blood sample should be taken. This time using microscopy to determine whether the medicine was appropriate for the *Plasmodium* species (Krampa et al., 2017; Mfuh et al., 2017).

1.2.4. Management and Prevention of Malaria

Most malaria deaths occur in rural areas. Quick progression from illness to death can be prevented by administering fast acting and effective medication. *P. falciparum* and *P. vivax* have been confirmed to be resistant (in some areas) to many antimalarial drugs. For example, the chloroquine-resistant strain of *P. falciparum* has spread across endemic areas. The following are

drugs recommended by National Malaria Control Programs for the treatment of malaria; artemether-lumefantrine, artesunate-amodiaquine. Other drugs commonly used to treat malaria are, artemisinin-based drugs in combination with; atovaquone-proguanil, chloroquine, doxycycline, mefloquine, quinine, and sulfadoxine-pyrimethamine. Sulfadoxine-pyrimethamine is usually given to pregnant women on a monthly basis as from late in the second trimester of pregnancy as intermittent preventive treatment (IPT).

Prevention of malaria is achieved mainly through vector eradication activities, which include; sleeping under insecticide-treated bed nets, use of insecticides for spraying, use of insecticide repellants as well as environmental cleanup (Agbor and Apinjoh, 2017; Leke et al., 2010; Mbacham et al., 2010; Rogerson et al., 2007).

1.2.5. Immunity to malaria during pregnancy.

Malaria during pregnancy alters the baseline immune environment in the placenta causing an increase in the levels of predominantly Th1 produced inflammatory cytokines like TNF, interleukin (IL) 2, and interferon gamma(Rogerson et al., 2007). Levels of TNF have been associated with LBW and anemia, while IFN γ production *in vitro* by placental cells has been associated with decreased prevalence of placental malaria(Megnekou et al., 2015). Chemokines produced by monocytes and syncytiotrophoblast may be important in attracting monocytes to the placenta and infiltrates also include increased numbers of neutrophils and T cells(Megnekou et al., 2015). Although peripheral blood T cell responses may be decreased in malaria, this may be due to trafficking of memory T cells out of the circulation. How T-cell responses relate to pregnancy outcomes warrants further investigation (Rogerson et al., 2007).

In malaria endemic areas, pregnant women produce antibodies (Ab) to VAR2CSA that inhibit the binding of IE to CSA *in vitro* reduce maternal anemia(Babakhanyan et al., 2016; Tutterrow et al., 2012), lower placental parasitemia at delivery, increase the length of gestation, and improve infant birth weight. Thus, Ab to VAR2CSA play an important role in protecting pregnant women from the severe effects of PM (Babakhanyan et al., 2014; Fodjo et al., 2016).

Figure 8 below summarizes the unfavorable outcomes caused by malaria during pregnancy.

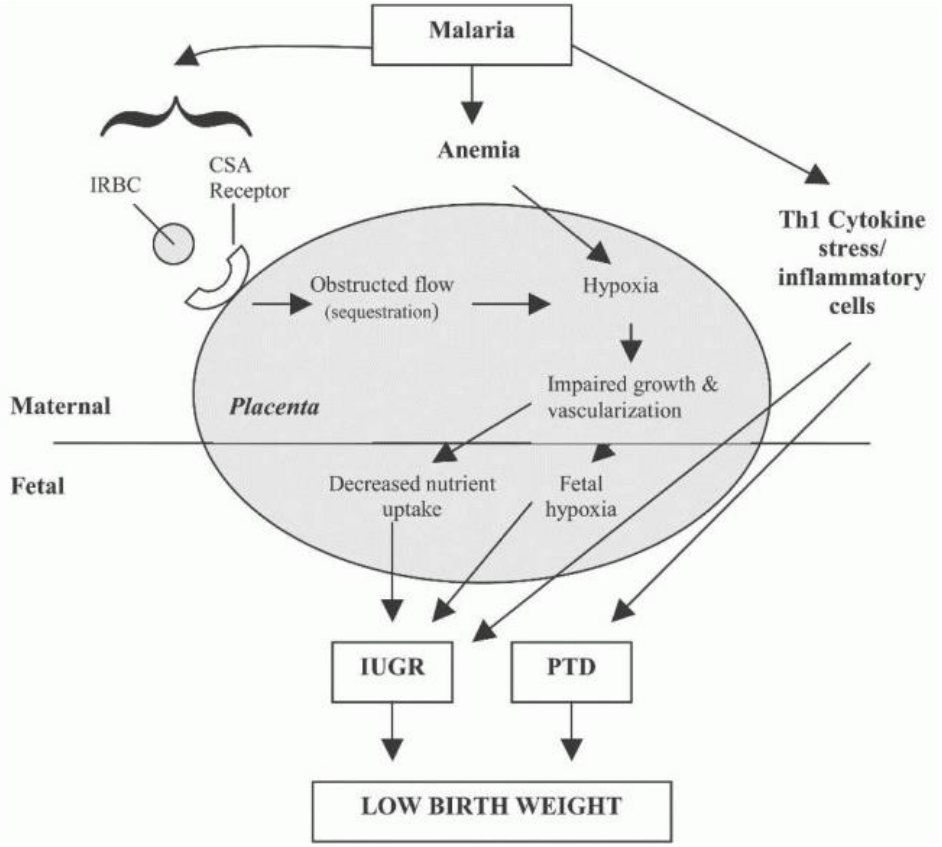

Figure 8: Some potential pathogenic mechanisms by which placental malaria affects placental function and results in IUGR or PTD.

Source: (Rogerson et al., 2007)

Legend: Some potential pathogenic mechanisms by which placental malaria affects placental function and results in IUGR or PTD, iRBC, infected red blood cell; CSA, chondroitin sulfate A; IUGR, intrauterine growth retardation; PTD, preterm delivery

1.3. The Epidemiology of Adverse Pregnancy Outcomes

In most developed countries, pregnancies are planned, complications are few and outcomes are generally favorable for both mother and infant. Adverse pregnancy outcomes are far more frequent in the developing world. The most severe adverse pregnancy outcome is the death of the mother or her offspring. Maternal death has become an extremely rare event in the developed world, with many countries reporting maternal mortality ratios of 5–10 per 100,000 live births. In the least developed countries, the ratios are 100 times higher. Disparities in infant deaths are not quite as wide but remain substantial, ranging from 4–5 to 100 per 1000 live births. Wide disparities probably also exist in the rate of late fetal deaths (stillbirths), although fetal deaths in developing countries are grossly underreported. Even if both the mother and infant survive, pregnancy

complications or problems at delivery or during the neonatal period can lead to severe maternal or infant morbidity. Despite the recognized importance of mortality and severe morbidity as measures of adverse pregnancy outcome, much of the published research in the area of adverse pregnancy outcomes, especially those outcomes related to maternal nutrition, are based on proxy outcomes for mortality and severe morbidity(ref). The most commonly studied of these have been low birth weight (LBW), including its constituents; preterm birth and intrauterine growth restriction (IUGR) and congenital anomalies(Aaron et al., 2012; Agbor et al., 2018; Kramer, 2013; Lambert et al., 2000; López et al., 2015).

Low birth weight is defined by the World Health Organization (WHO) as a birth weight below 2500 g. Birth weight, however, is determined by two processes: duration of gestation and the rate of fetal growth(Kramer, 2013). The WHO defines preterm birth as delivery before 37 weeks of gestation and small for gestational age (SGA) as a birth weight below the 10^{th} percentile for gestational age based on the sex-specific reference by Williams et al (Williams et al., 1982). The determinants of adverse pregnancy outcomes can be broadly classified as fetal, maternal and placental factors. Fetal causes include chromosomal abnormalities, multiple pregnancies, fetal structural anomalies, and fetal infections(WHO and UNAIDS, 1998). Maternal factors include nutritional deficiencies, especially of vitamin C and E. Maternal infections, especially HIV, malaria, and tuberculosis which can cause fetal growth restriction (FGR) (Krishna and Bhalerao, 2011).

Maternal HIV and placental malaria appear as the principal cause of this pathology especially in sub-Saharan Africa where the prevalence of these two infections among pregnant women is high(López et al., 2015; Rogerson et al., 2007).

1.3.1. Adverse Pregnancy Outcomes Associated with HIV/AIDS

Women of child-bearing age comprise approximately half of the estimated 36.9 million people living with HIV-1(Global HIV & AIDS statistics, 2019). Children born to HIV-infected women have an increased risk of adverse birth outcomes(Turner et al., 2000). Pregnancies in HIV-infected women are more likely to result in prematurity, intrauterine growth retardation, low birth weight spontaneous abortion, and prenatal death. In Africa, infection with HIV-1 and HIV-2 have been linked with spontaneous abortion. A study in HIV seropositive women was 1.47 times more likely

to have had a previous spontaneous abortion, and this rose to 1.81 in women in Uganda who were seropositive for HIV and syphilis (WHO and UNAIDS, 1998).

Preterm rupture of membranes may also be increased in HIV-positive women and *abruptio placentae* have been described as more common in HIV-positive women in South Africa and Kenya. Preterm labor and LBW in HIV positive women have been reported in some studies in developing countries. Increased stillbirth rates have been reported, especially from areas where the epidemic has been present for a long time. The risk appears to be lower in asymptomatic women, although stillbirth rates more than double those in HIV seronegative mothers have been shown in some African countries. A study in Thailand showed that LBW was significantly associated with maternal HIV status (Mitgitti et al., 2008). According to a study in Malawi in 2014, a strongly negative relationship was observed between both increasing peripheral HIV-1 viral load and LBW and placental HIV-1 viral load and LBW(Turner et al., 2013).

1.3.2. Adverse Pregnancy Outcomes Associated Placental Malaria

In endemic areas, pregnant women are more vulnerable to malaria infection and are more likely to develop hypoglycaemia and pulmonary oedema. Although they appear to be often asymptomatic, they are likely to spread malaria as gametocytes carriers. Infection due to *P. falciparum* can cause great damage to placenta and situations such as anaemia often occurs. This has been attributed to the fact that pRBC's adhere to placental receptors like chondroitin sulphate A (CSA) and hyaluronic acid. The sequestration of Plasmodium falciparum-infected erythrocytes in the placental intervillous spaces termed placental malaria can trigger the recruitment of inflammatory cells and production of cytokines, which are strongly associated with LBW (Umbers et al., 2011).

Each year more than 125 million pregnant women are at risk of malaria infection, which can have serious consequences for them and their offspring, especially in first and second- time mothers. In highly endemic settings up to 50% of low birth weight (LBW) deliveries can be attributed to malaria in pregnancy, leading to approximately 100, 000 infant deaths annually, many of which are consequences of FGR. Fetal growth and birth weight are determined by genetics, but both fetal and maternal insults (systemic or placental) can lead to FGR. Maternal causes of FGR that are common in malaria-endemic areas include young maternal age, anemia, HIV infection, undernutrition, chewing betel nut, chewing tobacco, and sexually transmitted infections(Umbers et al., 2011). In sub-Saharan Africa, 20% of LBW has been attributed to malaria in pregnancy (Kramer, 2003).

1.4.Fetal Growth Regulation

1.4.1. The Role of the Placenta in Fetal Growth Regulation

The placenta is the primary interface between the fetus and mother and plays an important role in maintaining fetal growth by performing several physiological functions, which, following birth, are controlled by the kidney, gastrointestinal tract, lungs, and endocrine glands. The main functions of the placenta include modulation of the mother's immune response to prevent immunological rejection termed tolerance, facilitating the exchange of respiratory gases, water, ions, nutrients and wastes between the maternal and fetal circulations, producing and secreting hormones, cytokines and other signaling molecules required to maintain pregnancy and to ensure placental and fetal development and growth(Zhang et al., 2015).

Maternal blood supply to the placenta is established at the end of the first trimester of pregnancy in humans, with maternal blood entering the intervillous space of the placenta from the transformed spiral arterioles for substrate transport to the fetus. The barrier between the maternal and the fetal circulations in the human placenta consists of three fetal cellular layers: (i) the syncytiotrophoblast, a multinucleated epithelial layer formed following the fusion of the mononucleated villous cytotrophoblasts; (ii) villous stromal tissue and (iii) the fetal capillary endothelium as depicted on Figure 9. The syncytiotrophoblast has two polarized plasma membranes: the maternal-facing microvillous plasma membrane (MVM) and the basal membrane (BM). The syncytiotrophoblast is the main regulator of substrate exchange and nutrient transporters are expressed on both plasma membranes (Forbes and Westwood, 2008).

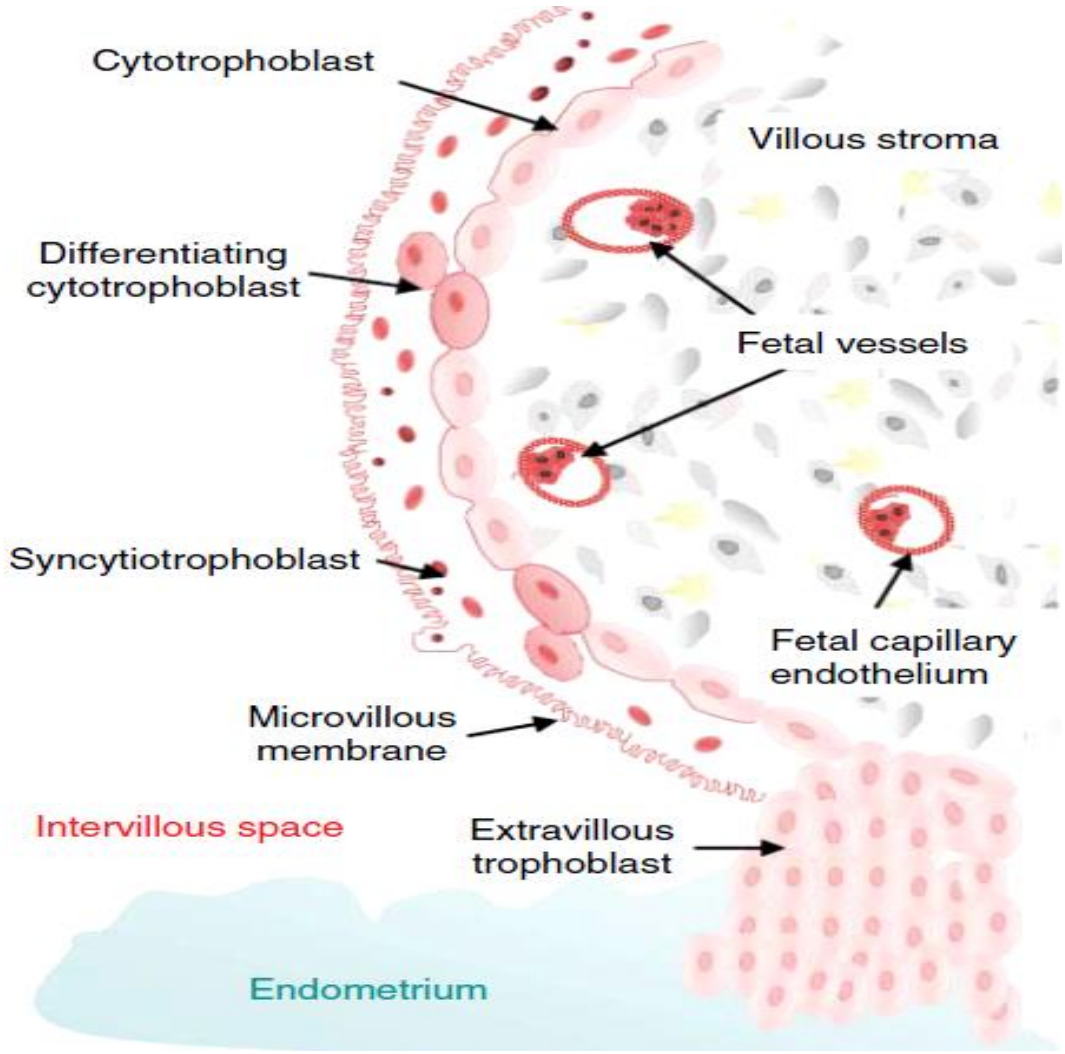

Figure 9: Schematic diagram of the first-trimester human placenta

Source: (Forbes and Westwood, 2008)

Legend: Cytotrophoblasts proliferate and differentiate into one of two subtypes, the invasive extravillous trophoblasts or the terminally differentiated non-proliferative syncytiotrophoblast. The syncytiotrophoblast functions as a protective barrier for the fetus and is the epithelial surface where there is exchange of nutrients and gases between the maternal and fetal circulations occurs. The villous stroma lies directly below the cytotrophoblast layer and contains numerous different cell types including placental macrophages (Hofbauer cells), fibroblasts, and endothelial cells.

Birth weight depends not only on maternal nutrition but also on the placenta's ability to transport substrates from the maternal to the fetal circulation. At any gestational age, placental efficiency measurements provide an indication of the conditions experienced *in utero* and the extent to which placental adaptations during intrauterine development have occurred in order to meet fetal growth demands. These morphological and/or functional adaptations determine placental substrate

transport capacity and efficiency to the fetus. The major substrates required for fetal growth include oxygen, glucose, amino acids, and fatty acids (Zhang et al., 2015)). While the placenta regulates the transport of nutrients to the fetus according to the mother's ability to deliver them, fetal demands as well as hormones and growth factors secreted by the placenta, the weight, size and shape of the placenta also affect its ability to transfer nutrients, vascularity as well as blood flow (Zhang et al., 2015).

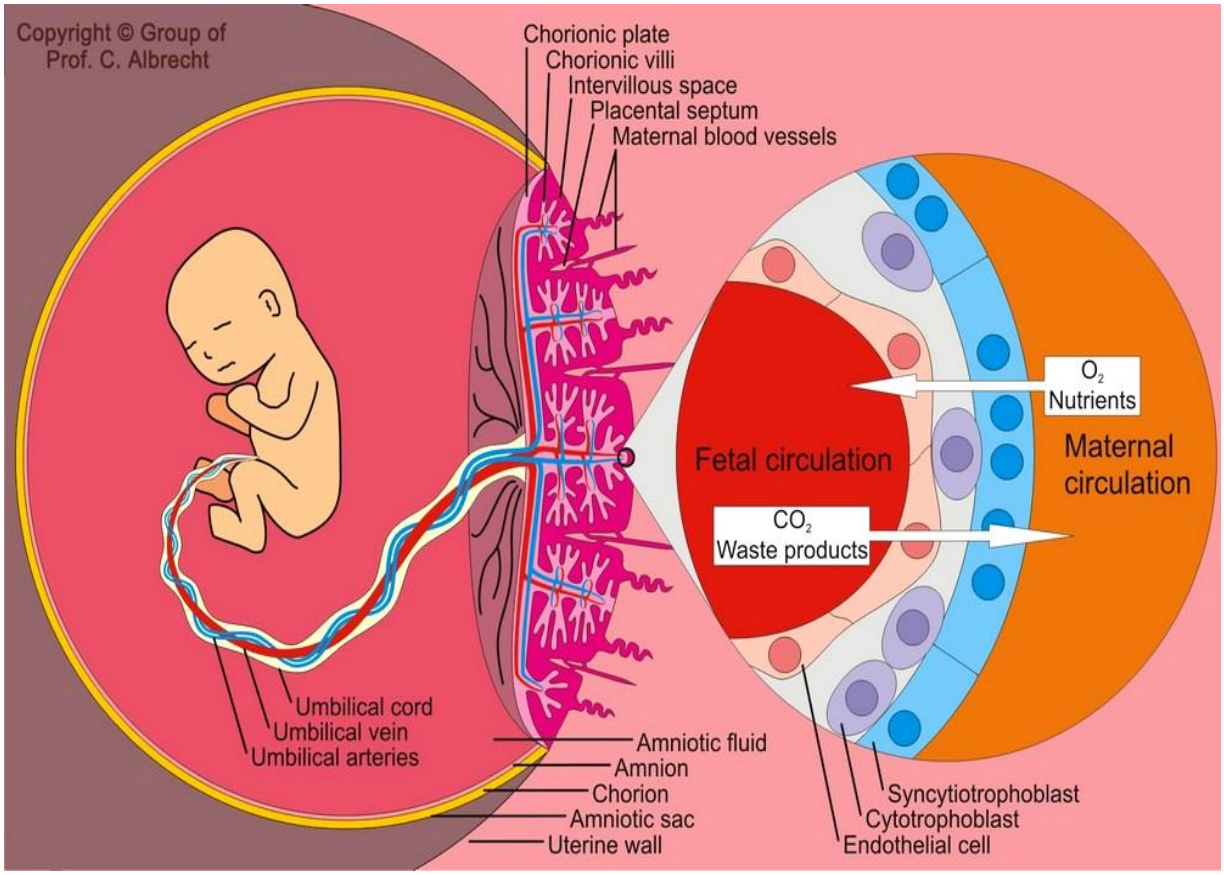

Figure 10: Maternal-fetal exchange

Source: (Derricott, 2016)

The transfer of highly permeable molecules, such as gases, oxygen and carbon dioxide, is influenced by blood flow and occurs via simple diffusion, whereas less permeable substrates are transferred through passive and active transport processes as shown on Figure 10 above. Glucose is transported across the placenta via facilitated diffusion and is orchestrated by hormones secreted by the placenta. In chorionic villi of the human placenta, cytotrophoblasts are a progenitor stem

cell population which continuously proliferate and differentiate into one of two subtypes; extravillous trophoblasts that migrate into the maternal decidualized endometrium and remodel the spiral arteries to optimize the supply of oxygen and nutrients to the placenta and fetus; or syncytiotrophoblast, a multinucleated epithelia which acts both to protect the fetus from the maternal immune response and as a nutrient and gas exchange membrane . As the growth and thus nutrient demands of the fetus increase with pregnancy progression, the syncytial surface area must also increase to ensure sufficient transfer of nutrients to the fetus. The villous syncytiotrophoblast layer has a short lifespan with terminally differentiated and apoptotic elements shedding continuously into the maternal circulation. A process to renew and expand the syncytial layer throughout pregnancy is therefore required. The syncytiotrophoblast layer has no transcriptional activity, and hence during pregnancy, it is maintained by the continual proliferation, differentiation, and fusion of cytotrophoblasts. Consequently, cytotrophoblast proliferation is important for placental growth, especially during the first trimester, when the tissue grows rapidly. Increased or decreased rates of trophoblast turnover have been associated with different tissue pathologies and are linked to enhanced (macrosomic) or reduced (FGR) fetal growth. In these conditions, the surface area available for the transfer of nutrients is altered. An increase in trophoblast proliferation results in enhanced placental nutrient transfer in macrosomia, while the converse occurs in FGR. Since extravillous trophoblasts invade the maternal circulation and are required to establish an oxygen supply to the fetus, it is unsurprising that alterations in this aspect of trophoblast function are also associated with pregnancy complications such as FGR and pre-eclampsia. Recently, several studies have suggested that soluble factors in the maternal circulation, including insulin-like growth factors and angiopoietins, can influence placental development and (Forbes and Westwood, 2008). The normal placental function can be affected by several factors; including anatomical and physiological causes; placental size and morphology, blood flow and vascularity as well as pathological causes; malaria and HIV during pregnancy and preeclampsia. Figure 10 above summarizes the potential role of the placenta during pregnancy.

1.4.2. The IGF Axis and Fetal Growth Regulation

The insulin-like growth factor (IGF) axis is the most influential growth-promoting factor in fetal life, with roles in regulating placental development and function, transplacental exchange of nutrients, and fetal growth A complex network of molecules, including its binding proteins, proteases, and receptors, which together comprise the 'IGF system', modulates the biological function of the IGFs. This system comprises the following components.IGF-1 and IGF-2 are two small, highly homologous single-chain polypeptides. In mice, genetic ablation of insulin-like growth factor 1 gene (*Igf1*) or insulin-like growth factor 2 gene (*Igf2*) decreases birth weight by 40%, and double knock-out of *Igf1* and *Igf2*, or of the IGF-1 receptor gene (*Igf1r*), further restricts fetal growth. The liver is the main source of circulating IGF-1 in postnatal life, but during pregnancy, the placenta and the fetus secrete IGFs and regulatory proteins. Fetal IGF-1 and IGF-2 promote fetal growth but have differential actions, which have been attributed to distinct interactions with receptors(Mynarcik et al., 1999; Suh et al., 2015; Umbers et al., 2011).

The IGF receptors (IGF-1R and IGF-2R) mediate IGF activity and are abundant in all placental cell types and in the microvillous plasma membrane of the syncytiotrophoblast. Activation of IGF-1R stimulates cell signaling cascades that lead to proliferation, survival, and fetal growth promotion, whereas IGF-2R lacks the cell signaling domain, acting as a sink to sequester free IGF-2, and is considered anti-mitogenic (see Figure 11 below). Alterations in the absolute level and bioavailability of the IGFs in maternal, fetal, and placental compartments are implicated in other causes of FGR(Umbers et al., 2011).

Although IGF-2 can bind to the type-2 IGF/mannose-6-phosphate receptor (IGF2R/M6PR) or the insulin receptor, the classical actions of both IGF-1 and IGF-2 are mediated by binding to the type-1 IGF receptor, IGF1R. There is also the Maternal-fetal termed IGF-binding proteins (IGFBPs)-1–6. Unsurprisingly, IGFBP levels, particularly IGFBP-1 and IGFBP-3 that are abundant at the maternal–fetal interface, are also correlated with fetal growth. The regulation of the bioavailability of IGFs is controlled by IGFBPs (Gibson, 2001).

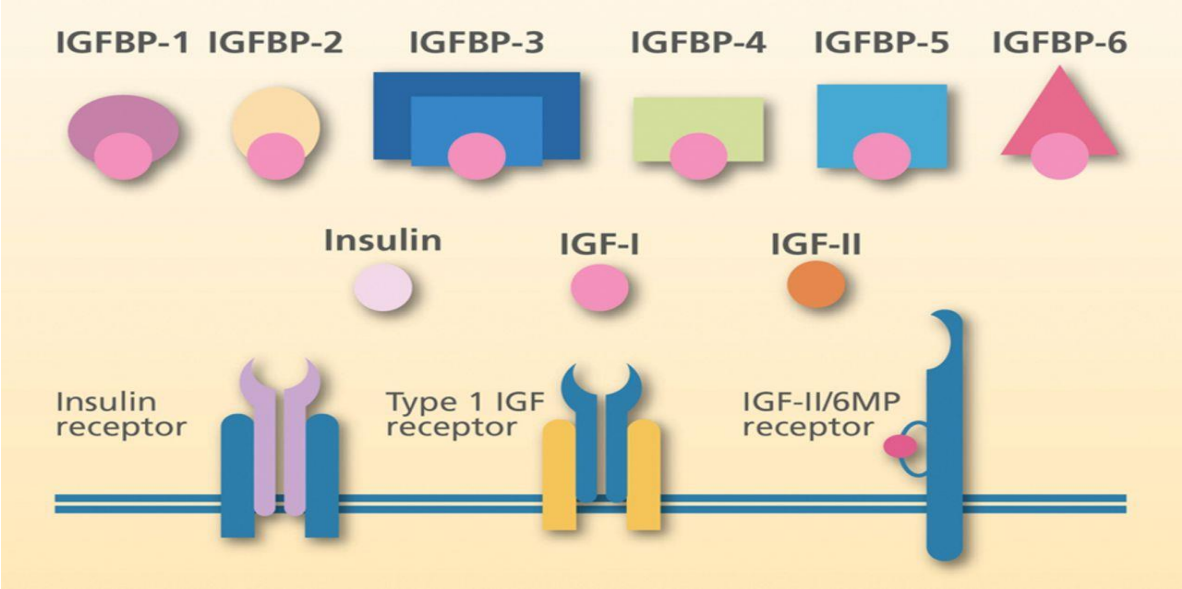

Figure 11: Interaction between IGFs, their receptors and binding proteins

Source: (Livingstone, 2013)

At term, maternal and fetal IGFBP-1 concentrations are negatively correlated with birth weight and in pregnancies complicated by IUGR and preeclampsia, raised maternal and fetal IGFBP-1 concentrations have been observed. These studies all point to a role for IGFBP-1 as a local modulator of IGF action in fetal growth. It has been shown that in the circulation, IGFBP-1 normally exists in a highly phosphorylated state (pIGFBP-1). However, non-phosphorylated isoforms of IGFBP-1(npIGFBP-1) have been found in maternal circulation. Since pIGFBP-1 has a high affinity for IGF-1 than npIGFBP-1, changes in IGFBP-1 phosphorylation does not affect the affinity of IGFBP-1 for IGF-1 and hence promoting the presence of npIGFBP-1 at the maternal-fetal interface will not affect the actions of the predominant IGF at this site. Another mechanism for releasing IGFs from binding proteins is proteolysis. Pregnancy-associated proteolysis of several other binding proteins, particularly IGFBP-3, has been comprehensively described. These binding proteins are cleaved within the maternal circulation by enzymes that appear to have no effect on IGFBP-1. However, plasmin, stromelysin-3 and amniotic fluid do have IGFBP-1 proteolytic activity though little is known of the relevance of an IGFBP-1 protease (Forbes and Westwood, 2008; Gibson, 2001).

The relationship between placental malaria and the IGF axis has been explored in some studies. It has been shown that both fetal and maternal plasma levels of IGF-1 levels are reduced in samples from women with placental malaria and associated inflammation as compared with those in

uninfected pregnancies(Mitgitti et al., 2008) though the consequence on LBW has not been well outlined. Such mechanisms in HIV-associated pregnancy have not been studied.

1.4.3. Angiopoietins and Fetal Growth Regulation

Successful placentation is dependent on the establishment of a competent vascular network formed by two processes: vasculogenesis, which involves the *de novo* formation of vessels from endothelial progenitor cells and branching and nonbranching angiogenesis, which is the remodeling of the pre-existing vessels. The imbalance between proangiogenic and anti-angiogenic factors can lead to impaired placentation, causing major pregnancy complications, such as preeclampsia (PE) and intrauterine growth restriction (IUGR), which can lead to poor obstetric outcomes. The role of various angiogenic factors in the pathophysiology of these conditions in pregnancy has been investigated. In recent years, in the field of angiogenesis research, studies have focused on the serum levels and placental expression of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) and its receptors in normal and pathological pregnancies (Charnock-Jones et al., 2004; Conroy et al., 2017; Kappou et al., 2015).

The angiopoietin system includes four ligands (ANG-1, ANG-2, ANG-3, and ANG-4), of which the best characterized are ANG-1 and ANG-2, and two corresponding tyrosine kinase receptors (TIE-1 and TIE-2). During pregnancy, angiopoietins are mainly produced by the placenta and play a critical role in endothelial cell survival and the remodeling of vessels (see figure 12 below). In particular, these factors seem to act complementary to the VEGF system and contribute to the later stages of angiogenesis. Both ANG-1 and ANG-2 bind to TIE-2, an endothelial cell-specific tyrosine kinase receptor with similar affinity. Although ANG-1 and ANG-2 share a similar protein structure (ANG-2 is 60% homologous to ANG-1), their biological activities differ significantly. ANG-1 acts as a paracrine agonist to TIE-2 leads to receptor dimerization and induces its phosphorylation on several cytoplasmic residues to activate downstream signaling pathways, including the phosphoinositide 3 (PI3)-kinase/Akt and extracellular signal-regulated kinase (ERK) pathways. The activation of the Akt pathway leads to the inhibition of FOXO transcription factors and down-regulates the expression of ANG-2, endothelial cell-specific molecule 1 (ESM1) and PlGF. Apart from endothelial cells, previous studies have indicated that a distinct population of monocytes, known as TIE-2 expressing monocytes (TEM) and early hematopoietic cells also express the TIE-2 receptor (Kappou et al., 2015). The other known Tie receptor (TIE-1; tyrosine kinase with immunoglobin and epidermal growth factor homology domains) seems to have weak

kinase activity and its functional role has not yet been fully elucidated. ANG-1 promotes the reorganization of endothelial cells and the structural integrity of blood vessels by recruiting and interacting with peri-endothelial cells. An additional role of ANG-1 is to inhibit the activation of the vascular endothelial barrier and reduce the leakage and leucocyte migration into tissues induced by inflammatory agents. Despite the fact that the basic functioning of the pathway has been explored, there is no consistency as to the role of ANG-2 in certain conditions of pathological vascular remodeling, such as inflammation. Several lines of evidence suggest that ANG-2 binds to TIE-2, but primarily acts as an antagonist of ANG-1 signaling. In particular, ANG-2 disrupts the connections between the endothelium and perivascular cells and promotes cell death and vascular regression. In addition, ANG-2 renders endothelial cells more accessible to VEGF that promotes neovascularization (Kappou et al., 2015). However, in the case of insufficient angiogenic stimuli, ANG-2 causes endothelial cell apoptosis and vessel regression (Figure 12).

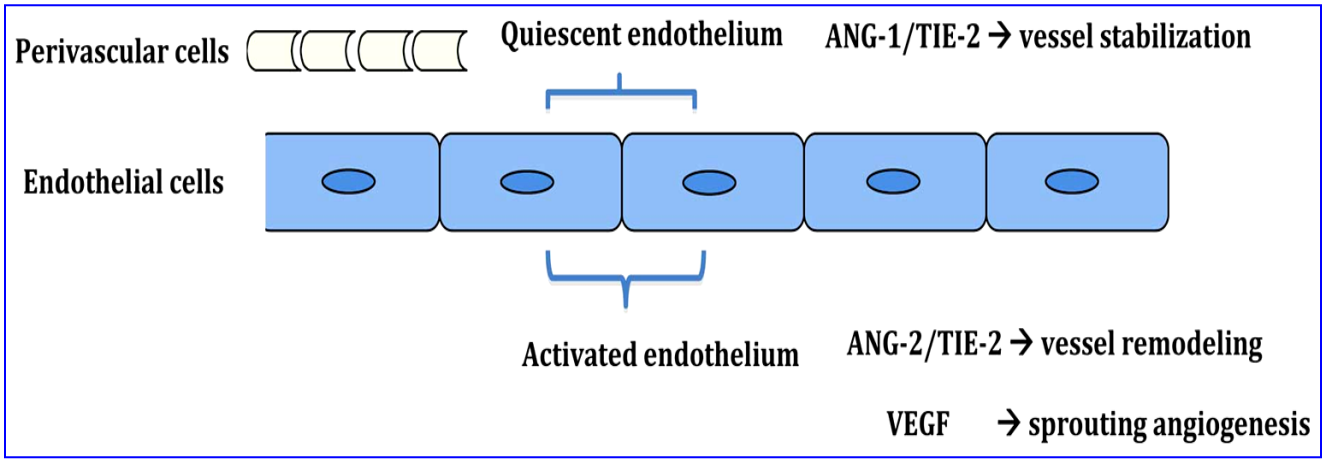

Figure 12: Role of the Angiopoietin/TIE system in vessel remodeling and stabilization

Source: **(***Kappou et al., 2015)*

1.4.4. The processes of vasculogenesis and angiogenesis in fetal growth

Vasculogenesis involves the *de novo* formation of blood vessels from endothelial progenitor cells. Vasculogenesis lays down the initial pipelines of the network. Structures called blood islands formed by cellular differentiation of precursor cells (hemangioblast), into endothelial and RBC. Next, the capillary plexus/bed (interconnected network in which a single atriole gives rise to dozens of capillaries that empty into several venules of the venous system) forms as endothelial cells migrate outward from blood islands to form a random network of continuous strands.

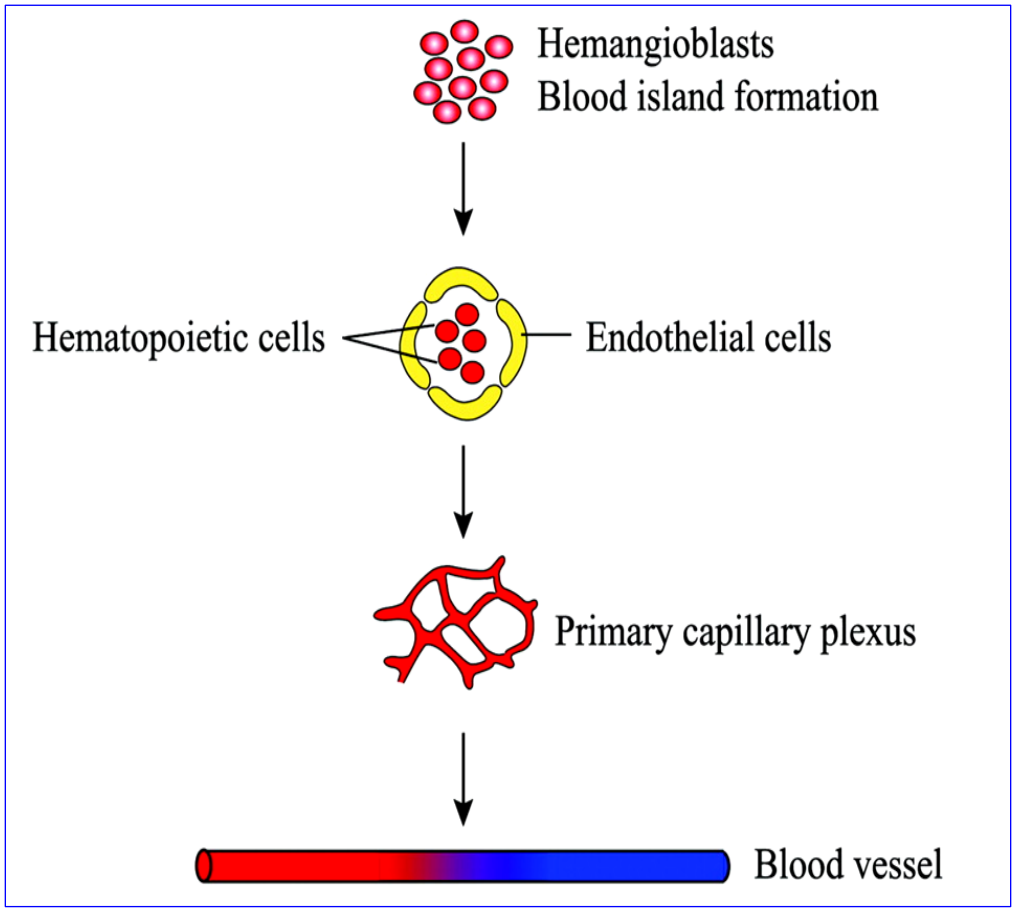

Figure 13: Vascular remodeling in normal pregnancy

Source: **(***Kappou et al., 2015***)**

These strands then undergo a process called lumenization, the spontaneous rearrangement of endothelial cells from a solid cord into a hollow tube (blood vessel).

The building and remodeling of blood vessels is a critical event in the formation of every organ, and the relationship between the blood vessels and the tissues they serve is tightly balanced between stasis and growth, and regression as shown on Figure 13 (Kappou et al., 2015).

Angiogenesis is the formation of more blood vessels from pre-existing ones, contributing to the complexity of the initial network established by vasculogenesis. Endothelial buds are formed with the expression of VEGF (vascular endothelial growth factor) and ANG2 and grow away from the parent vessel to form smaller, daughter vessels that spread into new territory (Kappou et al., 2015).

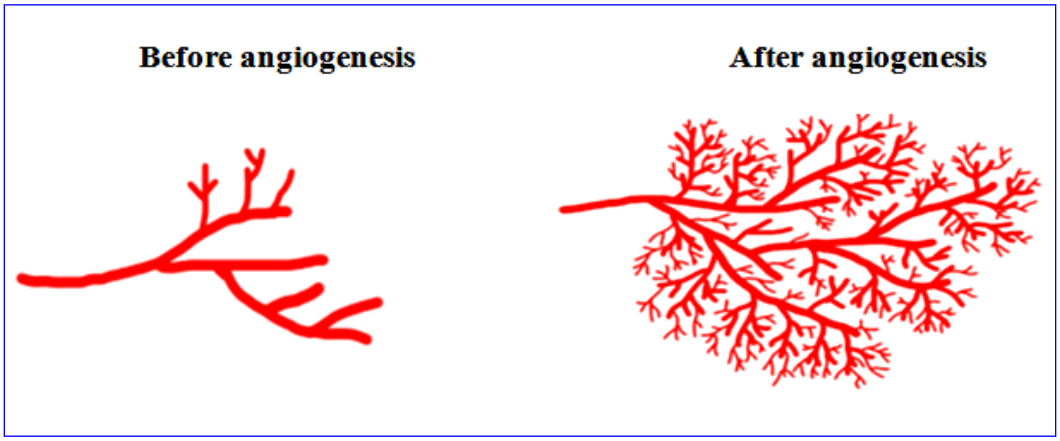

Figure 14: The effect of angiogenic factors on blood vessels

Source: (Silver et al., 2010)

A majority of published studies on the role of angiogenic factors on the development of pregnancy disorders are related to impaired placentation, such as PE and IUGR have focused on the expression profiles of VEGF, PlGF and its receptors. However, few studies have examined the expression pattern of angiopoietins in the above-mentioned pregnancy disorders.

Few studies have tried to define the precise mechanisms by which PM induces LBW based on the essential role of angiopoietins (ANG)-1 and -2 in normal placental vascular developments as shown on Figure 14 above. In a study by Silver et al, in 2010, Plasmodium falciparum infection was associated with a decrease in maternal plasma ANG-1 levels and an increase in the ANG-2/ANG-1 ratio. In the same study, it was demonstrated that angiopoietins dysregulation was associated with PM and LBW. Women with PM who delivered LBW infants had increased ANG-2/ANG-1 compared to uninfected women delivering normal birth weight infants(Silver et al., 2010).

Little is known about the mechanisms of the dysregulation of angiopoietins in pregnant women infected with HIV as well as women co-infected with HIV and malaria and their consequence on LBW.

1.4.5. Matrix Metalloproteinases (MMP) 2&9

Matrix metalloproteinases (MMPs) are produced by many cell types and in normal adult tissue, they had very low expression levels when measured using immunohistochemistry, however, in situations of injury and pregnancy, for example, their levels are elevated. MMPs have the ability to break down several proteins of the extracellular matrix (ECM) and they participate actively in remodeling the ECM by degrading important matrix scaffold macromolecules. Together with their

tissue inhibitors (TIMPs) they form a balance to maintain normal early pregnancy and placental development (Nissi et al., 2013).

The gelatinases MMP-2 and MMP-9 are especially involved in successful cytotrophoblast invasion in early pregnancy as they are considered key enzymes in degradation of basement membrane, which mainly consists of type IV collagen(Staun-Ram et al., 2004; Moore and Crocker, 2012). Tissue inhibitors for MMPs, such as TIMP-1 and TIMP-2, regulate protease activity.

MMP-2, MMP-9, TIMP-1 and TIMP-2 have been localized in the placental bed using immunohistochemistry and *in situ* hybridization. Immunoreactivity for MMP-2 was detected in both decidual cells and extravillous trophoblasts (EVT), but MMP-9 staining was only observed in areas with abundant EVT (Kizaki et al., 2008; Moore and Crocker, 2012; Seval et al., 2004; Staun-Ram et al., 2004). In early gestation weeks (weeks 6 and 7) the secretion of MMP-9 in placental bed is very low, but the secretion increases gradually after week 8, and in week 11 the cells produce a large amount of MMP-9 (Staun-Ram et al., 2004). In contrast, biosynthesis of MMP-2 is significantly higher in the early stages of the pregnancy(Kizaki et al., 2008). MMP-2 has been suggested to be the key regulator of trophoblast invasion in early pregnancy(Seval et al., 2004). MMP-2 is localized in the placental bed during early pregnancy and it is dominant over MMP-9 on the trophoblasts of 6–8 weeks of gestation(Onogi et al., 2011). During labor, MMP-9 is mainly responsible for gelatinolytic activity in the membranes. Trophoblasts of the human placenta can differentiate into extravillous trophoblasts (EVT) with invasive properties. Proteolytic enzymes such as MMP-2 and MMP-9 are essential for the invasion of EVT cells into endometrial stroma (Onogi et al., 2011). Figure 15 depicts the role of MMPs during pregnancy.

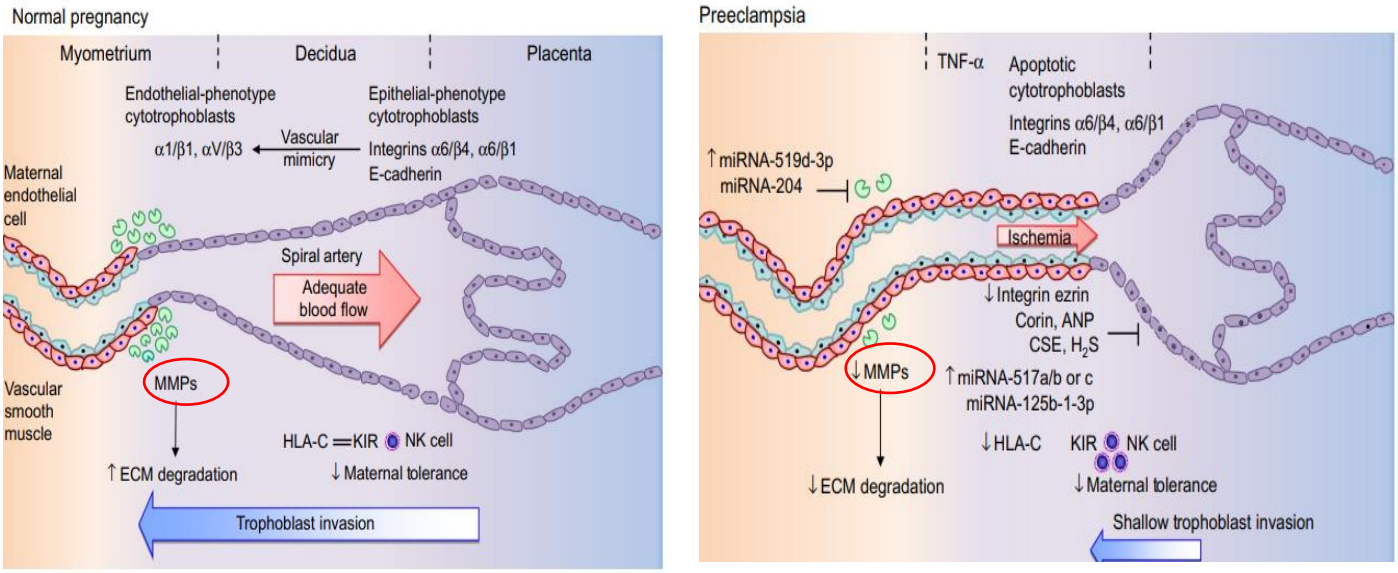

Figure 15: Matrix metalloproteinases in the Placenta

Source: (Possomato-Vieira and Khalil, 2016)

Finally, placental matrix metalloproteinases (MMP) are proteolytic enzymes that have been shown to have a vital role in trophoblast invasion, regulation of vascular endothelial cell functions and placental angiogenesis (Chen et al., 2013; Raffetto and Khalil, 2008). The MMP function by essentially degrading the extracellular matrix (ECM), releasing growth factors that allow the activation of signals that are important for angiogenesis(Sternlicht and Werb, 2001). For instance, MMP2 and MMP9 are secreted by placental trophoblasts and are critical in trophoblast invasion, vascular endothelial cell migration, attachment, proliferation and survival; therefore, supporting angiogenesis (Possomato-Vieira and Khalil, 2016).

1.4.6. Galectin 13

Galectin 13 also known as human placenta protein (PP)13 was discovered on the syncytiotrophoblast of human villous placental tissues at term using immunohistochemistry and immunofluorescence(Than et al., 2014a, p. 13). The presence of PP13 was also revealed in the Syncytiotrophoblast using *in situ* hybridization (Blois and Barrientos, 2014). Detecting PP13

mRNA expression in the same placental cells, further confirmed their existence. PP13 expression was found in the placentas of monkeys, suggesting that this protein was conserved during evolution. Moreover, studies using *in situ* hybridization show that *LGALS13* transcripts were present in the amnion and chorionic trophoblasts of the fetal membranes. These findings suggest that PP13 expression could predominantly be in microenvironments where there is interaction between maternal and fetal immune cells(Than et al., 2008).

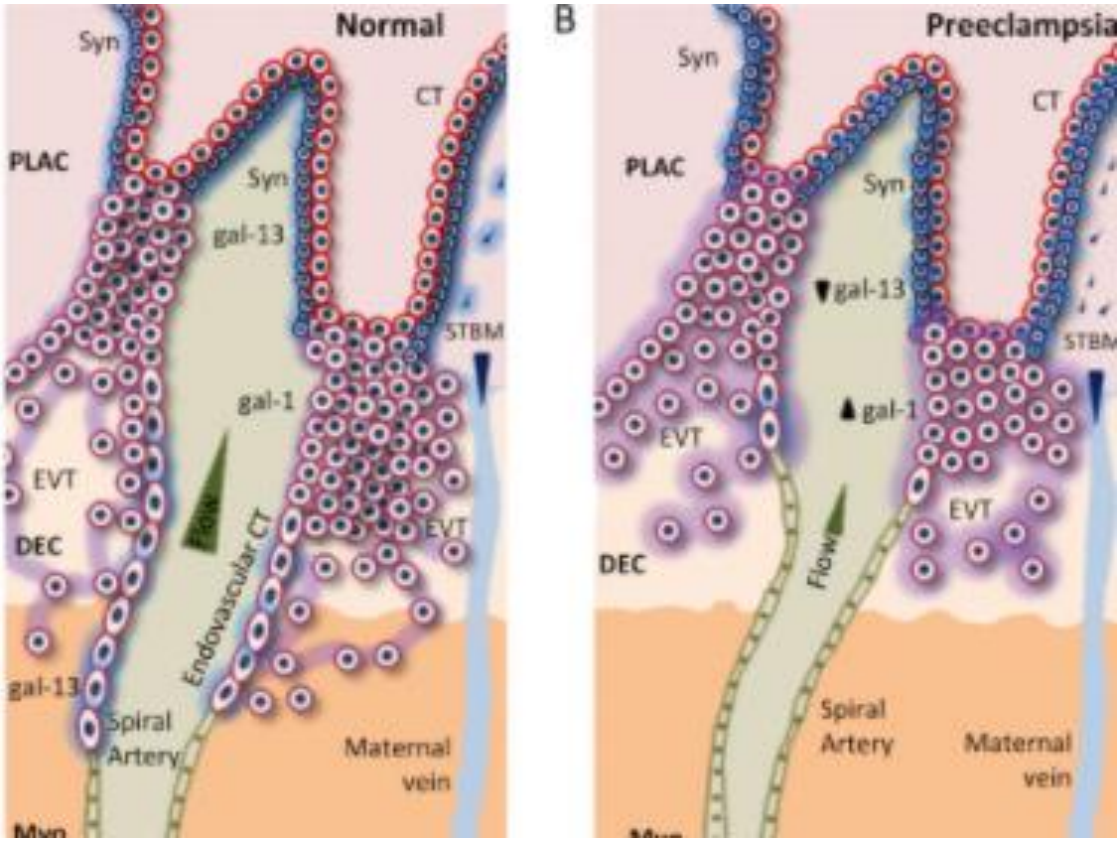

Figure 16: Galectin 13 in the placenta extravillous space

Source: (Blois and Barrientos, 2014)

During the first trimester, PP13 was detected using immunohistochemistry on the syncytiotrophoblast and multinucleated luminal trophoblasts that invade the decidual spiral arterioles (8). Interestingly, syncytiotrophoblastic staining intensity declined with gestational age, being the strongest between 6 to 8 weeks of gestation (Than et al., 2008).

Moreover, galectins are expressed in the placenta is thought to play key roles in placental formation and vascularization (Blois and Barrientos, 2014). Among the 19 galectins known, placental

galectin 13, Gal-13) has been shown to be expressed by the syncytiotrophoblast, endovascular trophoblast and decidual spiral arteries and is important in trophoblast invasion and vascular remodeling during placentation as shown on Figure 16 (Than et al., 2014b). Gal-13 is also regarded as an endogenous danger/damage signal, as its secretion from the syncytiotrophoblast is dramatically upregulated at the onset of preeclampsia and the hemolysis, elevated liver enzymes and low platelet count (HELEP) syndrome(Blois and Barrientos, 2014). However, its dynamics in infectious disease of pregnancy is not well known. This lectin likely also plays an important role in feto-maternal tolerance, as it has been shown to promote apoptosis of activated T cells and macrophages(Blois and Barrientos, 2014).

1.5. Methods used in the diagnosis of Malaria and HIV

1.5.1. Immunological Diagnosis

1.5.1.1. Rapid Diagnostic Tests (RDTs)

Majority of RDTs are based on the same principle. It involves the application of immunological techniques using antibodies (monoclonal) to detect antigens, in blood flowing along a membrane containing specific anti-malaria antibodies. For malaria diagnosis, some important soluble antigens secreted by erythrocytic forms of the parasite in the blood are targeted:

P. falciparum-specific histidine-rich protein-2 (PfHRP2) and *P. falciparum*-specific lactate dehydrogenase (Pf-pLDH), *P. vivax*-specific lactate dehydrogenase (PvpLDH), as well as, pan-pLDH and pan-aldolase (Mukkala et al., 2018).

For the diagnosis of HIV, many tests available. They can detect HIV earlier than antibody screening tests. They check for HIV antigen, a protein called p24 that's part of the virus that shows up 2-4 weeks after infection. (Sands, 2015)They also check for HIV antibodies. such as Alere Determine™ HIV-1/2 (Alere Medical Co. Ltd), HIV 1/2 STAT-PAK® (Chembio Diagnostic Systems, Inc.), HIV 1/2 STAT-PAK® Dipstick (Chembio Diagnostic Systems, Inc.), One Step HIV 1/2 Whole Blood/Serum/Plasma Test (Guangzhou Wondfo Biotech Co., Ltd), Uni-Gold™ HIV (Trinity Biotech Manufacturing Ltd), Anti-human immunodeficiency virus (HIV) antibody diagnostic kit (colloidal gold) (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd) Immunofiltration RDTs, INSTI™ HIV-1/HIV-2 Antibody Test (bioLytical™ Laboratories), Reveal® Rapid HIV Antibody Test (MedMira Laboratories Inc). They either detect HIV specific antigens or antibodies or both(Sands, 2015) .

1.5.1.2. Serological tests

Serological tests rely on the detection of antibodies against asexual stages of the parasite. A positive test reveals exposure to malaria since antibodies remain in the plasma for long.

The first serological test used for the detection of malaria antibodies was the immunofluorescence assay (IFA). The principle of IFA is that, following infection with any *Plasmodium species*, specific antibodies are produced within 2 weeks of initial infection, and persist for 3-6 months after parasite clearance. This technique is not good for routine diagnosis as antibodies fail to distinguish past from current infections (Ambroise-Thomas et al., 1974; Tangpukdee et al., 2009).

In the case of HIV infection, these tests detect antibodies in response to HIV specific protein synthesized by the virus 2-8 weeks post infection. They are generally very accurate.

Usually, a technician will take a small blood sample and send it to a lab for testing. Some immunoassay tests check urine or oral fluids, however, the paucity of antibodies in these bodily fluids make diagnosis difficult. Antibodies to HIV can be measured using a variety of techniques. None of these detect HIV itself, but rather detect an immune response to the virus, which generally take some time to be mounted and to become reactive (or positive) after HIV infection has been acquired. Antibodies to HIV-1 and HIV-2 are detected using the enzyme-linked immunosorbent assay (ELISA). Most commercially available ELISAs have a high sensitivity and specificity and are able to detect all subtypes of HIV-1 and HIV-2. A wide range of HIV serological assays are available, and it is therefore important to identify the most suitable assays for a given set of programmed circumstances. These tests are often used as confirmation tests (Fearon, 2005; Sands, 2015).

1.5.1.3. Luminex

The sample is added to a mixture of beads labeled with specific spectral addresses and, pre-coated with analyte-specific capture antibodies. The antibodies bind to the analytes of interest. Biotinylated detection antibodies specific to the analytes of interest are added and form an antibody-antigen sandwich. Phycoerythrin (PE)-conjugated streptavidin is added. It binds to the biotinylated detection antibodies. Polystyrene beads are read on a dual-laser flow-based detection instrument, such as the Luminex® 100™, Luminex 200™ or Bio-Rad®Bio-Plex® analyzer. One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. In addition to the Luminex 100, Luminex 200 or Bio-Rad Bio-Plex dual-laser, flow-based analyzers, magnetic beads can be read using the Luminex MAGPIX® Analyzer. A magnet in the MAGPIX analyzer captures and holds the magnetic beads in a monolayer, while two

spectrally distinct light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and, the second LED determines the magnitude of the PE-derived signal. Each well is imaged with a CCD camera (Luminex Assay Principle: R&D Systems).

1.5.2. Molecular diagnostic methods

1.5.2.1. Polymerase Chain Reaction (PCR)

A basic PCR set-up requires several components and reagents: Sterile nuclease free distilled water; a DNA template that contains the region (target) to be amplified; two primers (short DNA fragments); DNA polymerase with a temperature optimum at around 70ºC; deoxynucleoside triphosphates (dNTPs) i.e dATP, dTTP, dCTP, dGTP; buffer solution; divalent cations (Mg^{2+} or Mn^{2+}); controls: a positive one containing DNA fragments of known molecular weight and a negative one.

The PCR is commonly carried out in a reaction volume of 20 -100 µl in small tubes (0.2-0.5 ml volumes) in a programmable thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles each cycle consisting of three basic temperature steps; *1 Initialisation*; *2 Denaturation*; *3 Annealing; 4 Extension/elongation*; 5 *Final elongation; 6 Final hold.*

PCR-based techniques that have been developed for the detection and identification of malaria parasites have proven to be more sensitive and specific than conventional microscopy, with some reported to be able to detect as few as one parasite per µl of blood. However, to attain such a high sensitivity, the DNA isolation technique must be fast and yield pure products, which must be stored under ideal conditions to remain intact. The assay used for malaria parasite detection is a nested PCR which is a variant of the basic one. The nested PCR assay increases the sensitivity of DNA amplification by reducing background due to non-specific amplifications. Two sets of primers are used in two successive runs. In the first reaction (nest-1), one pair of *Plasmodium* specific primers is used to generate PCR products which besides the intended target, might still consist of non-specifically amplified fragments. The products of this nest 1 then serve as DNA templates in four separate second (nest-2) amplifications tube with completely different primers which are specific for each of the 4 human malaria species (Mfuh et al., 2017).

Although this method appears to have overcome the two major problems of malaria diagnosis - sensitivity and specificity - its utility is limited by complex methodologies, high cost, and the need for specially trained technicians. Quality control and equipment maintenance are also essential.

PCR therefore, is not routinely implemented in developing countries, but it is highly recommended to be coupled to basic microscopy or RDTs in epidemiological studies in order to obtain accurate data (Lloyd et al., 2018; Mfuh et al., 2017).

1.5.2.2. Real-time PCR

This is a technique that is similar to the conventional PCR but for the detection which is acquired in real-time. Dyes which either bind to the gene of interest or intercalates the double-stranded DNA of the amplified gene are used (Hwang et al., 2011). In this technique the thermal cycler is modified with a cooled charged coupled device which monitors the amplification in real time.

During viral load quantification of HIV-1 virus, an *in vitro* reverse transcription-polymerase chain reaction (RT-PCR) assay is used either on whole blood spotted on cards as dried blood spots (DBS) (i.e. obtained via venipuncture or capillary blood) or human plasma from HIV-1 infected individuals. Then it is followed by a Real-Ti*m*e HIV-1 assay which is intended for use in conjunction with clinical presentation and other laboratory markers for disease prognosis and for use as an aid in assessing viral response to antiretroviral treatment as measured by changes in plasma HIV-1 RNA levels. This assay is not intended to be used as a donor screening test for HIV-1 or as a diagnostic test to confirm the presence of HIV-1 infection. During this assay, the primers used are specific for the highly conserved integrase region (Yukl et al., 2011)

Like HIV, the quantification of malaria parasites can also be done using real-time PCR from either parasite blood extracted from whole blood or red blood cell pellets. They can be used in quantifying gene expression levels of key genes targeted in the during the assessment of the efficacy of efficacy of some antimalarials or identify alterations in gene expression of the host as result of malaria infection (Gavina et al., 2017; Lloyd et al., 2018). Real-Time PCR has the advantage of being less laborious, faster and more sensitive than conventional PCR however, it is more costly and there is need for considerable knowhow in validating results from a run (Hwang et al., 2011)

1.5.2.3. Microarray

This is a laboratory tool used to analyze large numbers of genes or proteins at one time. In a microarray, biologic molecules such as DNA, RNA, or protein are placed in a pattern onto a surface such as a glass slide. Other substances are added to these slides to detect specific patterns of molecules. Microarrays are being used to help diagnose diseases, investigate differential gene expression analyse in several conditions like HIV and to develop treatments for them ("NCI Dictionary of Cancer Terms," 2011).

- **Applications of microarrays**
  - **Gene expression analysis**

The major application of DNA microarrays has been to estimate and quantify gene expression levels. In this application, RNA is extracted from the cells of interest and either, labeled directly, converted to a labeled cDNA or converted to a T7 RNA promoter tailed cDNA which is further converted to cRNA through using *in vitro* transcription. A wide variety of methods have been developed for labeling of the cDNA or cRNA (Beckert and Masquida, 2011) including: incorporation of fluorescently labeled nucleotides during the synthesis, incorporation of biotin labeled nucleotide which is subsequently stained fluorescently labeled streptavidin, incorporation of a modified reactive nucleotide to which a fluorescent tag is added later, and a variety of signal amplification methods. The two most frequently used methods are the incorporation of fluorescently labeled nucleotides in the cRNA or cDNA synthesis step or the incorporation of a biotin labeled nucleotide in the cRNA synthesis step (as is done by Affymetrix and Illumina) (Kaliyappan et al., 2012).

The labeled cRNA or cDNA are then hybridized to the microarray, the array is washed and the signal is detected by measuring fluorescence on each spot. In the case of biotin labeled samples, the array is stained post-hybridization with fluorescently labeled streptavidin. Laser induced fluorescence is typically measured with a scanning confocal microscope. The intensity of the signal(s) on each spot is taken as a measure of the expression level of the corresponding gene ("NCI Dictionary of Cancer Terms," 2011).

- **Transcription factor binding analysis**

Microarrays has also been used together with chromatin immunoprecipitation to identify the binding sites of transcription factors. Briefly, transcription factors (TFs) are cross linked to DNA with formaldehyde and the DNA is fragmented. The TF(s) of interest (with the DNA to which they were bound still attached) are affinity purified using either an antibody to the TF or by tagging the transcription factor with peptide that's amenable to affinity chromatography (for example a FLAG-, HIS-, myc or HA-tag). After purification, the DNA is released from the TF, amplified, labeled and hybridized to the array. This technique is commonly referred to as "ChIP-chip" for Chromatin Immuno-Precipitation on a "chip" or microarray (Kaliyappan et al., 2012).

As TF's often bind quite a distance away from the genes that they regulate, the design of the array and size distribution of the fragment length are interrelated. E.g. the array must contain probes that

will interrogate the region of DNA bound to the transcription factor. For bacteria or yeast, the intergenic regions are fairly small, and the same arrays used for gene expression work can be applied to ChIP. For mammalian genomes, the intergenic regions are large, and the TF often bind many kbp away from the gene of interest. Hence, for mammalian genomes, oligo arrays with oligo's spaced evenly across the entire genome are typically used for ChIP experiments ("NCI Dictionary of Cancer Terms," 2011).

- **Genotyping**

Microarrays have been widely used as single-nucleotide-polymorphism (SNP) genotyping platforms. Several alternative approaches have been used to detect SNP's but the most commonly used are allele discrimination by hybridization as used by Affymetrix, allele-specific extension and ligation to a "bar-code" oligo which is hybridized to a universal array (the Illumina "Golden Gate Assay" or approaches in which the arrayed DNA is extended across the SNP in a single nucleotide extension reaction) the Arrayed Primer Extension assay or the Infinium Assay of Illumina (Kaliyappan et al., 2012).

In brief, SNPs for their assay are selected to be between restriction sites that are <1kb apart. Genomic DNA is fragmented with a restriction enzyme, end repaired and adapters for PCR are ligated to the fragments. PCR is performed under conditions that selectively amplify products of <1kb in size. This method reduces genomic complexity by approximately 50fold and results in a corresponding increase in signal to noise on the array.

Both the Affymetrix and the Illumina methods for SNP genotyping have been highly successful and are highly used. Today SNP arrays capable of detecting >1M different human SNPs are available from both vendors. Call rates (the fraction of SNPs on the array that can be reliable called) and reproducibility of SNP calls exceed 99.5%. In addition, the same arrays or variations thereof can also be used to detect copy number variants (Bumgarner, 2013).

CHAPTER 2: METHODOLOGY

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2.1. Ethical considerations

Ethical clearance (No 2013/11/366/L/CNERSH/SP) and an administrative authorisation were obtained from the National Ethical Committee of Cameroon and the Ministry of Public Health respectively. All participants were informed on the purpose of the study and all anticipated risks and benefits were clearly explained to them in their first official languages. Participation in the study was strictly voluntary and they were free to withdraw from the study at any time. Written informed consents were obtained from the participants and from the guardians of participants under 18 years and assents were collected from pregnant women below 18 years. Laboratory analyses and treatment (where necessary) were free of charge and results handed back to each participant or to their health attendants in order to facilitate health care. The confidentiality of participants' information was respected by the use of coded numbers and access to data was restricted only to members of the research team.

2.2. Study site, duration and population

This pilot cross-sectional study was conducted in Yaoundé, the capital city of Cameroon. Yaoundé which is situated between 3º 52' 0" N; 11º 31' 0" E and has a population with heterogeneous ethnic background. It has a wet, tropical climate characterized by two wet seasons (March–June and September – November), and two dry seasons (December –February and July- August). The annual rainfall ranges between 1500-2000mm while the temperature ranges from 18ºC to 30ºC (mean 24ºC). Malaria transmission is perennial with its peak transmission (EIR>0.1-1.1 infected bites/person/month) occurring mostly during the rainy-dry transition seasons(Babakhanyan et al., 2014). The prevalence of malaria is as high as 24.26% in sub-urban areas (our unpublished data). The prevalence of HIV in Yaoundé is 4.4% , HIV is more prevalent among females than males and women of child bearing age bear the heaviest burden pregnant women bear (CAMPHIA, 2018)

2.3 Study Duration

Enrolment of study participants took place between January to November 2014 and the study spanned to October 2018.

2.4. Eligibility criteria

Inclusion criteria

Study participants included pregnant women in their late third trimester of pregnancy who were admitted at the Yaoundé Central Hospital for delivery and fulfill the following criteria:

- Willing to give informed consent.
- Women not on active labor.
- 15 -50 years of age.
- Willing to give information on their HIV status.

Non-inclusion criteria

Women suffering from pre-eclampsia, diabetes, hypertension, and diagnosed with any chronic or sexually transmitted infection were excluded.

2.5. Sample size justification

This study was part of the umbrella study titled "Infectious disease affecting pregnant Cameroonian women and newborns" by Dr. Anna Babakhanyan. The umbrella study recruited 107 pregnant women with 31 being HIV positive and 76 HIV negative. From the mother study we selected 102 women (31 HIV positive and 71 HIV negative women) for study based on sample volume.

In order to investigate the resulting dysregulations in gene expression by HIV-1 and malaria infections in the placenta, we adopted Lee's approach for sample size estimation for disease control designs for microarray studies (Lee, 2002, 2004). We computed the a false discovery rate of 1; our anticipated number of undifferentially expressed genes was 40000; our anticipated power was 0.8; the anticipated difference in the log-expression between disease and control condition as postulated under alternative hypothesis was 1; and, the anticipated standard deviation of the difference in log-expression between disease and control group was 0.566. The computation generated a sample size of 9 samples per group. Unforeseen deterioration of sample quality as a result of long storage, only 8 control and 5 malaria positive placenta were of sufficiently good quality to qualify for microarray studies.

2.6. Sample Collection

Samples were obtained from 102 mother/neonate pairs admitted at the Yaoundé Central hospital at delivery and gave their consent. Women with pre-existing health conditions [e.g. diabetes, preeclampsia and Hemolysis, Elevated Liver enzymes, Low platelet count (HELLP) syndrome] and/or had spontaneous abortions were excluded from the study. Information on each woman's demographic and clinical history including HIV status, ART intake, use of the intermittent preventive treatment (IPT) with sulfadoxine pyrimethamine (SP) and insecticide treated bed nets (ITN) during pregnancy was available. The birth weight, length and APGAR score of newborns

were also available. Gestational age was estimated based on date of last menstrual period or ultrasound scan data when available. Women with axillary temperature greater than 37.5°C were considered as having fever. Neonates born between 28 and 37 weeks were classified as premature. Singletons weighing less than 2,500 grams were considered LBW babies. Women were tested for HIV during pregnancy and vaccinated with tetanus vaccine according to national guidelines. All HIV positive women were placed on cART following national guidelines. From these women, venous maternal blood, 10ml, was collected into heparinized and EDTA tubes (Medilife, E.c Becton Inc, Lagos, Nigeria) before delivery and tested for malaria using an HRP2/pLDH Pf/Pan combo RDT test (CareStart ^{TM}, Access Bio Inc, Somerset, NJ, USA) as described by manufacturer. Hemoglobin levels were measured using Hemocue Hb 201^{+} hemoglobinometer (Empire Drive, Lake Forest, CA) and recorded. A person was considered anemic if their blood hemoglobin level was less than 11g/dl. Participants' temperatures were assessed using an electronic thermometer (Sejoy, China). HIV history and information were obtained from participants' consultation books. Immediately after delivery, 10 ml of placental blood (intravillous space blood) and 10 ml of cord blood were collected into heparinized and EDTA tubes respectively. 5mm × 5mm × 5 mm cube of villous tissue was excised, carefully rinsed 4 times in 50ml of 1X PBS to remove red blood cell as it is known that hemoglobin interferes with PCR, and immersed in 5 ml of RNA Later solution (Ambion ®, USA) for RNA preservation. Another portion of placenta biopsy was preserved in buffered formalin (10%) for histosections analyses. Small pieces of placenta tissues were also excised, cleaned with tissue paper and impressed on clean glass slides for parasitological analyses by microscopy. Neonatal growth parameters were also recorded in the case report forms.

2.7. Sample processing

General processing: Samples were transported from the maternity of the Yaoundé Central Hospital to hospital to the Immunology laboratory of the Biotechnology Center, University of Yaoundé I, Cameroon in a biohazard labeled cooler that contained ice packs. Upon arrival in the lab, the placenta biopsies preserved in RNA later was stored at -80^{o}C until they were shipped. A portion of whole maternal, cord and IVS EDTA blood from HIV positive, malaria positive, co-infected mothers and controlled were spotted on Whatmann filter paper 3(GE Healthcare Life Sciences, Buckinghamshire, UK), dried overnight and stored in Ziplock bags containing Calcium sulfate (Sigma, Missouri, USA) for future studies.

Fetal cell stain: EDTA IVS blood was used to determine the degree of purity of maternal blood using the Fetal Cell Stain Kit (SIMMLER Inc, High Ridge, Missouri, USA) with slight modifications. Briefly, 30 µl of 0.85% saline was mixed with 20 µl of maternal IVS blood and gently mixed. Ten µl of the mixture was used to prepare a thin blood smear and allowed to air dry. The smears were immersed in Fetal cell fixing solution (80% alcohol) for 5 minutes, immersed again Fetal cell buffer solution (citrate buffer, 0.081M) for 10 minutes and inserted in a clean vessel containing fetal cell stain (Erythrosine-B, Fast green) for 3 minutes. The slides were allowed to dry and read using immersion oil (ThermoFisher, Germany) under 100× objective (Motic BA210, California, USA).

Fetal cells stained dark-reddish pink while adult cells stained light pink with a darker center.

The fetal/adult RBC ratio was determined as thus:

$$\textbf{Fetal/adult RBC ratio} = \frac{\textbf{Number of Fetal RBCs counted}}{\textbf{Number of Adult RBCs counted}}$$

Thick, thin and impression smear preparation and staining: Seven µl and 3.5µl of whole maternal and cord blood were used to prepare thick and thin film respectively, on the same 25 x 75 mm slide but 10mm apart as described by(Fodjo et al., 2016). The thin blood film and impression smears were fixed with absolute methanol (Biopharma, Cameroon) for 5 minutes and the slides were stained using 10% buffered Giemsa (Paramount, Fesam Chemicals, Cameroon) for 10 minutes an read under the microscope (Motic BA210, California, USA).

The malaria parasite could be identified by its red chromatin dot surrounded by a purple/blue ring. In the thick smear, parasitemia was calculated as follows:

$$\textbf{Parasitaemia (parasites/ µl)} = \frac{\textbf{Number of parasites X 8000}}{\textbf{Number of WBCs count in thick smear (200 WBCs)}}$$

Were, 8000 is the approximate number of white blood cells (WBCs) in 1 µl of blood

The percentage of infected red blood cells (iRBCs) in the thin blood and impression smears were determined as thus:

$$\textbf{\% of iRBCs} = \frac{\textbf{Number of iRBCs}}{\textbf{Number of iRBCs} + \textbf{Number of non iRBCs}} \textbf{ X 100}$$

N.B. RBCs were counted in at least 80 fields, corresponding to 16000 RBCs counted in total as that 1 field is approximated to have 200 RBCs.

Placental biopsy specimens in formalin were stained with Hematoxylene and Eosin at the University Hospital Center Yaoundé for histological examination (Leke et al., 2010).

Plasma preparation: Whole peripheral, IVS and cord blood still residing in the tubes (Heparin and EDTA) were centrifuged (Powerspin^{TM} BX Centrifuge, New Jersey, USA)) at 10000 rpm for 10 minutes. Plasma was carefully separated from the cells, aliquoted and stored at -80^{o}C till assay time. Cell pellets found in EDTA tube were also aliquoted and stored at -20^{o}C for future use.

Placental tissue examination by histology assay: The placental tissue was fixed by immersing in tissue biopsies in 10% buffered formalin. This was, then transferred into 70% ethanol, dehydrated, embedded in paraffin and 4µm section cut using a microtome. Sections were stained with haematoxylin and eosin, and slides examined by light microscopy. Haemozoin pigments were examined under polarized light to increase their visibility and minimize false positivity by formalin crystals. The presence of infectious pathogens, immune cells and placental integrity is can be validated using microscopy (Megnekou et al., 2015).

2.8. Measuring MMP 2, MMP 9, ANG1 and ANG2, IGFBP 1, IGFBP 3, MMP 2, MMP 9 using the MAP assay

2.8.1. Principle

Analyte-specific antibodies are pre-coated onto color-coded magnetic microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. A final wash removes unbound Streptavidin-PE, the microparticles are resuspended in buffer and read using the Luminex MAGPIX Analyzer. A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and the second LED determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Each well is imaged with a CCD camera.

2.8.2. Procedure

The MAP assay was carried out as described by manufacturer (R&D Systems Inc, NE, USA). In short, 50 µl of Microparticle Cocktail (prepared by adding 500 µl of Microparticle Cocktail in 5

mL of Diluent RD2-1 for 96 wells in container provided) was incubated with 50 µl of plasma diluted at 1:2 with Calibrator Diluent RD6-52 in microplate for 2 hours at 25ºC on a microplate shaker at 800 rpm (Orbital Shaker MP4, BioExpress, USA). Microspheres were washed twice with 100 µl of Wash Buffer (prepared by Adding 20 mL of Wash Buffer Concentrate to deionized water to prepare 500 mL of Wash Buffer) using a magnetic plate separator (Magnetic Plate Separator, Luminex, Austin, Texas, USA). Fifty µL of diluted Biotin Antibody Cocktail (prepared by adding 500 µl of Biotin Antibody Cocktail in 5 mL of Diluent RD2-1 for 96 wells) was added to each well and incubated in the dark for 1 hour at room temperature on the shaker set at 800 rpm. Microspheres were washed as described above. Then, 50 µL of diluted Streptavidin-PE (prepared by diluting 100X Streptavidin-PE to a 1X concentration by adding 55 µl of Streptavidin-PE to 5.5 ml of Wash Buffer) to each well and incubated for 30 minutes in the dark at 800 rpm. Wells were washed as described above and microspheres were re-suspended in 100 ml Wash buffer and placed on a microplate shaker at 800rpm for 2 minutes. Fifty µl of the microsphere suspension was analyzed using a MAGPIX analyzer (Luminex, Austin, Texas, USA)). The reader was programmed to read a minimum 50 beads per spectral address, Doublet Discriminator gates at approximately 8000 and 16,500 at a flow rate of 60µl/minute. The results were expressed as median fluorescence intensity (MFI). Standards (prepared by adding 200 µL of Calibrator Diluent RD6-52 into 5 test tubes labeled 2-6. Standard 1was used to produce a 3-fold dilution series for each assay. The standards were used to plot a curve where concentrations of the samples could be obtained via their respective MFIs. Calibrator Diluent RD6-52 served as the blank.

2.9. Measuring IGF-1 using ELISA

Among the samples collected only 17 HIV+, 17 HIV negatives and 8 malaria positives were enrolled for this assay.

2.9.1. Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IGF-I has been pre-coated onto a microplate. Standards and pretreated samples are pipetted into the wells and any IGF-I present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IGF-I is added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IGF-I bound in the initial step. The color development is stopped, and the intensity of the color is measured.

2.9.2. Assay Procedure

ELISA was conducted as described by manufacturer (R&D Systems Inc, NE, USA) on the R&D Systems ELISA kit. Briefly, samples were pretreated by adding 20 µl of plasma to 380 µl of Pretreatment A solution. Fifty µl of sample the resultant solution was then added to 200 µl of reconstituted Pretreatment B solution (prepare by adding 10 ml of deionized water to concentrate). 50µl of the pretreated samples was incubated with 150 µl of Assay Diluent RD1-53 at 4^{o}C in a 96 well plate pre-coated with monoclonal antibodies to IGF-1. The plate was washed four times with 400 µl of Wash Buffer (prepared by Adding 20 mL of Wash Buffer Concentrate to deionized water to prepare 500 mL of Wash Buffer). Two hundred µL of cold IGF-I Conjugate was added to each well and the plate was incubated at 4^{o}C for 1 hour. The plate was washed as described above and 200 µL of Substrate Solution was added to each well and incubated in the dark for 30 minutes. Stop solution (50 µl) with an expected color change of blue to yellow. The plates were read using an ELISA reader (ELISA iMARK BioRad, S#13738, JAPAN) at 450 nm with wavelength correction at 540 nm. Results were expressed in optical density (OD). Standards were prepared by pipetting 360 µl Calibrator Diluent RD5-22 into tube containing 40µl of 60 ng/ml standard and serially diluting the solution seven folds by pipetting 200 µl of the solution into 200 µl of Calibrator Diluent RD5-22 to obtain concentration of 6 ng/ml, 3 ng/ml, 1.5 ng/ml, 0.75 ng/ml, 0.375 ng/ml, 0.188 ng/ml and 0.094 ng/ml respectively) which correlates OD. A five-parameter software the standard curve was plotted from which concentrations of our analytes were derived.

2.10. Measuring Human placenta protein (Galectin) 13 using ELISA:

2.10.1. Principle

This assay is based on biotin double antibody sandwich technology to assay Human placenta protein13 (PP13). Placenta protein13 (PP13) in samples and standards is added to wells that are pre-coated with placenta protein13 (PP13) monoclonal antibody and then incubated. After incubation, anti PP13 antibodies labeled with biotin are added to unite with streptavidin-HRP (Horse Radish Peroxidase), which forms the immune complex. Unbound enzymes are removed after incubation and washing, then substrate A and B are added. The solution will turn blue and change to yellow with the effect of acid. The shades of solution and the concentration of Human placenta protein13 (PP13) are positively correlated.

2.10.2. Assay Procedure

The ELISA assay was conducted as described by manufacturer (MyBioSource). Briefly, 50µl of standard (biotin antibodies are already coupled to standards) and 50µl of streptavidin-HRP incubated in wells allocated to standards in a 96 well plate pre-coated with anti- PP13 antibody for 1hour at 37^{o}C. In the wells allocated to samples, 40µl of sample, 10µl boitinated PP13 antibodies and 50µl streptavidin-HRP were added, gently to mixed and incubate at 37 ^{o}C for 1 hour. The plate was washed 5 times using 400 µl of washing solution (prepared by diluting stock 30 folds with deionized water). Then, 50µl chromogen solution A and 50µl chromogen solution B were added into each well, the plate was gently shook and incubated in the dark for 10 minutes at 37 ^{o}C. Fifty µl of Stop Solution was added to stop the reaction and the plate was read using an ELISA microplate reader (ELISA iMARK BioRad, JAPAN) at 450nm and corrected at 540nm. Results were reported as absorbances (OD). Standards were prepared by serially diluting 120 µl of 2400 pg/ml standard to 5 tubes that contain 120 µl Standard Diluent to obtain concentrations of 1200pg/ml, 600pg/ml, 300pg/ml, 150pg/ml and 75pg/ml respectively. A five-parameter software the standard curve was plotted from which concentrations of our analytes were derived.

2.11. RNA Extraction

2.11.1. Principle

RNA from lyses tissue is trapped on the surface of a silica gel by the aid of a chaotropic agent, then eluted from the gel with a buffer solution.

2.11.2. Procedure

RNA extraction was performed using the Qiagen RNA extraction kit (Gmbh Hilden, Germany) as described by the manufacturer. Briefly, approximately 30 mg of placenta tissue was placed in an Eppendorf tube. Six hundred ul of DDT/RLT buffer was added to the tube and the tissues disrupted using a tissue disruptor (Qiagen, Germany) for 40 seconds. The lysate was centrifuged at 16000 rpm for 3 minutes and the supernatant was carefully collected. A volume of 70% ethanol was added to the supernatant which was homogenized and 700 ul of the mixture was transferred to the RNeasy spin column with 2ml flow-through tube. The columns were spun at 8000 x g, washed with 350 ul of Buffer RW1 and 80 ul of DNase I was added to the RNeasy column. The column was left to incubate for 15 minutes at room temperature. Next, the column was spun at 8000xg after 500 ul of Buffer RPE was added to them and spun for 15s at 8000 x g. Buffer (500 ul) was introduced a second time into the column and the column was spun at 8000 x g for 2 minutes. The column was then placed in a 1.5 ml collection tube and the purified RNA eluted with 50 ul of RNase free water.

The extraction yield was measured using a NanoDrop™ Lite Spectrometer (Thermo Scientifics, Delaware, USA).

2.12. Bioanalyses of RNA quality:

2.12.1. Principle:

Bioanalyzer works under the principle of capillary electrophoresis where molecules are estranged based on their molecular weight on a microgel under an applied voltage. The smaller molecules migrate faster than the larger molecules.

2.12.2. Procedure

The analyses were performed as described by the manufacturer. Briefly, 2ul of RNA ladder was heat denatured for 2 min at 70 °C and immediately cooled on ice. Next, 550ul of gel matrix was filtered at 1500 g for 10 minutes. Sixty-five ul of the filtered was transferred to an RNA-free Eppendorf tube and 1ul of vortexed RNA 6000 Nano dye concentrate added to the tube and homogenized and spun for 10 minutes at room temperature at 13000 g. Nine ul off this gel-dye mix was deposited at the bottom of well-marked **"G"** and circled in black, and well-marked "**G"** and circled in white of a new RNA Nano chip. Five ul of RNA 6000 Nano marker (green) was pipetted into the ladder well and each of the sample wells. One ul of RNA ladder was then added into the ladder well and 1 ul of the sample was pipetted into each sample well. The chip was then vortexed for 1 minute at 2400 rpm. The chip was then placed in an Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) and read.

2.13. *In vitro* transcription

2.13.1. Principle

The amplification of RNA from a single strand to several copies through the use of reverse transcriptase from mRNA to cDNA followed by the conversion from cDNA to RNA using synthetic RNA polymerase (Beckert and Masquida, 2011).

2.13.2. Procedure

First-strand cDNA Synthesis

Extracted RNA was pre-treated as described in Table II below.

Table II: Sample prep-preparation for 1st strand synthesis

	Reagent	Volume(ul)
1	RNase-Free Water	1
2	Total RNA sample	1
3	T7-Oligo (dT) Primer	1
	Total	3

After preparation, the mixture was heated at 65°C for 5 minutes in a water bath. Next, the preparation was chilled on ice for 1 minute. The preparation was set for the 1st strand synthesis.

Table III: Mastermix composition for 1st strand synthesis

	Reagent	Volume(ul)
1	1st-Strand cDNA PreMix	1.5
2	DTT	0.25
3	SuperScript III Reverse Transcriptase (200 U/µl)	0.25
	Total	2

Two ul of the mixture on Table III was then added to the sample preparation tubes. The tubes were thoroughly mixed and incubated 50°C for 30 minutes in a water bath.

Second-strand cDNA Synthesis

Table IV: Sample preparation for 2nd strand synthesis

	Reagent	Volume(ul)
1	2nd-Strand cDNA PreMix	4.5
2	2nd-Strand DNA Polymerase	0.5
	Total	5

The Mastermix prepared on Table IV was gently mixed and 5ul was introduced into the tubes containing the synthesized 1st strand products. This mixture was then 65°C for 10 minutes in a water bath. The tubes were later incubated at 80°C for 3 minutes and chilled, pulsed and chilled on ice.

In Vitro Transcription of Biotin-aRNA

The T7 RNA Polymerase and other reagents were brought to room temperature. The Constituents of the kit were mixed as described in Table V below.

Table V: Sample preparation for *in vitro* Transcription

	Reagents	Volume (ul)
1	T7 Transcription Buffer	2
2	UTP / Biotin-UTP	3
3	NTP PreMix	10
4	DTT	3
5	T7 RNA polymerase	2
	Total	20

Twenty ul of this mix prepared on Table V was then introduced into each of the 2nd strand synthesis sample tubes and the tubes incubated 42°C for 4 hours in a thermocycler (Abbott 3700, USA). Next, 2 µl of RNase-Free DNase I to each reaction tube which was gently mixed and incubated each at 37°C for 15 minutes.

2.14. Microarray

2.14.1. Principle

The basic principle behind the DNA microarray is "nucleic acid hybridization". In this process, two complementary strands of a DNA are bound together by hydrogen bonds to form a double-stranded molecule. This helps researchers to compare and analyze the DNA or RNA molecules of identical sequences (Bumgarner, 2013).

2.14.2. Procedure

In vitro transcribed RNA was preheated at 65°C for 5 minutes vortexed and allowed to cool to room temperature. Five ul of RNA free water and 10 ul of Hybridization Buffer (HYB) was added into the tube. Next, 200ul of Hybridization Chamber Buffer (HCB) was added to the HYB chamber gasket. Next, 15 ul of the pre-prepared sample was introduced into the Microarray hybridization BeadChip (HT12v4, Illumina, California, USA). The sample-laden BeadChip were then placed into the hybridization gasket which was then sealed and placed into a preset illumine hybridization oven (Illumina, California, USA) at 58°C for 15 hours. After hybridization, the BeadChip was then submerged into a dish containing 250ml of E1BC solution where the cover seal of the BeadChip was removed. The BeadChip was then transferred into a Hybex water bath containing High-temp wash buffer and incubated for 10 minutes. The BeadChip was then plunged 10 times into 250 ml of E1BC wash buffer and shook for 5 minutes in the buffer at room temperature. The BeadChip was then plunged 10 times into 250 ml of absolute ethanol and shake for 10 minutes. The Beadchip underwent a second wash with 250ml of E1BC buffer at room temperature. Reaction on the BeadChip was blocked by immersing the chip into 4 ml of Block E1 buffer and rocking it at

maximum speed for 10 minutes. The Beadchip was then immersed in Cy3-streptavidin dye for 10 minutes, washed in E1BC buffer for 5 minutes and dried by centrifugation at 1400 rpm for 4 minutes and prepared for scanning. The Beadchip was scanned using an Illumina iScan (Illumina, California, USA). The iScan reader used lasers to excite the fluor of the single-base extension products on the beads on the BeadChip. The light emitted from the fluor were then recorded as high-resolution images of the BeadChip sections and analyzed to determine SNP genotypes using Illumina's GenomeStudio Gene Expression software.

2.15. Data analyses

Demographic and clinical variables were summarized using descriptive statistics, means and standard deviations or median and interquartile range (IQR), for continuous variables such as age or hemoglobin levels; and frequencies and percentages for categorical variables, e.g., maternal anemia status (yes or no) and HIV-1 infection status (yes or no). Two-sample t-tests or Mann-Whitney U-tests for continuous variables, while Chi-square tests or Fisher's exact tests was used to compare the categorical variables between women with and without HIV-1. The biomarker values were log transformed into natural logarithm scales. The effects of maternal HIV-1 infection on levels of each of biomarkers were evaluated through linear regression models, controlling for the selected demographic and clinical variables. All p values less than 0.05 were considered significant. All statistical analysis was performed using SAS 9.4 and GraphPad Prism 7.0. For the microarray analyses, Genomestudio was used to get raw expression data and the Neqc function in R was used to normalize the data. The Limma package in R was used to analyze the data for the identification of differentially expressed genes (DEGs) in HIV-1 and malaria positive women. DEGs with Fold change>1.5 and p-values <0.05 were then transferred to the Ingenuity pathway analyses(IPA) where pathways and gene networks specifically dysregulatd by HIV-1 and malaria were identified based on best matching scores with established canonical pathways. The NormFinder tool was used to identify suitable reference genes for qPCR.

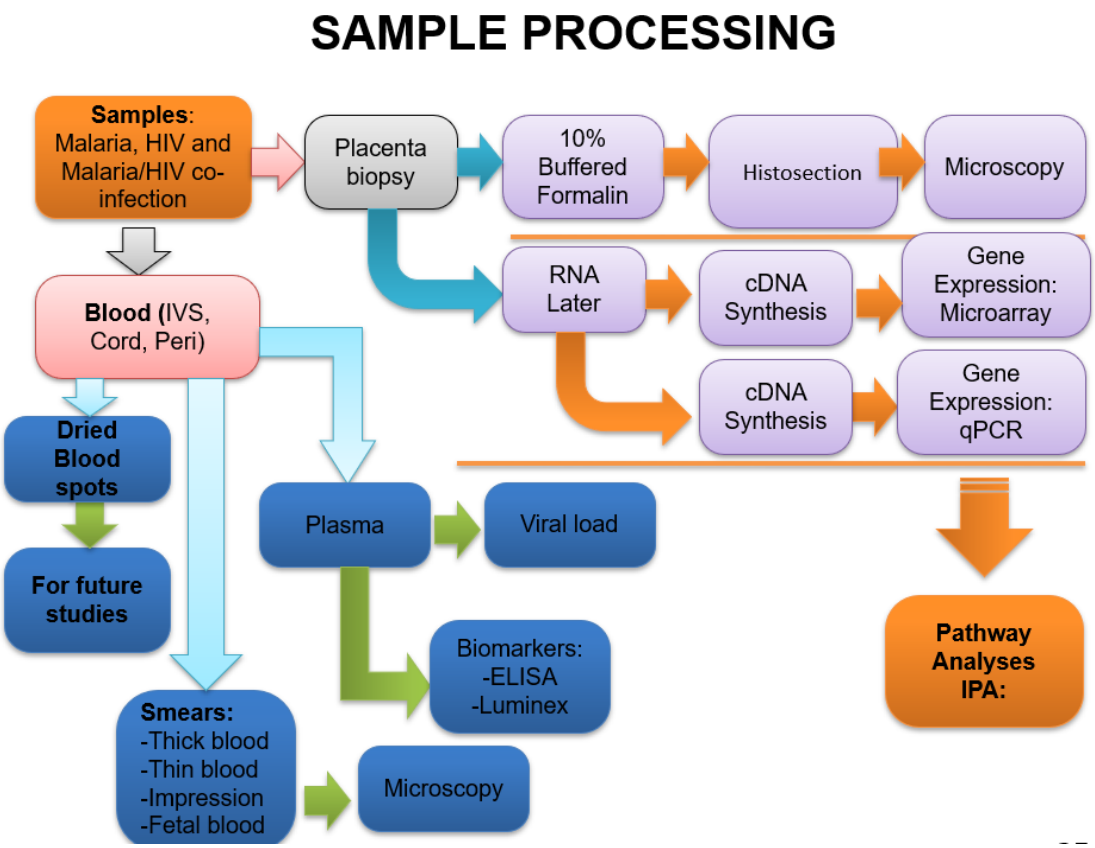

Figure 17: Flowchart of the study

CHAPTER 3: RESULTS

CHAPTER 3: RESULTS

3.1. Characteristics of Study Participants

Demographic and clinical characteristics of study participants at delivery are summarized in Table VI. Overall, 102 women were enrolled in the study, 31 women were HIV-1 positive and 71 women were HIV-1 negative. HIV-1 positive and negative women were similar for the following characteristics: IPT use, hemoglobin level, temperature, blood pressure, peripheral malaria status, parity and pregnancy outcomes: length of gestation, proportion of singleton deliveries and C-section, neonate sex, neonate weight and prevalence of LBW babies (all p-values>0.05). However, HIV-1 positive women were older compared to their healthy counterparts (p=0.027) with average age of 30.0 ± 5.1 vs. 27.3 ± 5.8 years, respectively. Majority (83.9%) of the HIV-1 positive pregnant women were receiving ART, and most of the women were on Tenofovir Lamivudine and Efavirenz tritherapy. HIV viral load was available for 14(47%) HIV-1 positive women with median (25th, 75th) of 683 (0, 130,680) copies/µl. CD4 counts were available for 9 (30%) HIV-1 positive women; median (25th, 75th) of 350 (248, 675) cells/µl. Four (13%) HIV-1 positive pregnant women were also infected with placental malaria.

Table VI: Sociodemographic and clinical characteristics of the mothers

Characteristic	HIV-1 (-)	HIV-1 (+)	p-value
Number of enrolled participants, n	71	31	-
Age in years, mean ± SD	27.3 ± 5.8	30.0 ± 5.1	**0.027**
Maternal fever, n (%)	18 (25.4)	6 (19.4)	0.35
Maternal weight in kg, mean ± SD	75.7 ± 12.5	73.9 ± 12.9	0.64
Maternal BMI in kg/m^2, mean ± SD	29.1 ± 4.3	28.6 ± 3.7	0.72
Maternal hemoglobin level in g/dL, mean ± SD	12.1 ± 1.6	11.7 ± 1.7	0.41
Maternal anemia, n (%)	13 (18.3)	6 (19.4)	0.73
ART use by pregnant women, n (%)	0	26 (83.9)	-
Maternal viral load, median, (25^{th}, 75^{th})	0	683 (0, 130,680)	-
Maternal CD4 Count median, (25^{th}, 75^{th})	N/A	350 (248,675)	-
Maternal IPT use, n (%)	60 (84.5)	30 (96.8)	0.18
Number of SP doses pregnant women took, median, (25^{th}, 75^{th})	2 (1, 3)	2 (2, 2)	0.92
Maternal bednet use, n (%)	52 (73.2)	26 (83.9)	0.41
Maternal heart rate in beats per minute, mean ± SD	84.7 ± 13.9	88.5 ± 15.9	0.39

Maternal blood pressure in mmHg, mean ± SD			
Systolic	120.9 ± 17.6	119.7 ± 8.8	0.70
Diastolic	75.2 ± 13.3	76.1 ± 8.7	0.74
Maternal peripheral malaria by blood smears, n (%)	11 (15.5)	4 (12.9)	0.75
Maternal parasite density in peripheral blood[£] in parasites/uL, median (25^{th}, 75^{th})	1,880 (400, 15,940)	1,080 (440, 12,490)	0.61
Malaria by RDT on maternal **peripheral blood**	14 (19.7)	4 (12.9)	0.44
Placental malaria, n (%)	10 (14.1)	3 (9.7)	0.33
Parasitemia[£] in %, median (25^{th}, 75^{th})	5.35 (0.06, 26.0)	0.23 (0.03, 0.61)	0.11
Parity including current child, median (25^{th}, 75^{th})	2 (1, 3)	3 (1, 4)	0.40
Primigravidae, n (%)	12 (16.9)	4 (12.9)	0.46
Multigravidae, n (%)	41 (57.8)	22 (71.0)	0.46
Length of gestation in weeks, mean ± SD	39.2 ± 3.0	38.9 ± 2.6	0.66
Preterm deliveries, n (%)	10 (14.1)	6 (19.4)	0.59
C-section, n (%)	6 (8.5)	5 (16.1)	0.28

Similar to the mothers, there was no major difference between clinical parameters between children born to HIV-1 positive women and healthy controls as depicted by Table VII below

Table VII: Demographic and clinical characteristics of neonates

Characteristic	HIV-1 (-)	HIV-1 (+)	p-value
Singleton deliveries, n (%)	67 (94.4)	29 (93.6)	0.59
Male neonates, n (%)	38 (53.5)	20 (64.5)	0.38
Placental weight in g, mean ± SD	616 ± 155	609 ± 177	0.85
Neonate weight in g, mean ± SD	3169 ± 587	3127 ± 497	0.74
Low birth weight, n (%)	6 (8.5)	2 (6.5)	1.00
APGAR at 1min, mean ± SD	7.9 ± 1.5	8.4 ± 1.0	0.17
APGAR at 5min, mean ± SD	8.8 ± 1.4	8.9 ± 1.0	0.81
Cord malaria infection by blood smears, n (%)	0	0	-

3.2. Angiopoietin 1 and 2 are not dysregulated in HIV-1 positive women on antiretroviral therapy.

The placental levels of ANG1 and ANG2 biomarkers in natural logarithm scales by HIV-1 status are presented in Table 3. There was no significant difference between HIV-1 positive and HIV-1 negative women in ANG1 ($p = 0.68$) and ANG2 ($p = 0.20$). In the general linear regression models

adjusted for maternal age and malaria status (Table IX) HIV-1 infection did not have significant impact on ANG1 (p = 0.93) and ANG2 (p = 0.33). In order to determine whether angiopoietins are dysregulated during HIV-1 infection, ANG1, ANG2, as well as ANG2/ANG1 ratio were measured in placental intervillous space plasma from HIV-1 positive PM-negative and HIV-1 negative PM-negative women. No significant differences in ANG1 or ANG2 (all p>0.05) were observed between women with HIV-1 and their healthy counterparts (Figure 18). No significant differences between HIV-1 negative PM positive women and their healthy counterparts were observed for either ANG1 or ANG2 (all p>0.05, Figure 18). Although, ANG1 was lower in 3 co-infected pregnant women compared to healthy women, the difference was not statistically significant (p = 0.09, Figure 18).

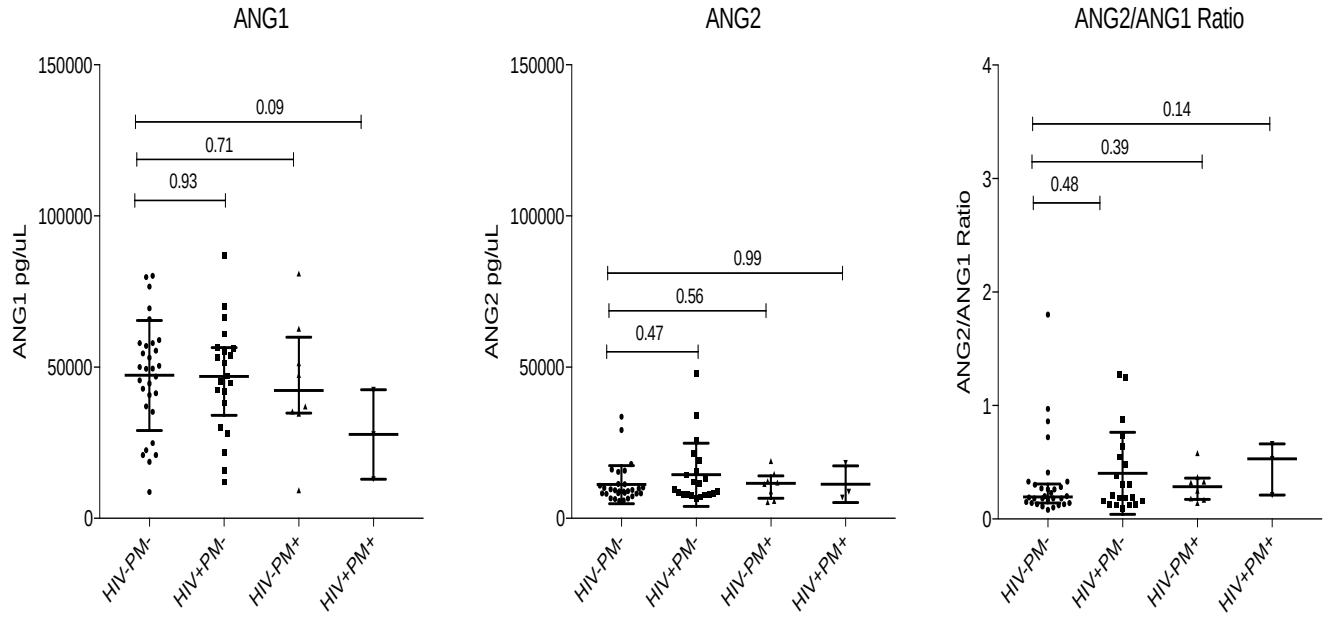

Figure 18: Angiopoietin levels in placental intervillous space

Legend:ANG1 and ANG2 levels, as well as ANG2/ANG1 ratios were measured in placental intervillous space in healthy (HIV-&PM-, n=30), HIV-infected (HIV+&PM-, n=21), PM-positive (HIV&PM+, n=8) and co-infected (HIV+&PM+, n=3) women. Median and interquartile ranges (IQR) are plotted; differences between the healthy and infected women were assessed using the Mann-Whitney test. HIV: Human Immunodeficiency Virus; ANG1: Angiopoietin 1; ANG2: Angiopoietin 2; PM: Placenta Malaria positive mothers.

3.3. IGF axis is not dysregulated in HIV-1 infected women receiving antiretroviral therapy

The placental biomarkers levels of IGF axis in natural logarithm scales by HIV-1 status are presented in Table VIII. There was no significant difference between HIV-1 positive and HIV-1 negative women in IGF1 (p = 0.76) and IFGBP1 (p = 0.92). In linear regression models adjusted

for maternal age and malaria status (Table IX), IGF-1 was not significantly reduced as a result of HIV-1 (p = 0.12) but due to malaria status (p = 0.0038), while no effect of HIV-1 on IFGBP1 was identified (p = 0.84). The impact of HIV-1 on the IGF axis was evaluated by probing placental intervillous space plasma obtained from HIV-1 positive PM-negative, HIV-1 negative PM-negative and HIV-1 negative PM-positive women for IGF-1 and IGFBP1 and IGFBP3. Lower but not significant levels (p = 0.3) of IGF-1 were observed in HIV-1 positive PM-negative women compared to healthy women (Figure 19). No significant differences in IGFBP1 were observed between HIV-1 infected and healthy women. In linear regression models adjusted for maternal age and anemia status no significant effect of HIV-1 on IGFBP1 was identified (Table VIII). IGFBP3 was not detected in any of the samples and thus excluded from the analysis

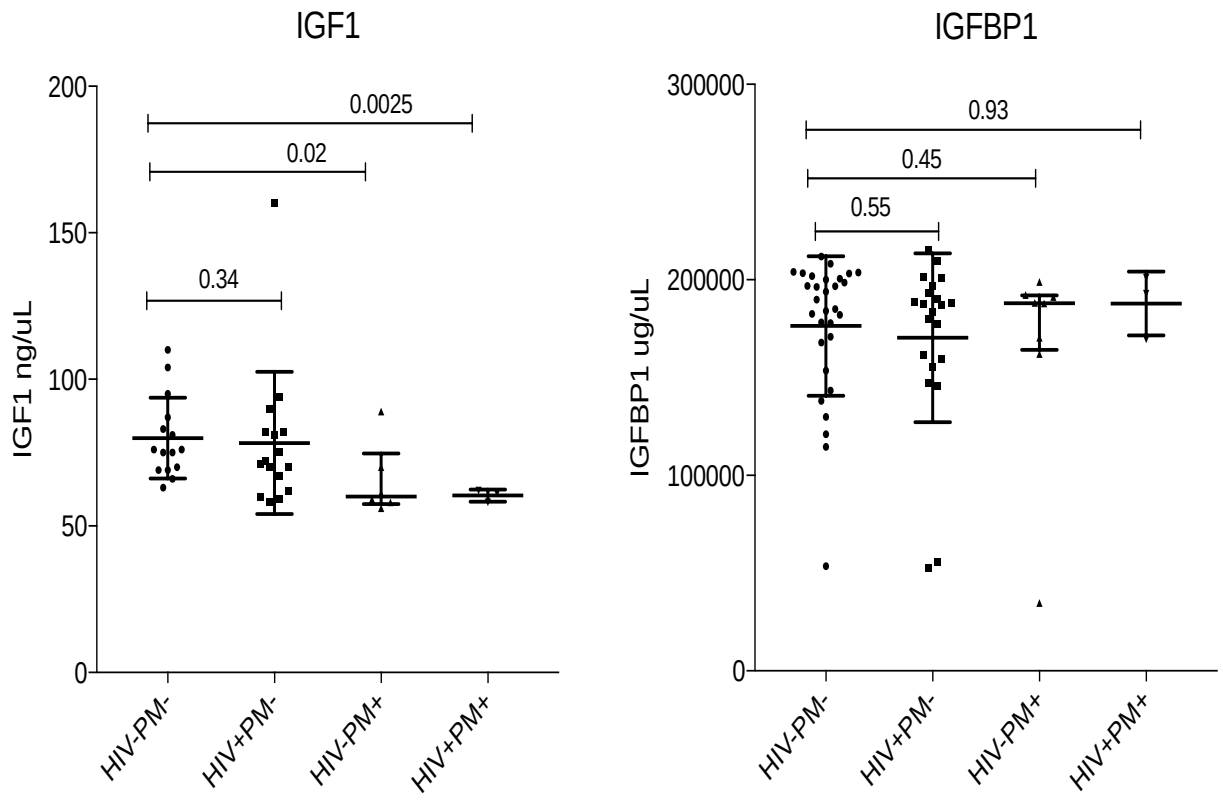

Figure 19: IGF1 and IGFBP1 levels in placental intervillous space.

Legend: IGF1 levels were measured in placental intervillous space plasma of healthy (HIV-&PM-, n=15), HIV-infected (HIV+&PM-, n=16), PM-positive (HIV-&PM+, n=6) and co-infected (HIV+&PM+, n=3) women. IGFBP1 and IGFBP3 levels were also measured in healthy (HIV-&PM-, n=30), HIV-infected (HIV+&PM-, n=21), PM-positive (HIV-&PM+, n=8) and co-infected (HIV+&PM+, n=3) women. Median and interquartile ranges (IQR) are plotted; differences between the healthy and infected women were assessed using Mann-Whitney test. HIV: Human

Immunodeficiency Virus; IGF1: Insulin Growth Factor 1; IGFBP1: IGF Binding Protein 1; PM: Placenta Malaria positive mothers.

3.4. HIV-1 is not associated with decreased levels of MMP2, MMP9 and Gal-13

The placental levels of MMP2, MMP9 and Gal-13 biomarkers in natural logarithm scales by HIV-1 status are also presented in Table xxx. There was no significant difference between HIV-1 positive and HIV-1 negative women in MMP9 ($p = 0.91$), but marginally significant in MMP2 ($p = 0.066$) and Gal-13 ($p = 0.060$). After adjusting for maternal age and malaria status, HIV-1 status had no significant impact on MMP2, MMP9 and Gal-13 (all $p>0.05$, Table VIII). The impact of HIV-1 on additional biomarkers of placental formation and vascularization were also explored, including MMP2, MMP9 and Gal-13. No significant differences were observed between HIV-1 positive PM-negative and healthy women for MMP2 and MMP9 levels in intervillous space plasma (all $p>0.05$, Figure 20and Table VIII). No significant differences in MMP2 and MMP9 levels were observed between HIV-1 negative PM-positive women and their healthy counterparts (all $p>0.05$, Figure 20). Intervillous space plasma Gal-13 levels were not significantly different between HIV-1 positive and healthy women (Figure 20), and linear regression model showed HIV-1 had no significant impact on Gal-13 levels (Table IX).

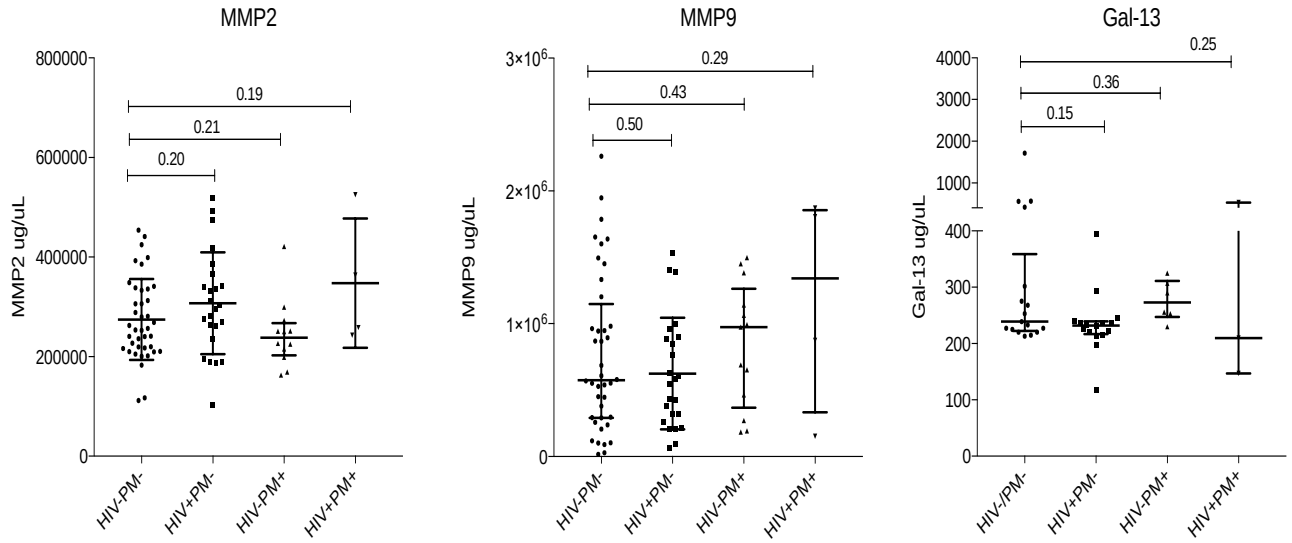

Figure 20: MMP and Gal 13 levels in plasma from placental intervillous space plasma.

Legend: MMP2 and MMP9 levels were measured in placental intervillous space in uninfected (HIV-&PM-, n=40), HIV-infected (HIV+&PM-, n=24), PM-positive (HIV-&PM+, n=12) women and co-infected (HIV+&PM+, n=4) women. Gal-13 were measured in intervillous space plasma

of healthy (HIV-&PM-, n=17), HIV-infected (HIV+&PM-, n=16), PM-positive (HIV-&PM+, n=6) and co-infected (HIV+&PM+, n=3) women. Median and interquartile ranges (IQR) are plotted; differences between the healthy and infected women were assessed using Mann-Whitney test. HIV: Human Immunodeficiency Virus; MMP: Matrix Metalloproteinase; Gal-13: Galectin-13; PM: Placenta Malaria positive mothers.

3.5. Validation of intervillous space blood collection and placental histopathology.

A total of 9 random intervillous blood samples were tested for fetal blood contamination. The average proportion of fetal erythrocytes in intervillous space blood was 1.7 ± 0.3%, which shows that the level of contamination was extremely low (Figure 21). Thus, the sample collection methodology was validated, and the experiment results are reflective of what occurs on the maternal side of the placenta. Placental weight was not significantly different between HIV-1 positive and HIV negative women (p = 0.85, Table VIII). In placentas from HIV-1 positive mothers, lesions and syncytial knots were occasionally observed; placentas from HIV-1 and PM co-infected women had lesions, fibrinoid tissue (Figure 22). Prevalence of placental malaria was not significantly different between HIV-1 positive (9.7%) and uninfected (14.1%) women (p = 0.33, Table VI). Except in women coinfected with malaria and HIV, women with PM did not have placental inflammation (Figure 21).

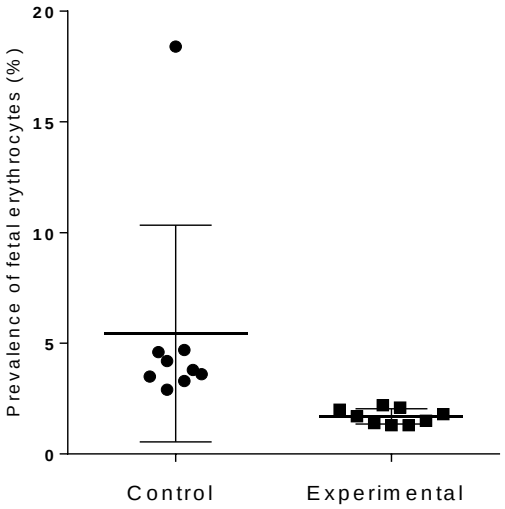

Figure 21: Fetal blood contamination of intervillous space blood. Nine randomly selected maternal intervillous blood samples were screened for the presence of fetal erythrocytes (experimental). In addition, a known amount of cord blood was mixed with corresponding maternal intervillous space blood as a positive control. Percentage of fetal erythrocytes in each intervillous blood sample was determined; mean and standard deviation for the samples are presented in Figure 21.

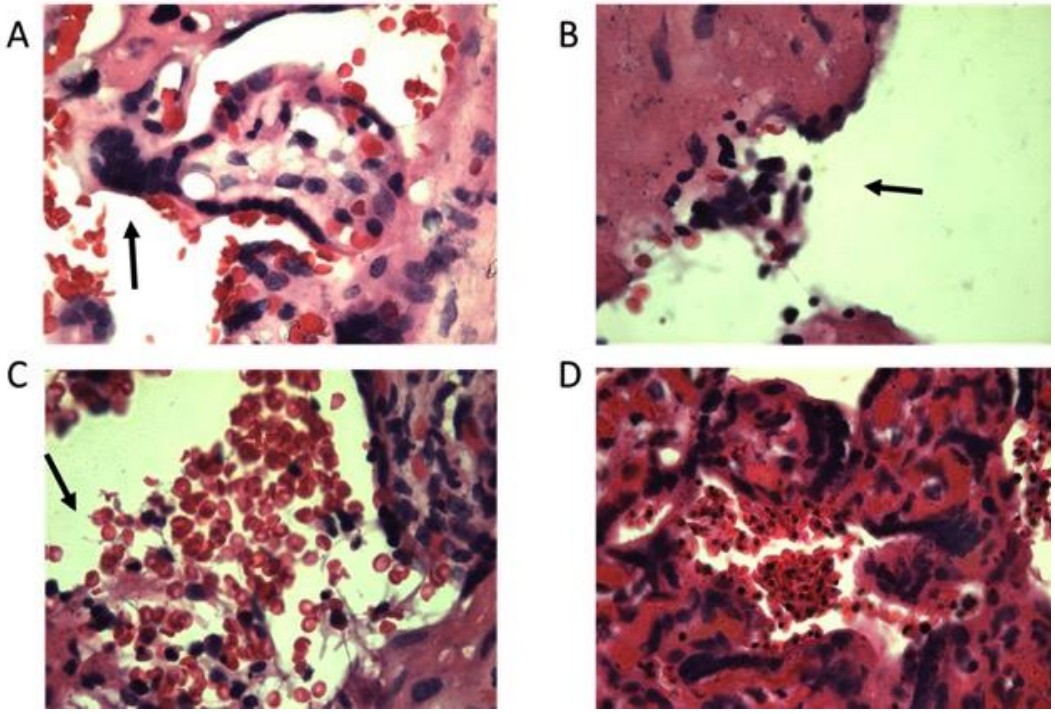

Figure 22: Placental histology.

Legend: A. HIV-1 infected woman. Arrow points to a syncytial knot. B. HIV-1 and placenta malaria co-infected woman. Arrow points to a lesion. C. HIV-1 and PM co-infected woman. Arrow points to fibrinoid tissue. D. HIV-1 negative placenta malaria-positive woman. Infected erythrocytes are present in large numbers, no monocytes. 400x magnification.

Table VIII . Placental biomarker levels by HIV-1 status

Biomarker	HIV-1 (-) n=71	HIV-1 (+) n=31	p-value
ANG1(pg/ul)	10.64 ± 0.53	10.6 ± 0.52	0.68
ANG2(pg/ul)	9.22 ± 0.42	9.38 ± 0.54	0.20
IGF1 (ng/ul)	4.31 ± 0.19	4.29 ± 0.24	0.76
IGFBP1(ug/ul)	12.02 ± 0.36	12.01 ± 0.36	0.92
MMP2(ug/ul)	12.45 ± 0.30	12.60 ± 0.36	**0.066**
MMP9(ug/ul)	13.18 ± 1.05	13.15 ± 0.87	0.91
Gal-13(ug/ul)	5.70 ± 0.47	5.45 ± 0.31	**0.06**

*Biomarker levels were log transformed and the data were summarized by mean ± SD, based on non-missing values

Table IX: Placental biomarker level changes due to HIV-1

		HIV-1 (+) vs. HIV-1 (-)		Malaria (+) vs. Malaria (-)		Age	
Biomarker	R^2	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
ANG1(pg/ul)	0.081	0.012 (-0.26, 0.29)	0.93	-0.22 (-0.55, 0.11)	0.19	-0.020 (-0.043, 0.004)	0.11
ANG2(pg/ul)	0.036	0.14 (-0.14, 0.41)	0.33	-0.038 (-0.37, 0.29)	0.82	0.011 (-0.015, 0.031)	0.49
IGF1(ng/ul)	0.27	-0.086 (-0.19, 0.022)	0.12	-0.19 (-0.32, -0.067)	**0.0038**	-0.004 (-0.014, 0.006)	0.44
IGFBP1(ug/ul)	0.0038	0.10 (-0.23, 0.19)	0.84	0.12 (-0.29, 0.21)	0.75	-0.002 (-0.019, 0.016)	0.84
MMP2(ug/ul)	0.039	0.12 (-0.042, 0.28)	0.15	0.092 (-0.21, 0.15)	0.74	0.007 (-0.012, 0.016)	0.79
MMP9(ug/ul)	0.029	0.018 (-0.48, 0.52)	0.95	0.28 (-0.19, 0.93)	0.20	0.021 (-0.055, 0.028)	0.53
Gal-13(ug/ul)	0.089	-0.25 (-0.55, 0.042)	0.090	0.17 (-0.33, 0.30)	0.82	0.013 (-0.031, 0.023)	0.79

3.6. Sociodemographic and clinical parameters of selected sample subset for microarray analyses

Amongst the subset of samples identified for this objective, maternal parameters were not significantly different between HIV-1 positive women under ART and HIV negative women but for systolic pressure which was reduced in HIV-1 infected women (p=0.04). Similarly, neonatal parameters were not significantly different between the two groups as shown on Table X.

Table X: Sociodemographic and clinical characteristics of study participants for HIV-1 positive samples selected for microarray

Characteristic*	HIV-1 (-)	HIV-1 (+)	p-value
Enrolled participants (n)	8	9	
Age in years (mean ± SD)	27.4 ±5.45	28.1 ±5.58	0.79
Alcohol use during pregnancy (n, %) **	4(50%)	2(22.2%)	0.33
Axillary temperature (°C) (median, IQR)	37.3(36.1-37.4)	37.1(36.6-37.3)	0.3
Fever (>37.5°C)	1(14.3%)	1(14.3%)	0.99
BMI (mean ± SD)	30.04(26.57-32.39)	27.23(26.55-28.44)	0.38
Hemoglobin levels (g/dL) (median, IQR)	12.1(11.45-12.9)	12.45(11.75-13.6)	0.46
Anemia (<11g/dL) (n, %)	1(12.5%)	1(12.5%)	0.99
ART use (n, %)	/	9/9(100%)	/
IPT use (n, %)	7(100%)	9(100%)	0.99
Number of SP doses (median, IQR)	2(1-3)	2(1.5-3)	0.97
Bednet use (n, %)	5(62.5%)	8(88.89%)	0.29
Maternal heart rate, beats per minute (median, IQR)	80(76-83)	70(60-100)	0.63
Maternal blood pressure in mmHg (median, IQR)			
Systolic	126(112.3-130)	120(110-120)	0.22
Diastolic	90(75.25-90)	80(70-80)	**0.04**
Primigravidae (n, %)	3(37.5%)	2(22.2%)	0.62
Multigravidae (n, %)	5(62.5%)	7(77.8%)	0.62
Length of gestation in weeks (median, IQR)	39 ±1.5	39.2±1.2	0.74
Preterm deliveries (<37 weeks) (n, %)	1(12.5%)	0(0%)	/
Singleton deliveries (n, %)	8(100%)	9(100%)	/
C-section (n, %)	1(12.5%)	4(44.4%)	0.29
Male neonates (n, %)	2(25%)	7(77.8%)	0.99
Placental weight in g (median, IQR)	560(525-610)	560(502.5-742.5)	0.62
Neonate weight in g (mean ± SD)	3090 ± 260.1	3417 ± 556.5	0.15
Low birth weight (<2500g) (n, %)	0(0%)	0(0%)	/
APGAR at 1min (median, IQR)	8(7-8)	9(6.5-9)	0.3
APGAR at 5min (median, IQR)	9(8.25-9.75)	9(9-10)	0.67
Cord malaria infection by blood smears (n)	0	0	-

*** Numbers may not add up to 17 due to missing responses.**

**** None of the study participants were smoking during pregnancy.**

3.6.1 Principal component analyses of samples

The principal component analyses on Figure 23 shows the interrelatedness between sample types. There is greater homogeneity in sample characteristic in the controls than there is between HIV-1 positive and malaria positive samples.

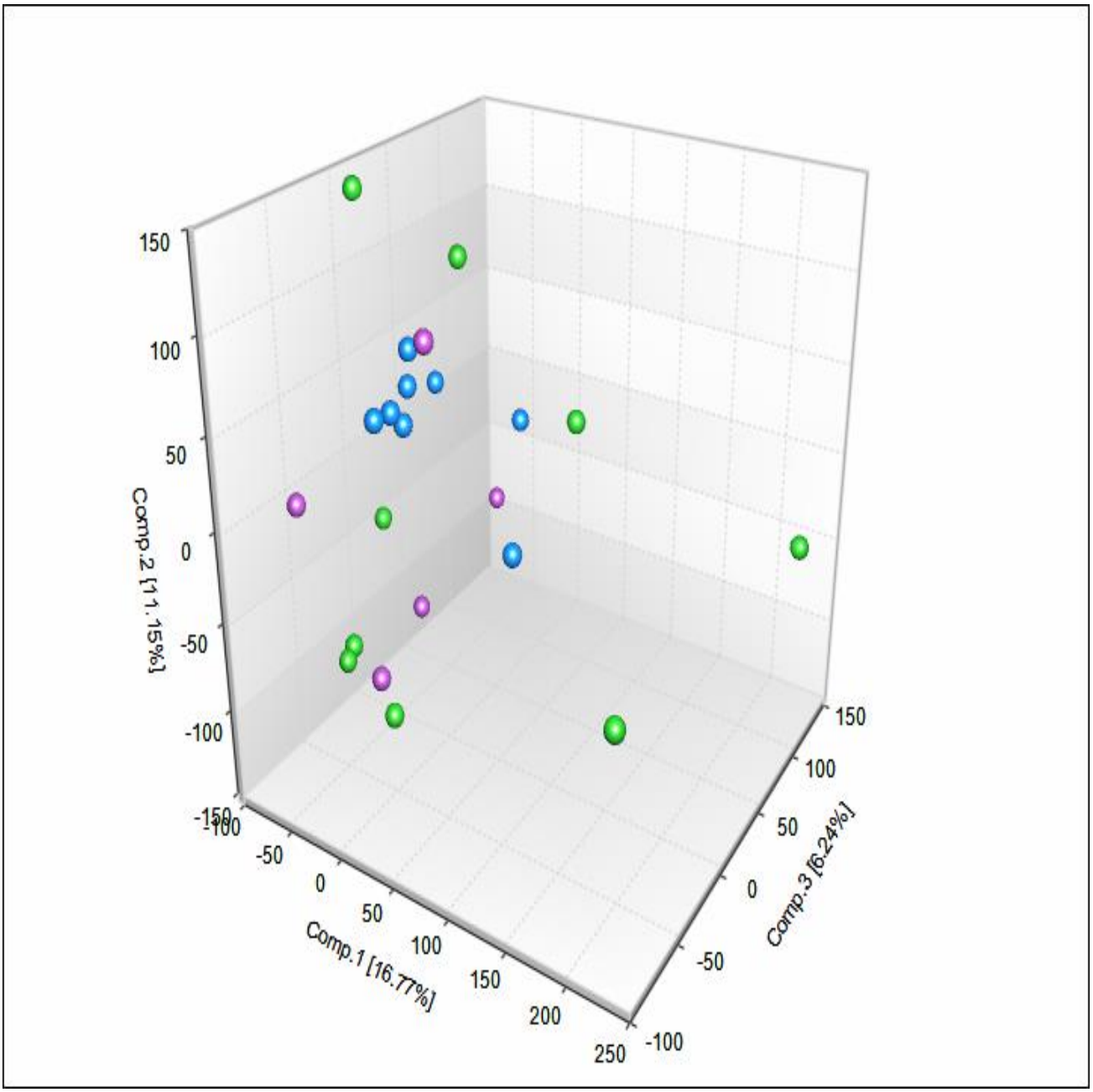

Figure 23: Principal Component Analysis (PCA) for malaria positive, HIV-1 positive, HIV-1/Malaria coinfected and healthy individuals

3.7. Differentially expressed genes in the placenta altered by HIV-1 and ART

The Limma function in R was used to identify differentially expressed genes in the placenta of HIV-1 infection under ART.

Table XI: Differentially expressed genes in the placenta of HIV-1 infected women

Attribute	HIV
Genes assessed	47310
Mapped genes	37435
Threshold transferred to IPA	p<0.05
Genes assed in IPA	349
Genes mapped in IPA	292
Threshold for p-values in IPA	0.05
Threshold for Fold change	1.5
Genes up regulated	166
Gene Down regulated	126

Table XI summarizes the number of differentially expressed genes identified after the set threshold was set. Out of the 47310 genes on the coupled to Illumina beadchip, 37435 genes could be mapped on Ingenuity Pathway Analyses (IPA). After setting a significant level of p<0.05 and fold change

Expr Fold Change up-regulated

Molecules	Expr. Value
RPS4Y1	6.676
EIF1AY	4.282
EPYC	3.686
KRT17	3.435
SERPINA3	3.254
ISG15	3.049
AHNAK2	2.985
KDM5D	2.855
TNNI2	2.746
INS-IGF2	2.376

Expr Fold Change down-regulated

Molecules	Expr. Value
XAGE1B/XAGE1E*	-5.182
MAGEA4*	-3.181
MUCL1	-3.103
SLC39A8	-3.035
IQCG	-3.027
ERV3-1	-2.810
LGSN	-2.661
SERPINI1*	-2.536
MAPK4*	-2.366
CDH3	-2.330

Figure 24: Top up-regulated and down-regulated genes by HIV-1 from IPA

of 1.5, 292 genes were dysregulated by HIV-1, of which 166 were up-regulated and 126 were down-regulated.

3.7.1. Top genes dysregulated in the placenta by HIV-1 infection

The best top ten up-regulated and down-regulated genes are shown on Figure 24 with RPS4Y1 as most upregulated gene and XAGE1B/XAGE1E is the most down-regulated gene.

3.8. Canonical pathways and gene networks affected by HIV-1

3.8.1. Canonical Pathways:

Overall, thirty-six pathways were identified to be affected by HIV-1 on IPA. However, only 3 pathways were significantly upregulated by HIV which are: Leucocyte Extravasation signaling, Go12/13 Signaling, and LXR/RXR activation pathway as depicted on Figure 25.

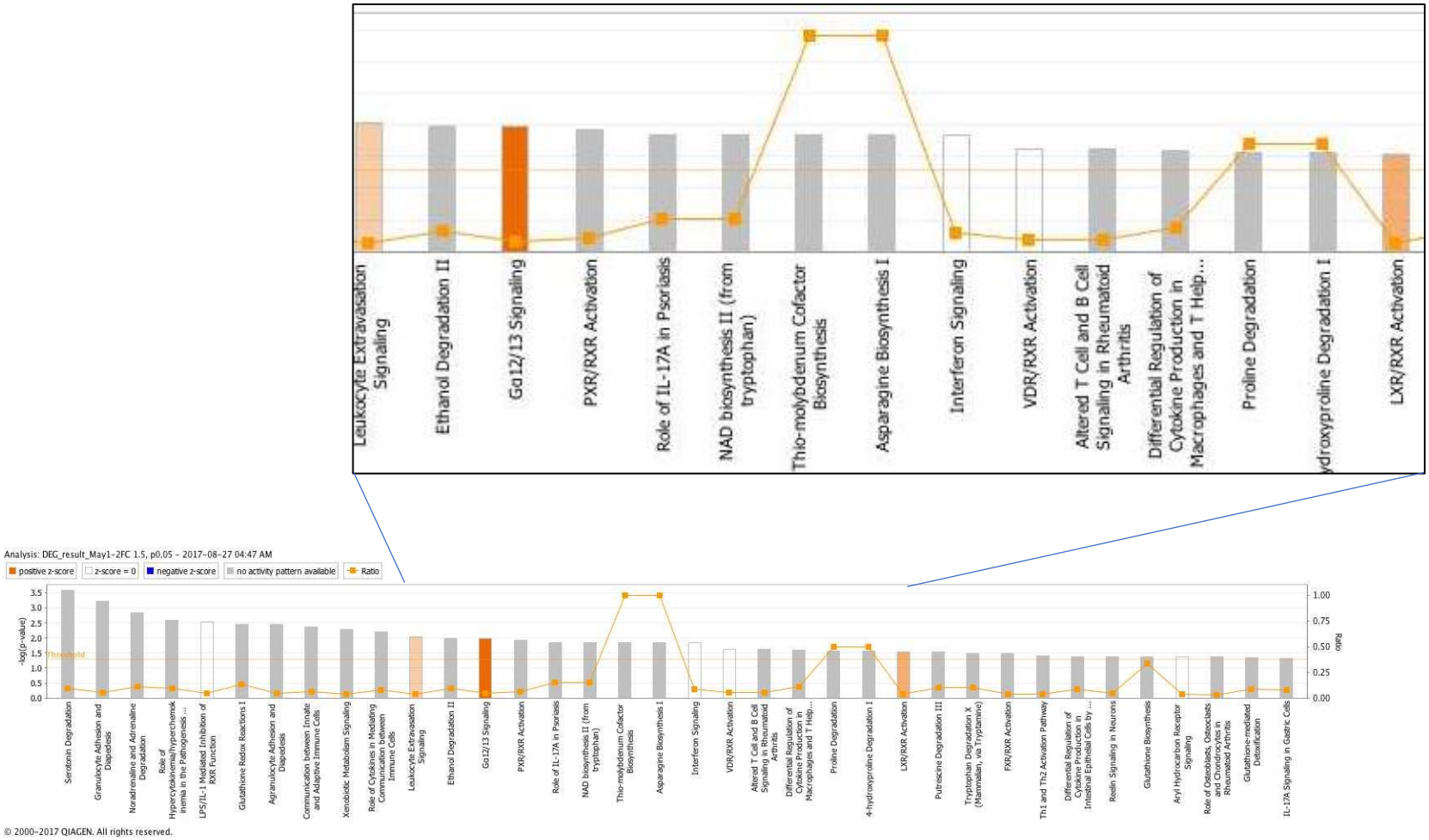

Figure 25: Canonical Pathways affected by HIV-1

3.8.1.1. Leucocyte extravasation pathway affected in placenta of HIV-1

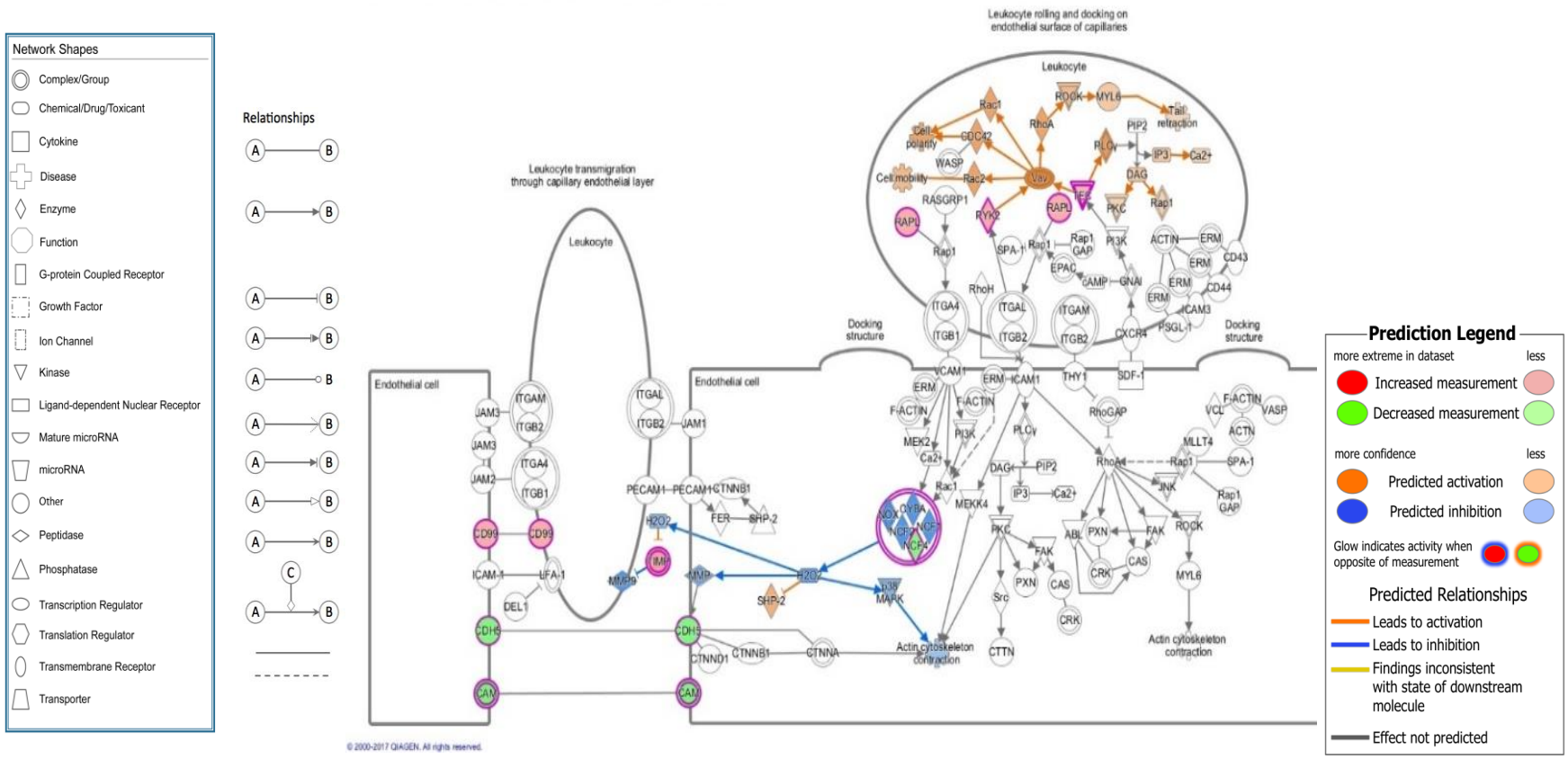

Figure 26: Leucocyte extravasation pathway affected in placenta of HIV-1 infected women

Key genes activated in this pathway are responsible for the recruitment of immune cells to the tissue. This process is modulated by molecules like into Vav as depicted on Figure 26 above.

3.8.1.2. G12/13 Pathway affected in Placenta of HIV infected women

HIV-1 affects molecular interaction in this pathway through G12/13 molecule, the activation of this molecule results in a cascade of reactions that activate cellular differentiation and proliferation as depicted on Figure 27.

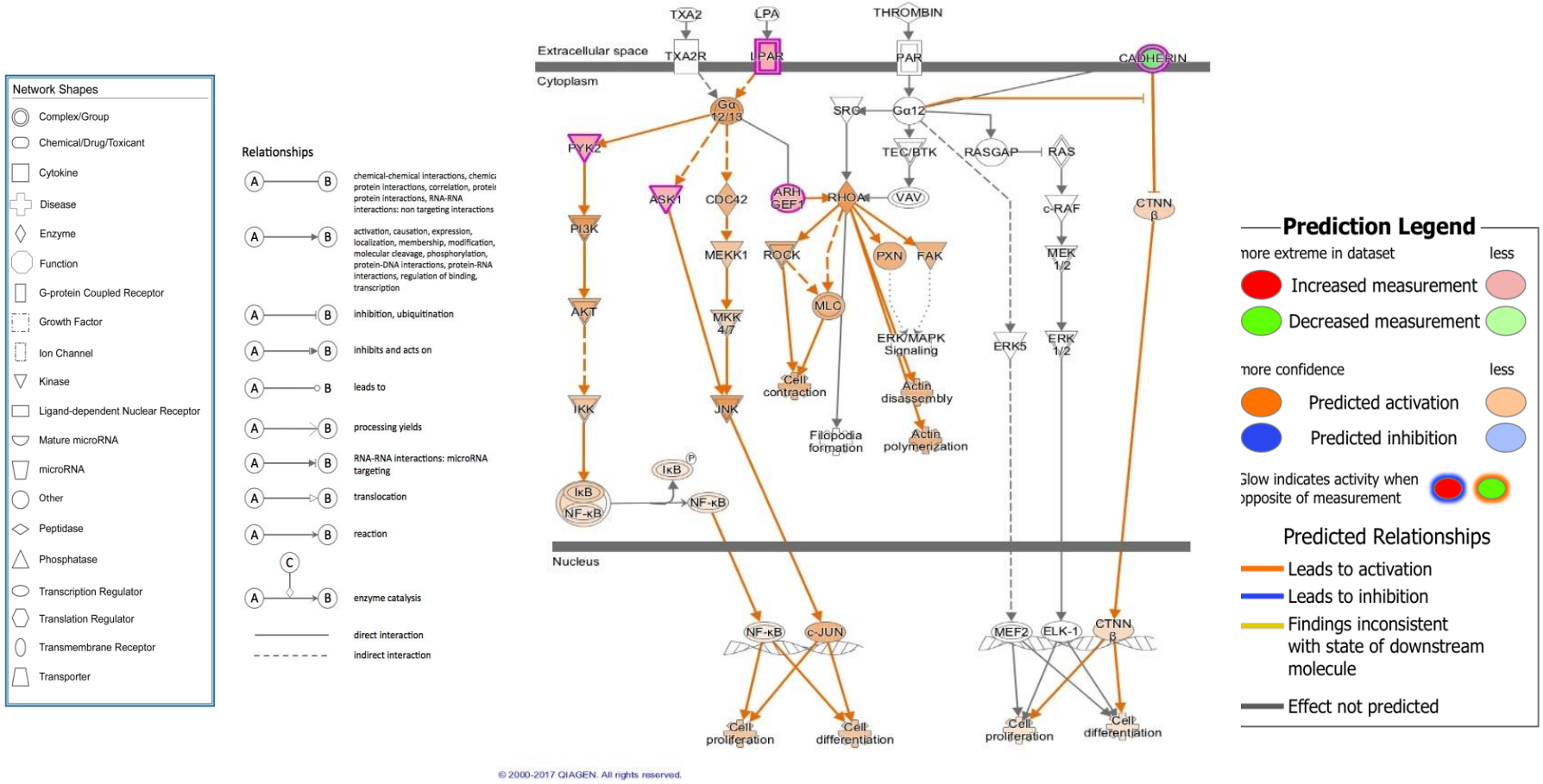

Figure 27: G12/13 Pathway affected in Placenta of HIV infected women

3.8.1.3. LXR/RXR signaling Pathway affected in Placenta of HIV infected women

HIV-1 affects molecules in the LXR/RXR signaling pathway as depicted on Figure 28 below

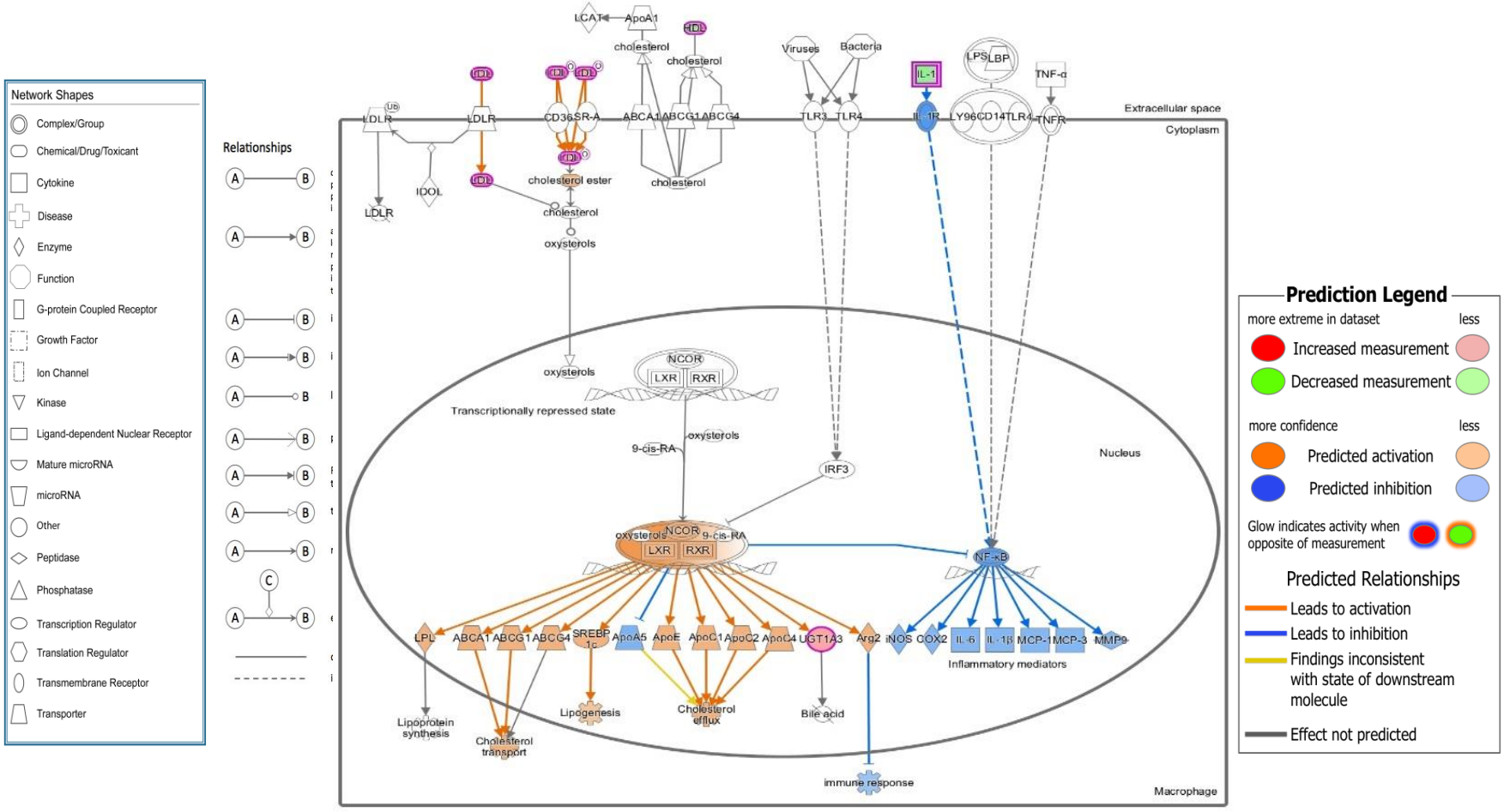

Figure 28: LXR/RXR signaling Pathway affected in Placenta of HIV infected women

3.8.2. Comparative analyses of gene networks

Four top gene networks affected by HIV-1 with scores above 20 were captured by analyses.

They are as follows:

3.8.2.1. Antimicrobial Response, Inflammatory Response, Cell Death and Survival

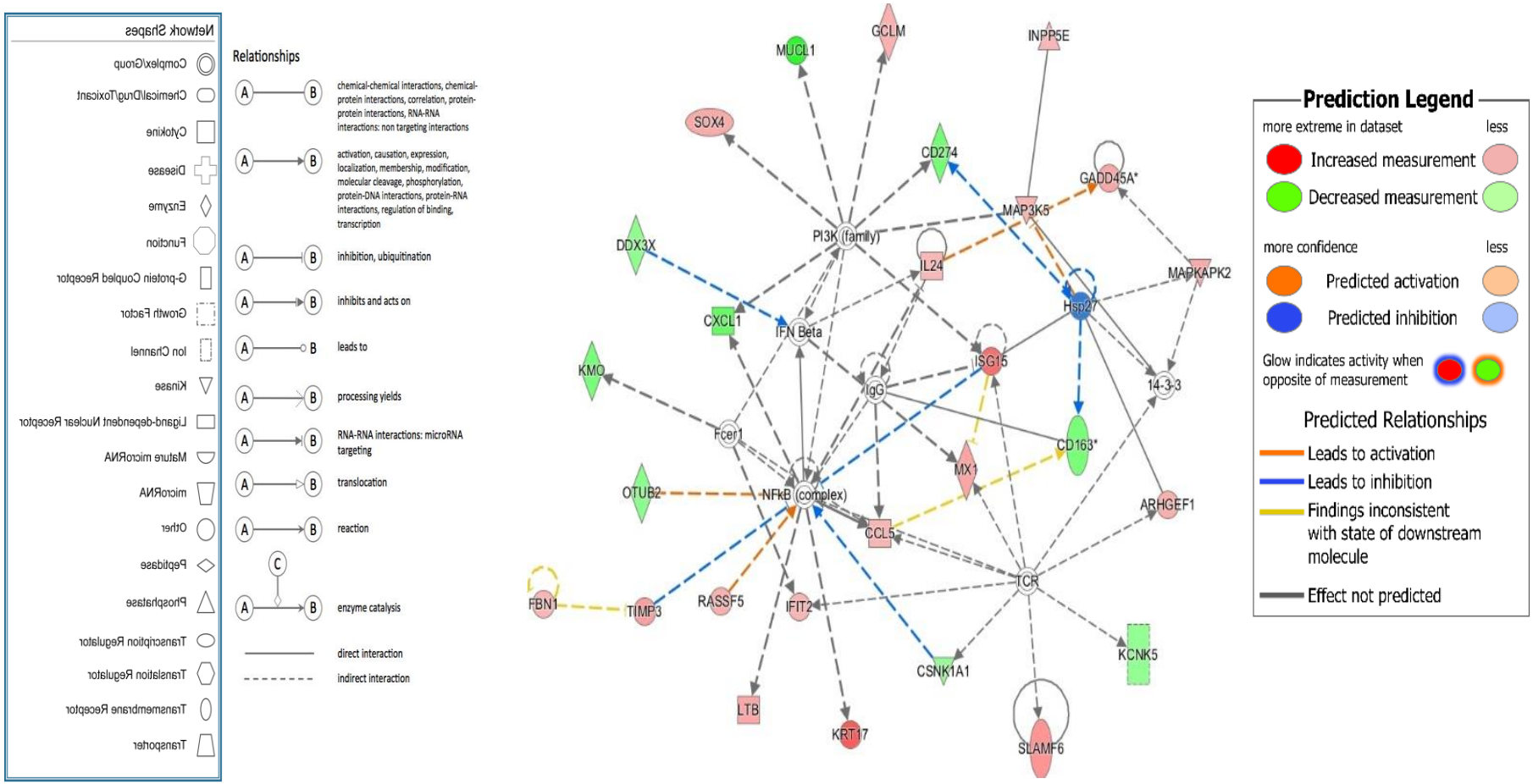

Figure 29: Network 1.

Legend: HIV-1-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio with regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

Network 1 had the highest score (43) and assembled 27 genes were specifically affected by HIV-Eighteen of these genes were upregulated whereas 9 were downregulated. This network has in central position NFkB complex, PI3 family, IFN beta, Fcer 1 and TCR. In this pathway, upregulated molecule (RASSF5) is predicted to indirectly activate NFk B complex whereas the concomitant upregulation of TIMP3, and down-regulation of CSNk1A1 are shown to indirectly inhibit NFkB. Elevated CCL5 levels were also observed to be elevated in HIV-1 positive placenta as a result of NFkB action. Similarly, the downregulation of DOX3Xgene is predicted to indirectly activate IFN beta pathway. This network was correlated with the following functions: Antimicrobial Response, Inflammatory Response, Cell Death and Survival as shown on Figure 29.

3.8.2.2. Cellular movement, cell death, and survival and cell morphology.

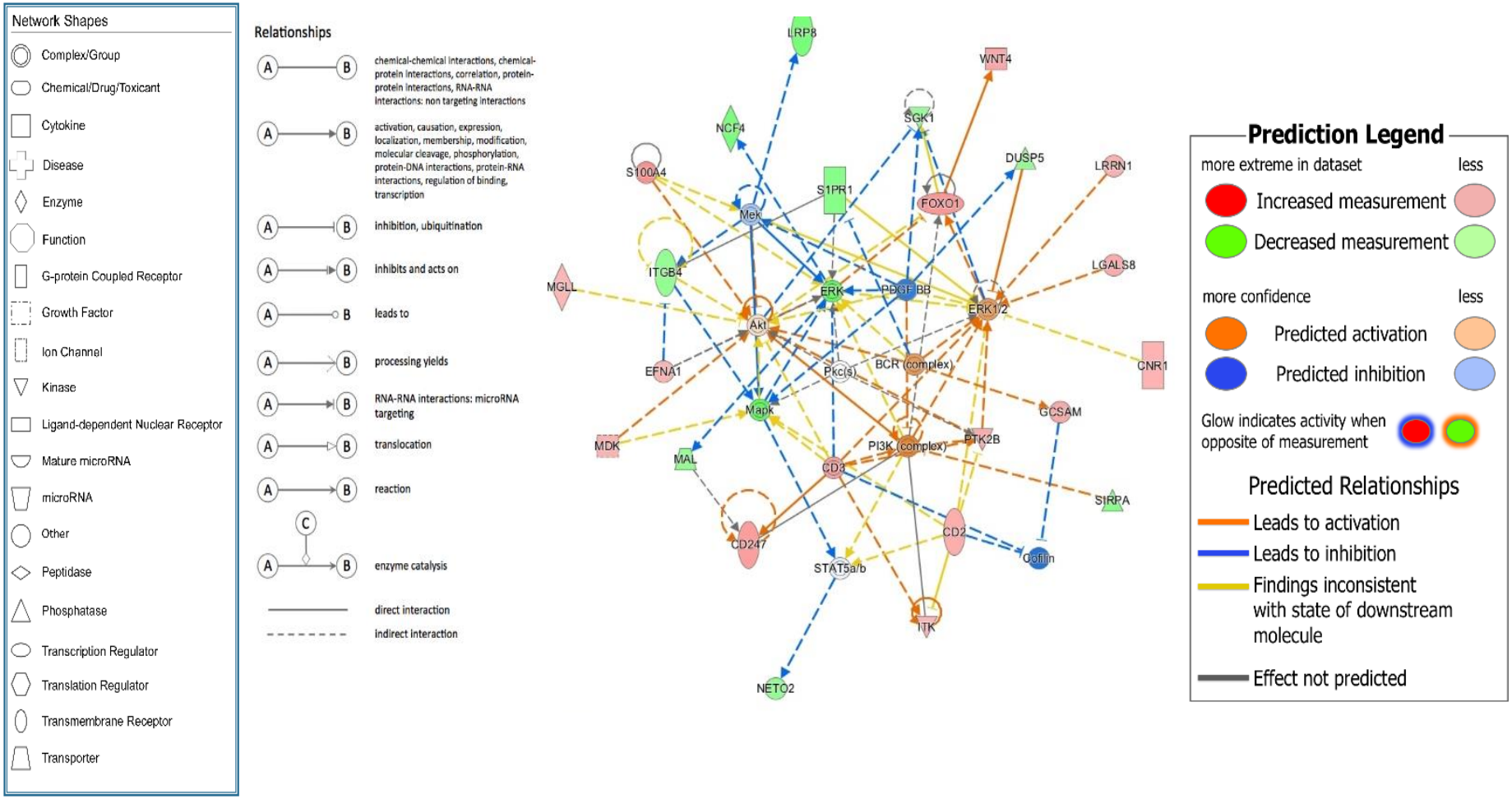

Figure 30: **Network 2**.

Legend: HIV-1-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

Figure 30 shows molecules activated in network 2 yielding a score of 34. In central position are PI3K complex, Akt, ERK ½, Mek, MapK, BCR, CD3 with 26 genes specifically affected by HIV-1. In this network, 10 genes were downregulated while 16 genes were upregulated. The PI3K complex, ERK ½ and BCR complex, and Akt were predicted to be activated whereas the Mapk ERK complex had decreased measurements levels. Upstream CD3 indirectly activated the PI3K complex which in turn indirectly activated. This network had a complex cascade of interactions of which many were found to be inconsistent with downstream molecules. However, it correlated with biological functions associated with cellular movement, cell death, and survival and cell morphology.

3.8.2.3. cell-to-cell signaling and interaction, cell death and survival, hematological system development and function

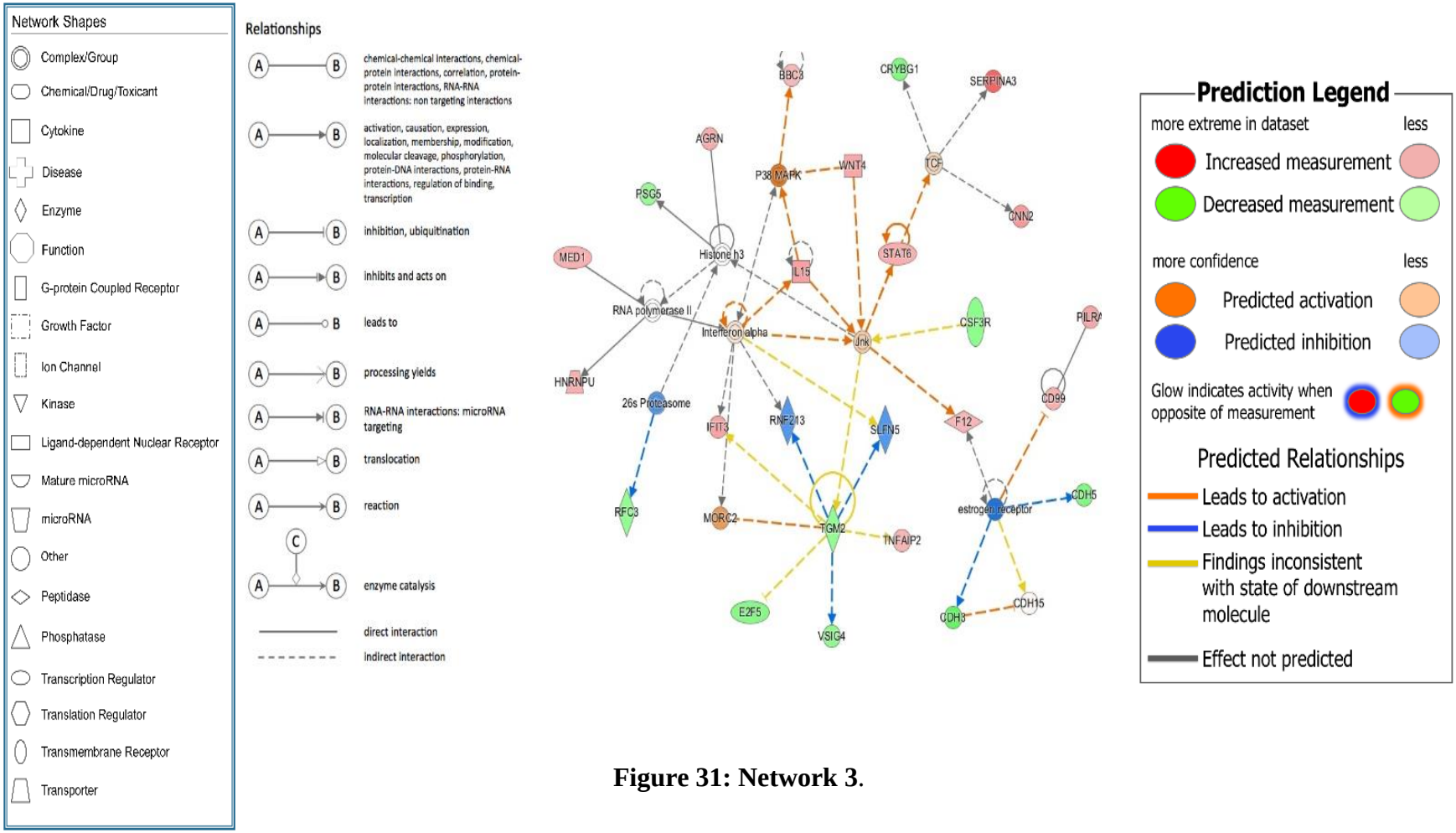

Figure 31: Network 3.

Legend: HIV-1-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

This network (3) has 26 molecules specifically altered by HIV-1 and summed up to a score of 34. Molecules P 38MAPK, TCF, interferon alpha and Jnk complexes had key roles in this network. Upstream, the upregulation of transcription factor MED1 activated the interferon alpha pathway also activated P38MapK pathway (pro-apoptotic) through the mediation of IL 15. The activated Interferon alpha pathway also indirectly activates the Jnk pathway (mitogen-activated protein kinase) which then indirectly activates SERPINA3(protein-encoding gene) through a cascade of transcription factors (STAT6) and intermediate pathways (transcription factor signaling-TCF). Estrogen receptors were predicted to be inhibited in the placenta of HIV-1 women as a result of the downstream molecules (CDH3 and CDH5-cadherins). This network is associated with the following functions: cell-to-cell signaling and interaction, cell death and survival, hematological system development and function (Figure 31).

3.8.2.4. Hematological disease, Immunological disease, Cell morphology

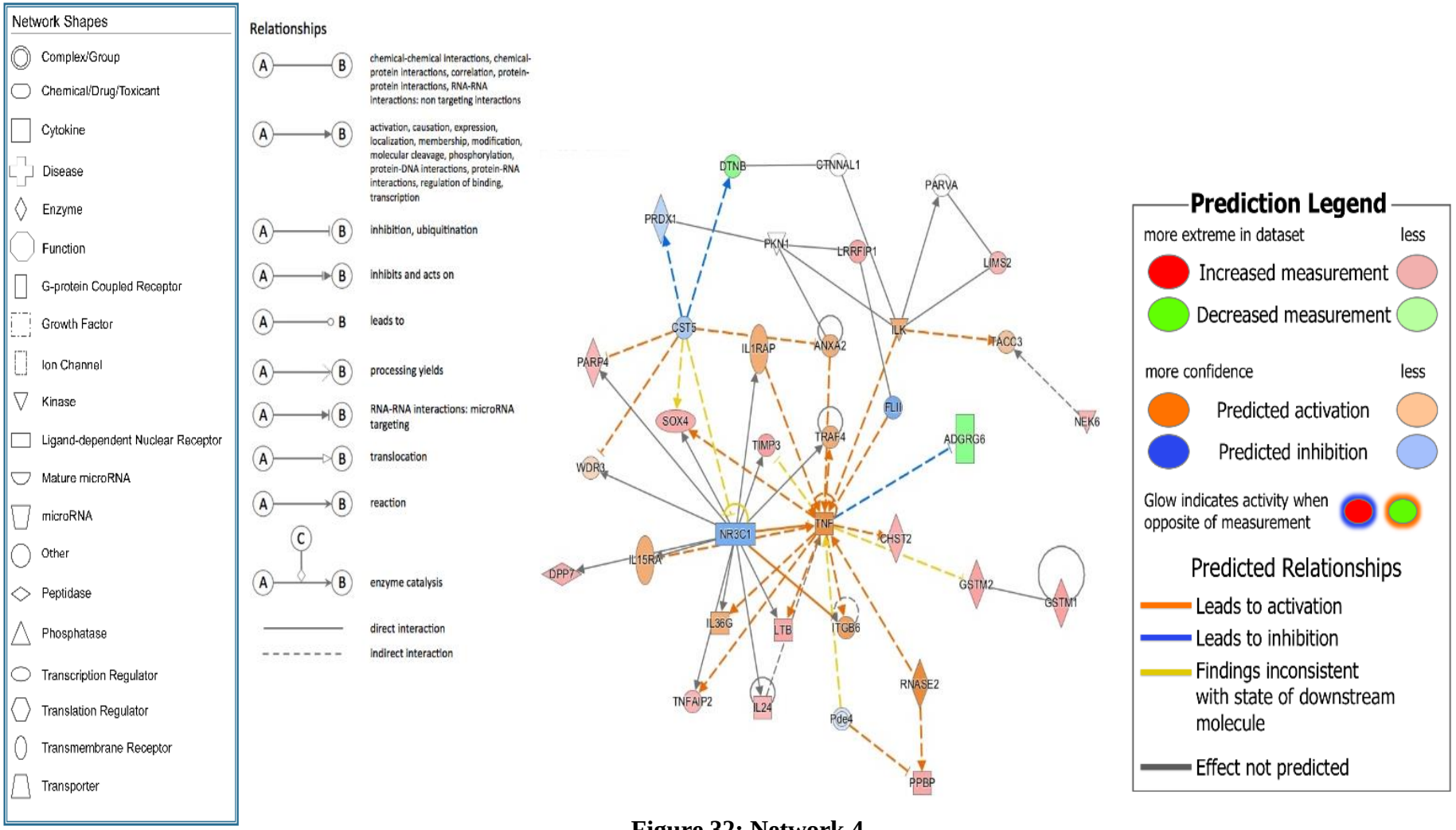

Figure 32: Network 4.

Legend: HIV-1-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

This network (4) has in central position the TNF cytokine which is indirectly activated by a number of upstream receptors (ILR1AP), enzymes (RNASE2), Kinases (ILK) and other molecules. It is predicted to be directly activated by the inhibition of the nuclear receptor NR3C1 whose downstream cascade is correlated with the following biological functions: hematological disease, immunological disease, cell morphology. Network 4 comprised 15 genes specifically altered by HIV-1 and had a score of 20. Figure 32 summarizes this network.

3.8.3. Selection of the most suitable reference genes for downstream confirmation assays

Reference genes were identified using Normfinder. After identifying DEGs, genes with the greatest p-values and fold change close to or equal to 1 were considered for these assays. Consequently, the expression values from 28 selected stable genes from the placenta of HIV-1 positive women were entered into the software and the most stable genes were selected after computation. The results of this computation identified the XKR3 and SERPINA12 genes as the most suitable pair to be used as reference genes when performing downstream confirmation of top dysregulated genes in the placenta by HIV-1. Table XI portrays the selected reference genes.

Table XII: List of genes suitable for reference gene in qPCR to confirm HIV-1 microarray data

Best gene	XKR3
Stability value	0.011
The best combination of two genes	XKR3 and SERPINA12
Stability value for the best combination of two genes	0.008

3.9. Demographic and clinical characteristics of study participants selected for the Microarray experiments on malaria

On Table XII below is the comparison of participant characteristics depicts higher temperature, and diastolic pressure in malaria positive women (p>0.05). However, a baby was born to a woman in the control group with low birth weight. There was no significant difference between fetal parameters between the children born to mothers with and without malaria.

Table XIII: Demographic and clinical characteristics of study participants selected for the Microarray experiments on malaria

Characteristic*	Mal (-)	Mal (+)	p-value
Enrolled participants (n)	8	5	
Age in years (mean ± SD)	26.5(24.25-32)	29(22.5-32.5)	0.97
Alcohol use during pregnancy (n, %) **	4(50%)	0(0%)	0.1
Axillary temperature (°C) (median, IQR)	37.3(36.1-37.4)	38.2(37.58-38.9)	**0.02**
Fever (>37.5°C)	1(14.3%)	3(75%)	0.09
BMI (median, IQR)	30.04(26.57-32.39)	30.41(24.22-31.22)	0.83
Hemoglobin levels (g/dL) (median, IQR)	12.1(11.45-12.9)	10.2(9.05-13.8)	0.29
Anemia (<11g/dL) (n, %)	1(12.5%)	3(60%)	0.57
ART use (n, %)	/	/	/
IPT use (n, %)	7(100%)	4(100%)	0.99
Number of SP doses (median, IQR)	2(1-3)	2.5(2-3)	0.42
Bednet use (n, %)	5(62.5%)	3(60%)	0.99
Maternal heart rate, beats per minute (median, IQR)	80(76-83)	110(100-120)	0.055
Maternal blood pressure in mmHg (median, IQR)			
Systolic	126(112.3-130)	120(105-123)	0.35
Diastolic	90(75.25-90)	60(52.5-69)	**0.03**
Peripheral malaria by blood smears[§] (n, %)	/	/	/
Parasite density (parasites/uL)[£] (mean ± SD)	/	4120(520-47020)	/
Placental malaria[§λ] (n, %)	/	5	/
Parasitemia (in %) (mean ± SD)[£]	/	6.8(2.707-24.05)	/
Parity, including current child (median, IQR)	3(1-4.75)	2(1-3)	0.46
Primigravidae (n, %)	3(37.5%)	2(40%)	0.99
Multigravidae (n, %)	5(62.5%)	3(60%)	0.99
Length of gestation in weeks (median, IQR)	39(38.25-40)	40(38-40.5)	0.76

Preterm deliveries (<37 weeks) (n, %)	1(12.5%)	0(0%)	**0.001**
Singleton deliveries (n, %)	8(100%)	5(100%)	/
C-section (n, %)	1(12.5%)	0(0%)	0.99
Male neonates (n, %)	2(25%)	4(80%)	0.99
Placental weight in g (median, IQR)	560(525-610)	545(340-705)	0.88
Neonate weight in g (mean ± SD)	3110(2818-3333)	3000(2760-3400)	0.72
Low birth weight (<2500g) (n, %)	0(0%)	0(0%)	/
APGAR at 1min (median, IQR)	8(7-8)	8(4.25-8.75)	0.99
APGAR at 5min (median, IQR)	9(8.25-9.75)	9(7-9)	0.52
Cord malaria infection by blood smears (n)	0	0	-
*** Numbers may not add up to 13 due to missing responses.**			
**** None of the study participants were smoking during pregnancy.**			
§ Peripheral blood smears were tested by microscopy for presence of *P. falciparum, P. ovale, P. malariae, P. vivax.* Only *P. falciparum* was detected.			
£ Calculated for only smear positive individuals.			
λ Woman was considered placental malaria positive if malaria-infected erythrocytes were found in intervillous blood smears, impression smears or histological slides. Percent placental parasitemia is reported based on impression smear data.			

Table XIV summarizes the number of differentially expressed genes identified after the set threshold was set. Out of the 47310 genes on the coupled to Illumina beadchip, 37435 genes could be mapped on Ingenuity Pathway Analyses (IPA). After setting a significant level of $p<0.05$ and fold change of 1.5, 372 genes were dysregulated by malaria, of which 218 were up-regulated and 154 were down-regulated

Table XIV: Differentially expressed genes in the placenta of women with pregnancy associated malaria

Characteristics	Number
Genes assessed	47310
Mapped genes	37435
Threshold transferred to IPA	p<0.05
Genes assed in IPA	426
Genes mapped in IPA	372
Threshold for p-values in IPA	<0.05
Threshold for Fold change	>1.5
Genes up regulated	218
Gene Down regulated	154

3.10 Top genes affected by in the placenta women with placenta malaria

Expr Fold Change down-regulated

Molecules	Expr. Value
XAGE1B/XAGE1E*	↓ -7.007
HLA-DRB5	↓ -6.494
MAGEA4*	↓ -4.953
RN7SK*	↓ -3.779
MUCL1	↓ -3.631
CTAG2	↓ -3.220
CTNND1	↓ -2.999
EMX2	↓ -2.979
SGSM1	↓ -2.766
TREML2	↓ -2.738

Expr Fold Change up-regulated

Molecules	Expr. Value
LILRA3*	↑ 11.622
CXCL9	↑ 8.806
FN1*	↑ 7.886
HLA-G	↑ 6.380
CXCL10	↑ 4.697
ADA	↑ 4.694
IL18BP	↑ 4.591
EPYC	↑ 4.300
HLA-DQA1	↑ 4.027
CXCL8	↑ 4.000

Figure 33: Top up-regulated and down-regulated genes in the placenta malaria

The best top ten up-regulated and down-regulated genes are shown on Figure 33 with LILRA3 as most upregulated gene and XAGE1B/XAGE1E is the most down-regulated gene in the placenta of malaria positive women.

3.11. Canonical Pathways and Gene Networks affected by Malaria

3.11.1. Canonical pathways

Up to 100 pathways were dysregulated by malaria. Amongst them 14 were activated while 4 were inhibited as depicted on Figure 34 below.

Legend: Malaria-induced specific pathways in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

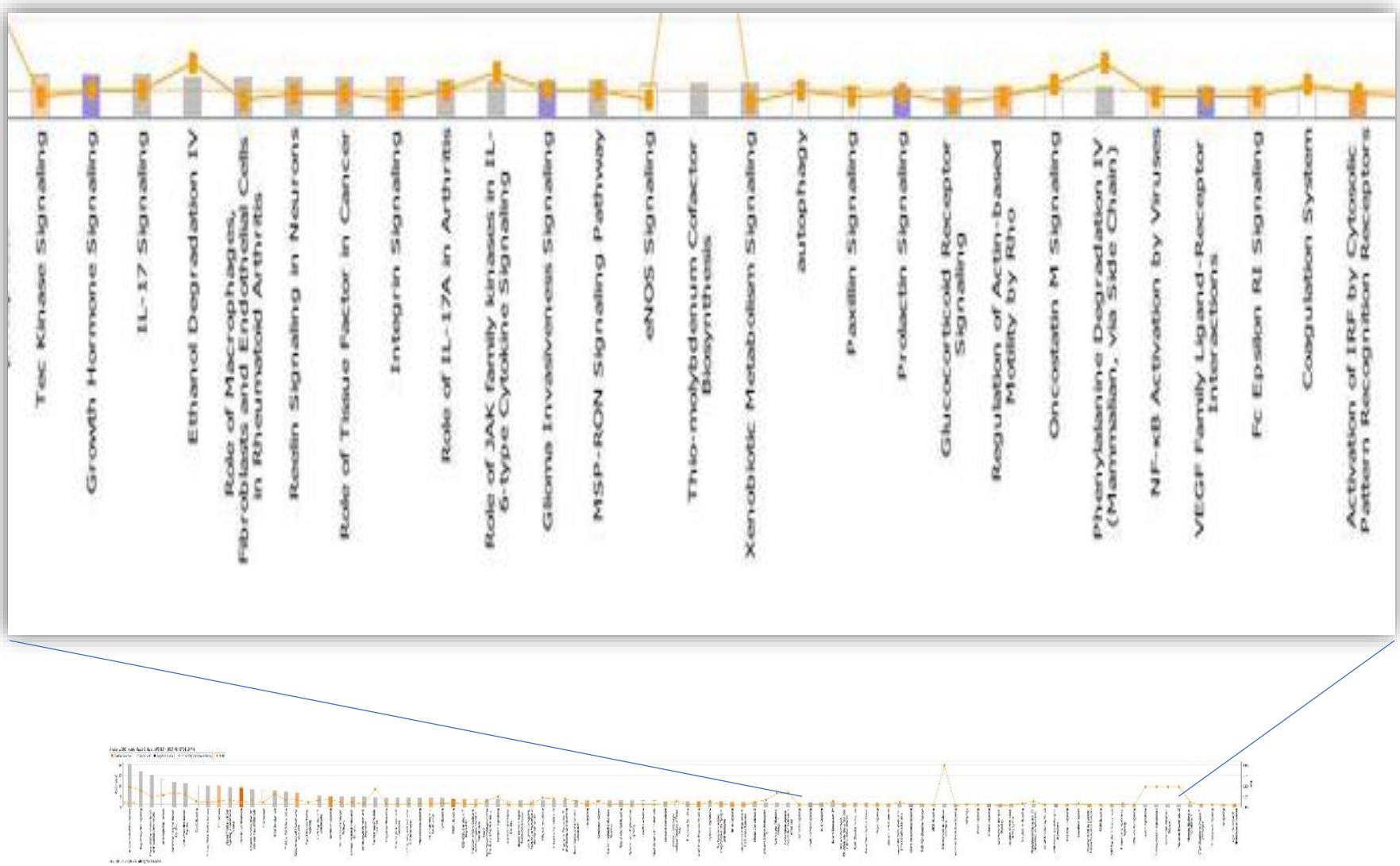

Figure 34a: Canonical Pathways affected by malaria.

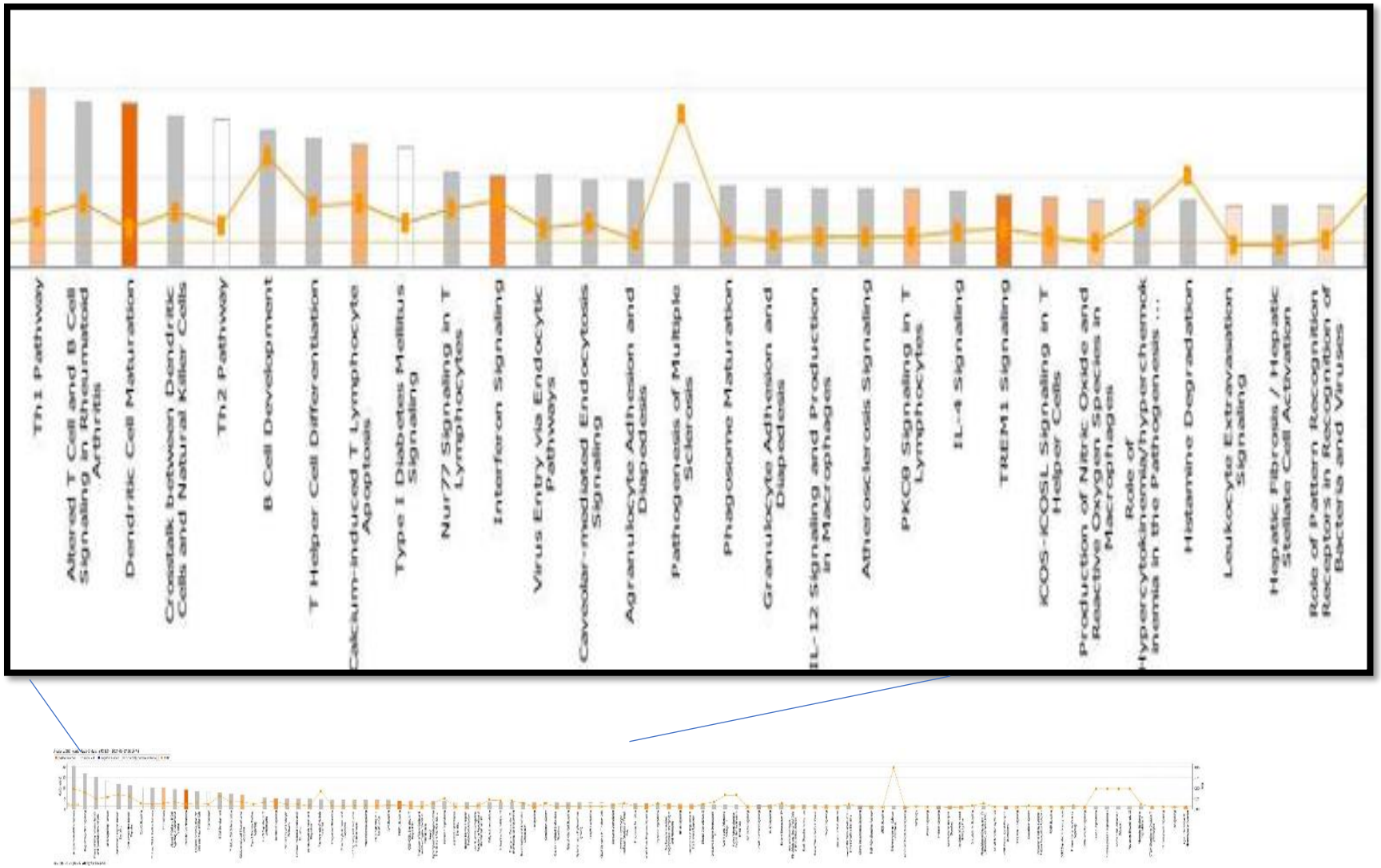

Figure 35b: Canonical Pathways affected by malaria.

3.11.1.1. The TH1 Response pathway

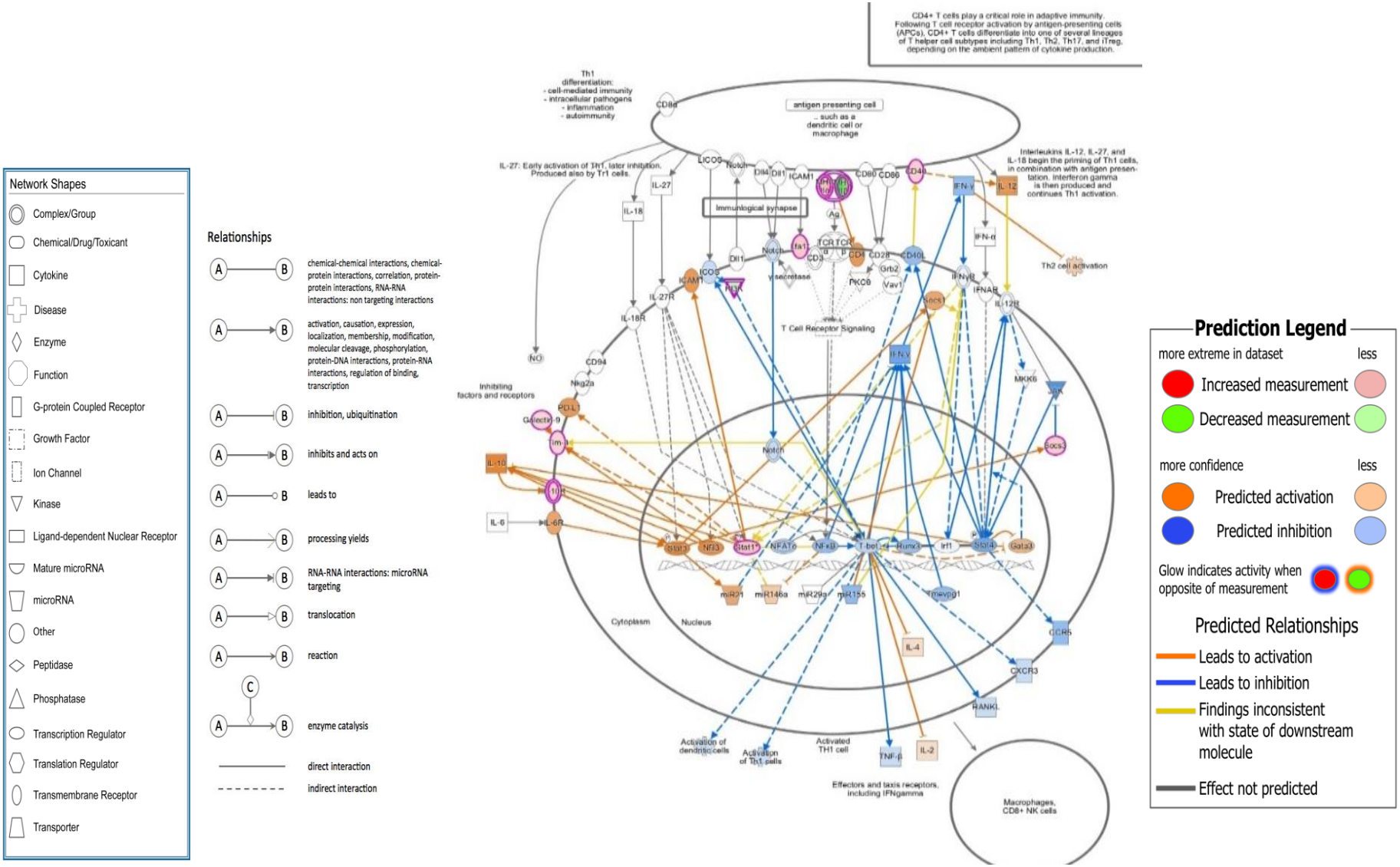

Figure 36: TH1 Response pathway is activated in the placenta malaria positive women

During pregnancy associated malaria, the T helper 1 response pathway is activated in the placenta when in contact with APCs. The activation of this cell types causes the release of numerous cytokines including IL-10, IL-2, IL-4. Certain receptors are also activated such as the IL-6R, PD-L1 and ICAM receptors. The production of certain cytokines is inhibited in these T cells such as TNF-b, INF-g and certain cytokine and chemokine ligands are inhibited as well, such as CXCR3, CCR5, ICOS and CD-40L as shown on Figure 35.

3.11.1.2. The Interferon Signaling Pathway

In pregnant women, malaria causes the activation of transcription factors responsible for the production of interferons and their co-stimulatory molecules. This phenomenon is depicted on Figure 36.

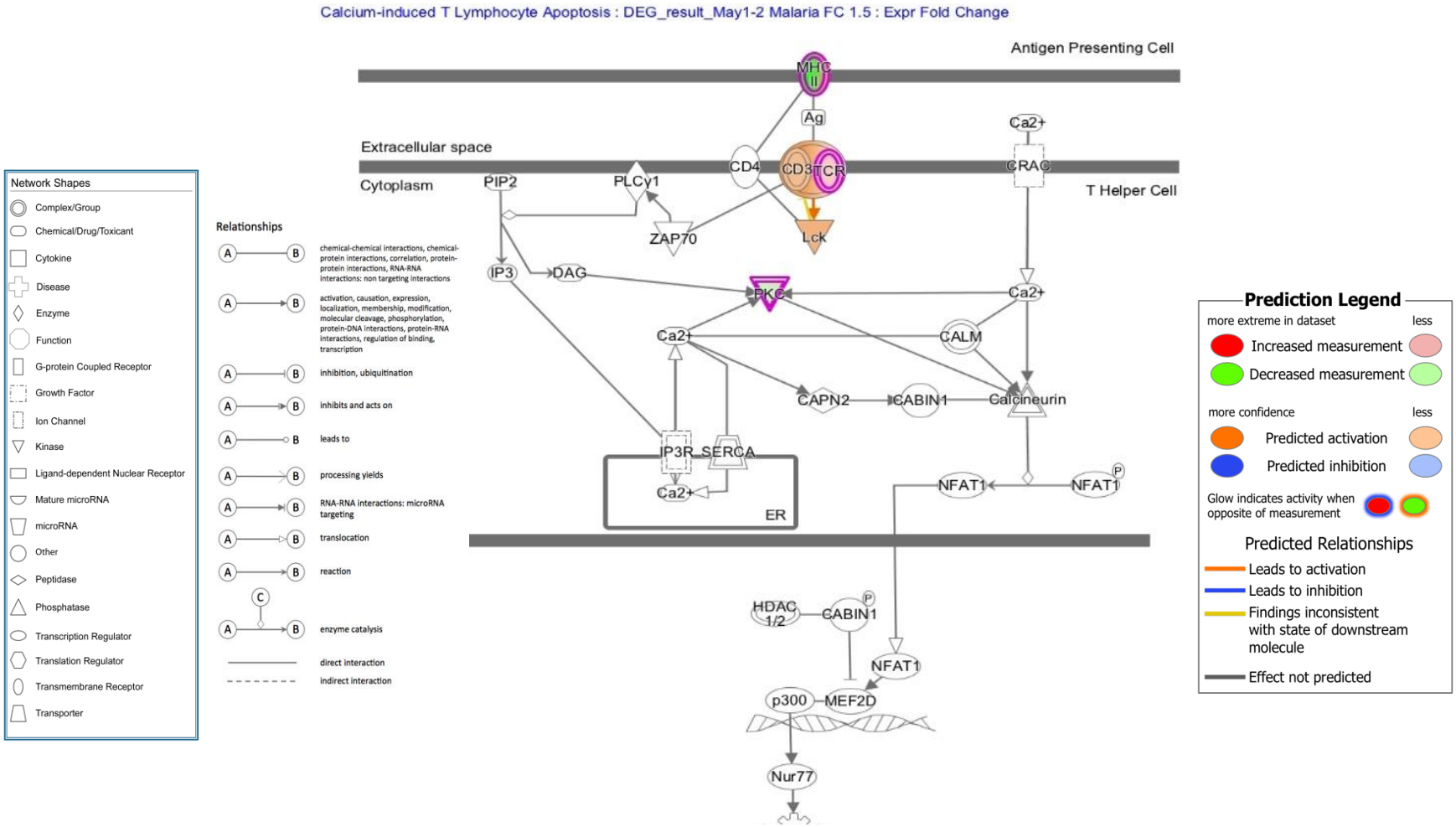

Figure 37: Interferon Signaling Pathway is activated in the placenta of malaria infected women

3.11.1.3 Calcium-induced T-Lymphocyte Apoptosis pathway

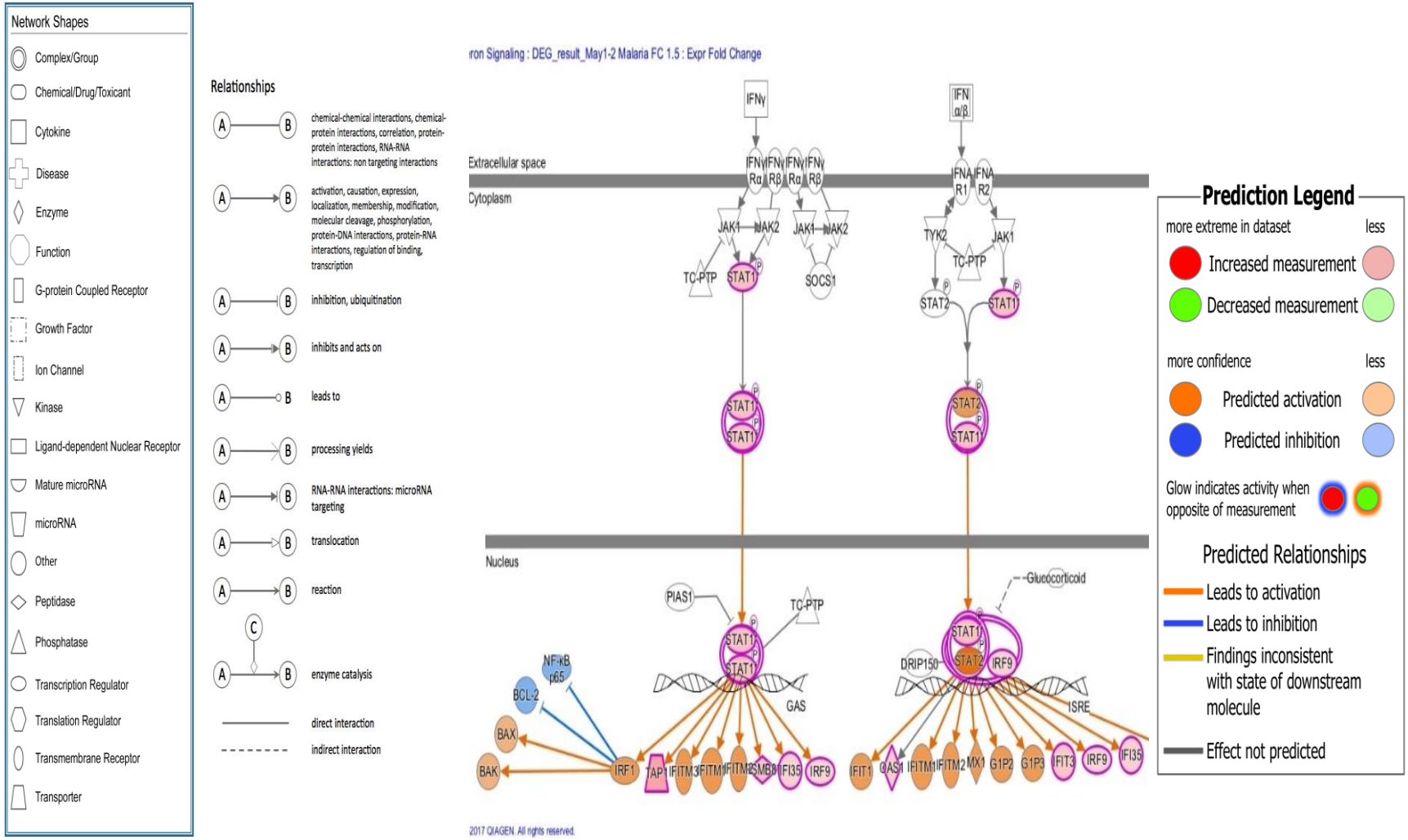

Figure 38: Calcium-induced T Lymphocyte Apoptosis activated in the placenta of malaria positive women

Malaria in the placenta is predicted to induces the apoptosis in the T lymphocytes in a calcium dependent manner as depicted on Figure 37 above. Here CD3 and TCR are co-stimulated by and APC. This leads to the activation of the Lckinase. The downregulation of P kinase initiates the cascade of calcium related intermediates which initiates the process of apoptosis in these cells.

3.11.1.4. The Growth Hormone Signaling pathway

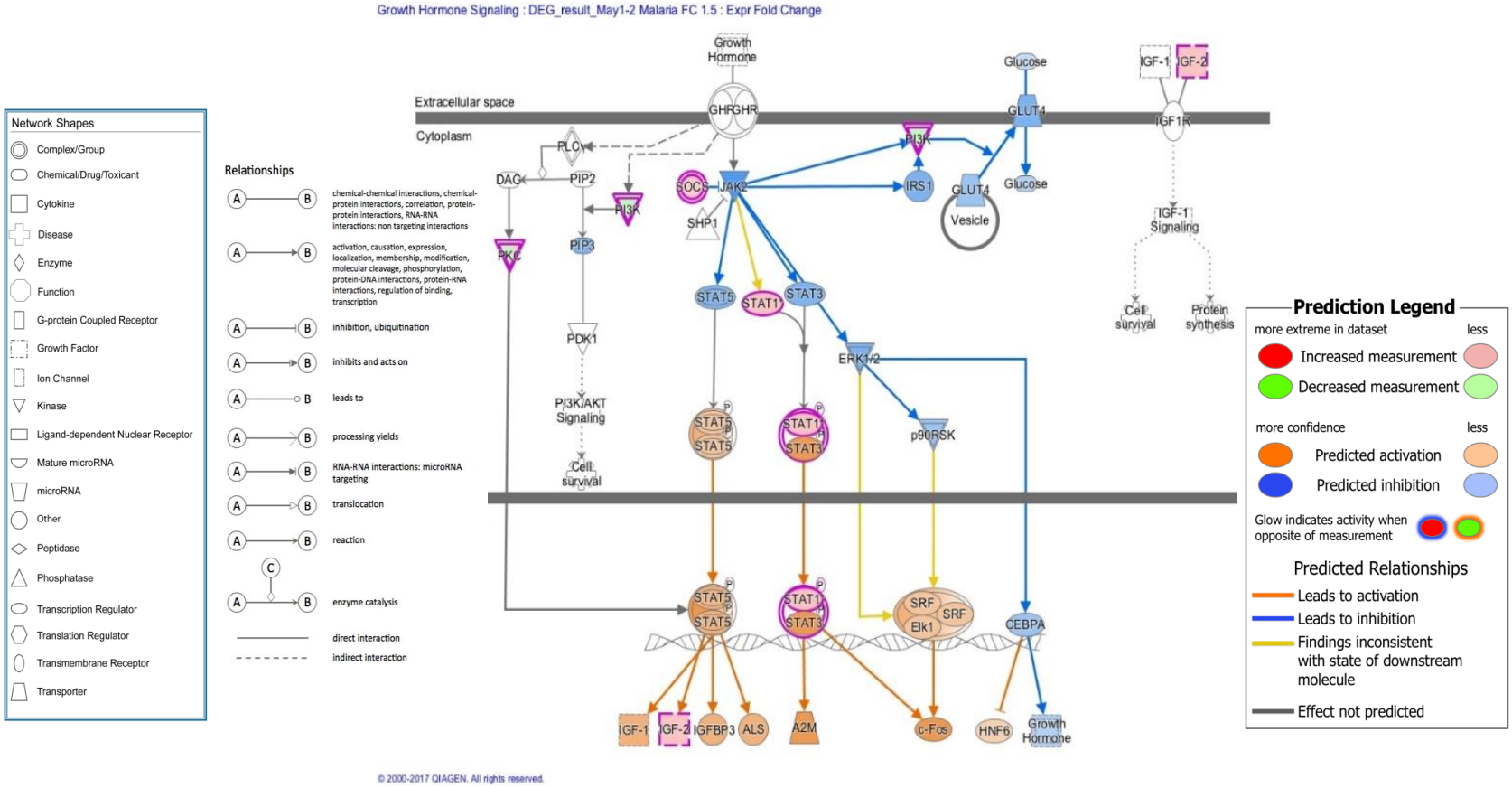

Figure 39: The Growth Hormone Signaling pathway is inhibited in the placenta of malaria positive women

Malaria during pregnancy disrupts placental function by inhibiting transcription factors (CEBPA) necessary for growth hormone synthesis by the placenta. IGF synthesis is also observed to be activated in this pathway through the mediation of the STAT5 transcription factor. However, the co-activation of its regulatory molecules IGF2 and IGFBP3 is observed as depicted on Figure 38.

3.11.1.5. VEGF Family Ligand Receptor Interactions

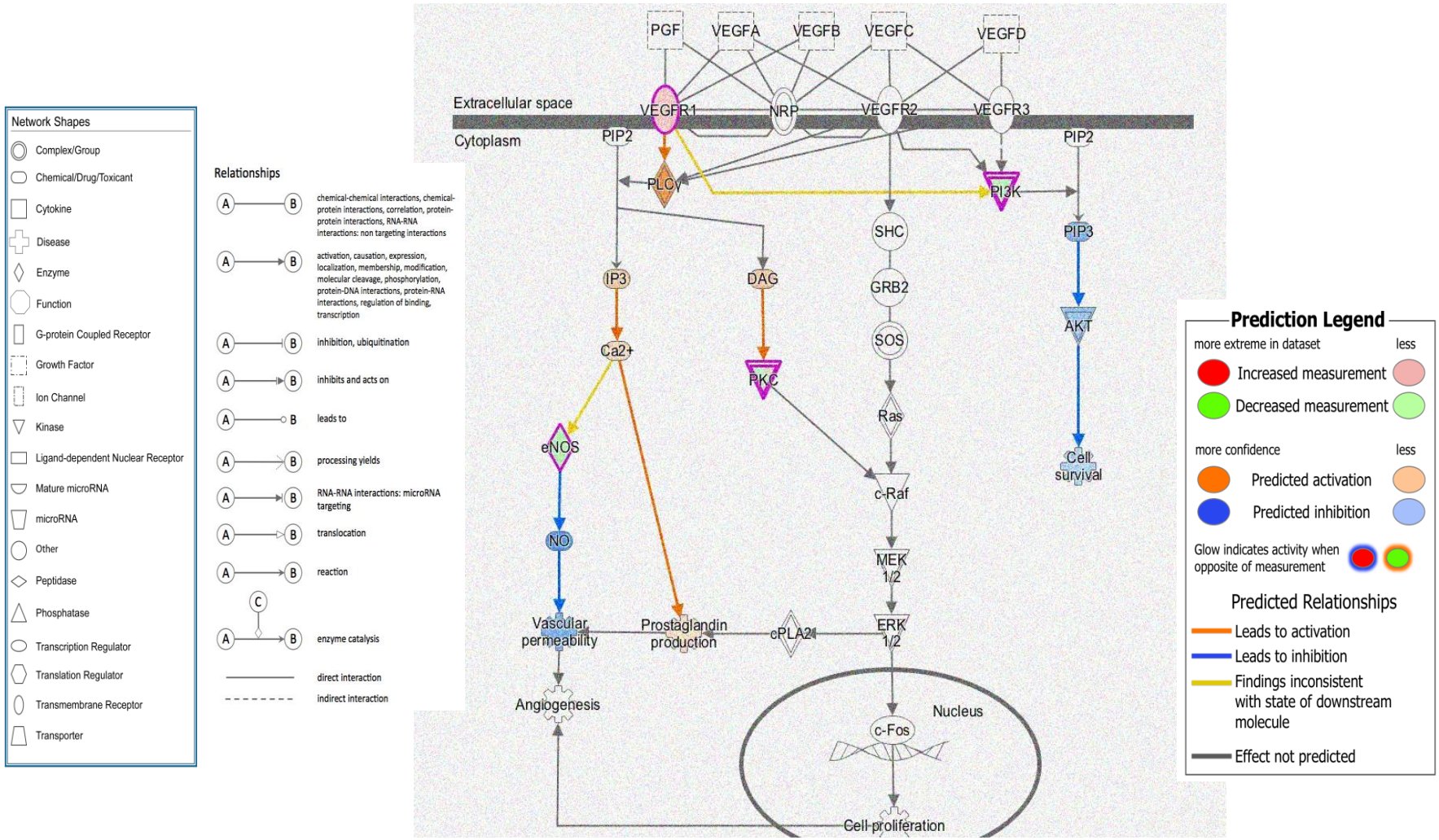

Figure 40: VEGF Family Ligand Receptor Interactions in the placenta is inhibited during malaria infection

During malaria infection, the placenta turns to inhibit vascular permeability through a cascade of reactions activated through the activation of VEGFR1 and the inhibition of eNOS enzyme to molecules while prostaglandins are produced are synthesized. In this pathway, molecules responsible for cell survival are inhibited. This is summarized on Figure 39.

3.11.1.6. The Prolactin signaling pathway

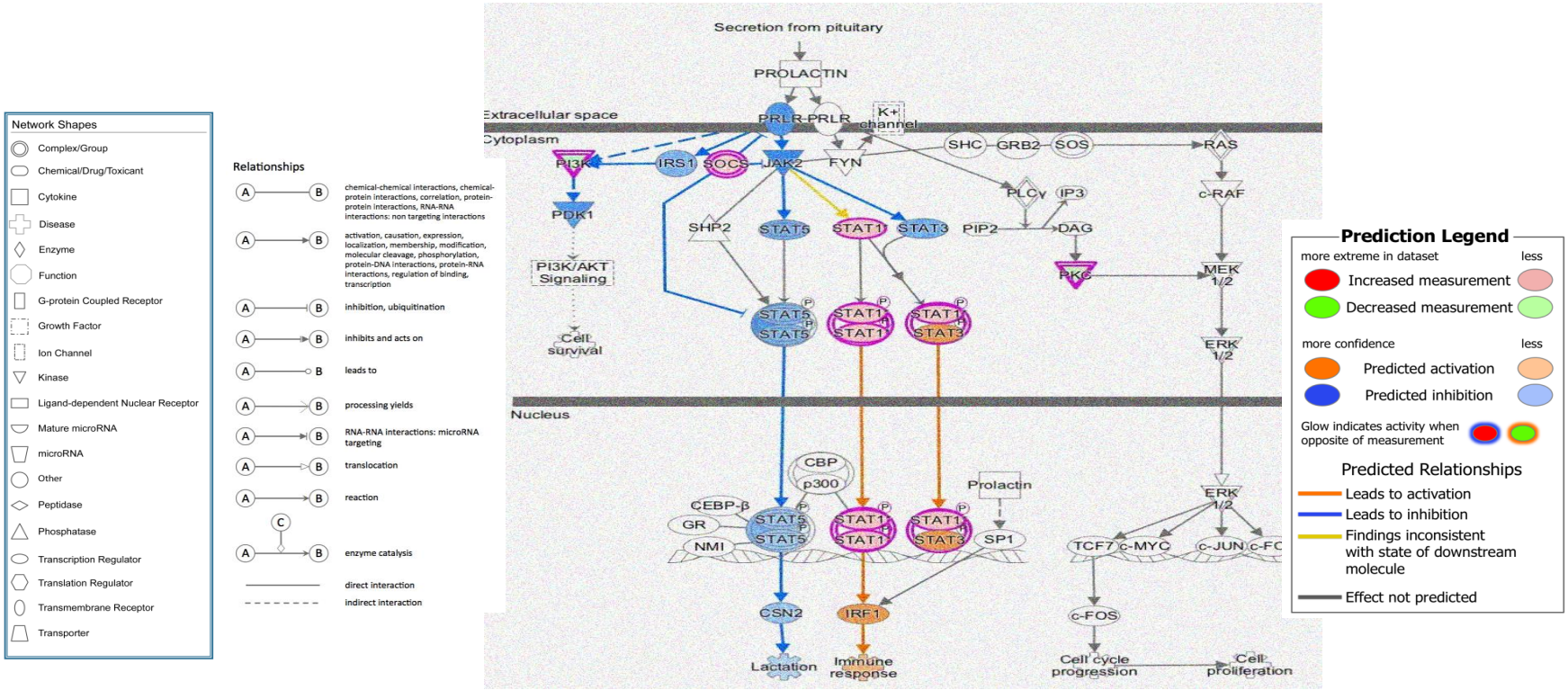

Figure 41: Prolactin signaling pathway inhibited in the placenta of malaria positive women.

The Prolactin signaling pathway generally regulates cell cycle progression, cell proliferation, lactation and immune response. Malaria infection in the placenta inhibits lactation via the CSN2 and activates immune response as through IRF1 as shown on Figure 40 above.

3.11.2. Gene networks altered in the placenta of malaria positive women

3.11.2.1. Neurological disease, organismal injury, and abnormalities, psychological disorders network

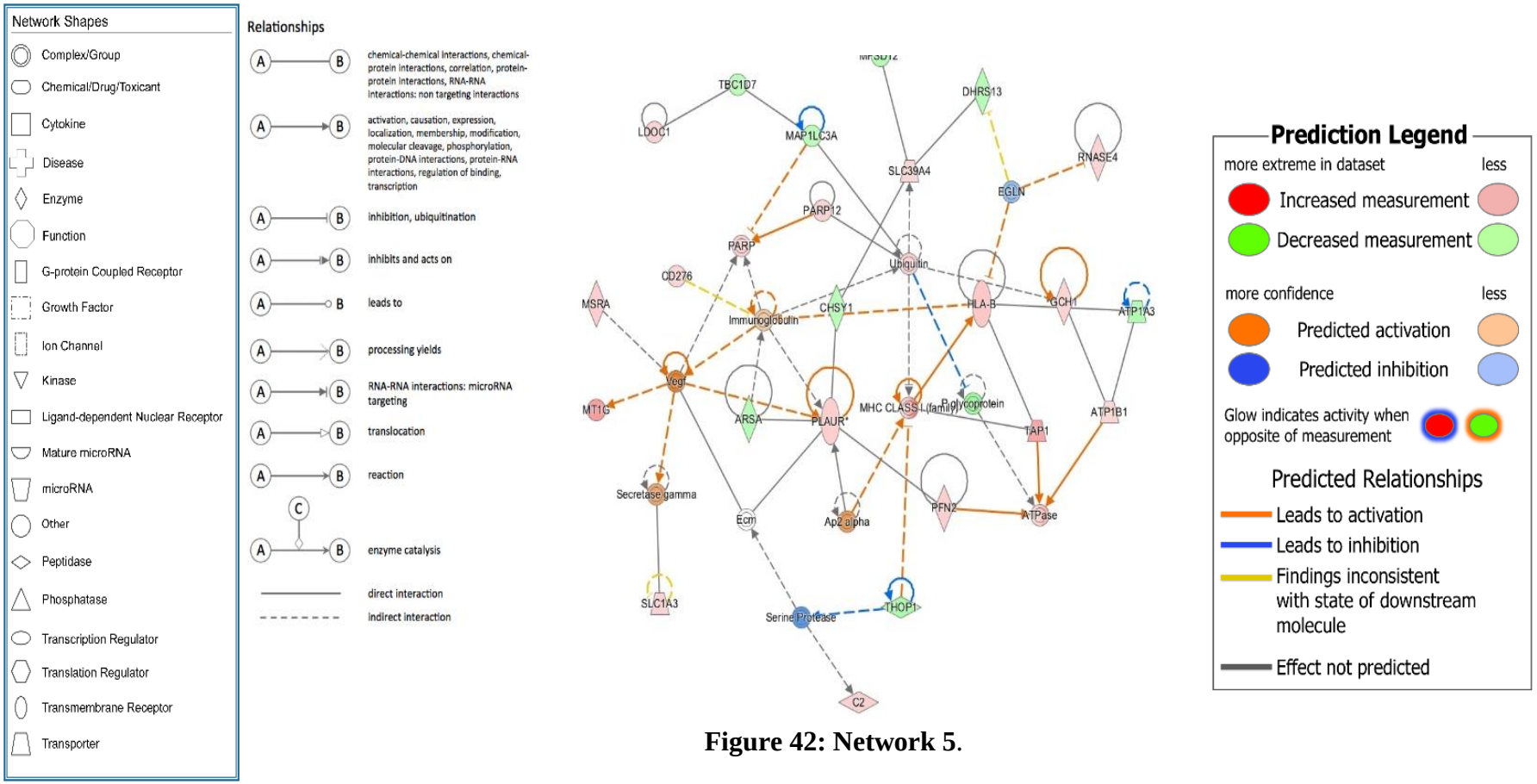

Figure 42: Network 5.

Legend: Malaria-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

The 27 molecules specifically dysregulated by malaria in this network yielded a score of 35 In dominant position are MHC Class I, Immunoglobulin, Ubiquitin, VEGF, PARP, EGLN, Secretase gamma. Ap2 alpha, Vegt, secretase gamma whereas predicted to be inhibited were the serine protease and EGLN pathways. Ubiquitin was predicted to inhibit the synthesis of p glycol proteins. The overexpression of MHC I family was predicted to cause overexpression of the ATPase, the activation of immunoglobulin production and VEGF. Neurological disease, organismal injury, and abnormalities, psychological disorders are biological functions that correlated with this network shown on Figure 41 above.

3.11.2.2. Cancer, organismal injury, and abnormalities, inflammatory disease

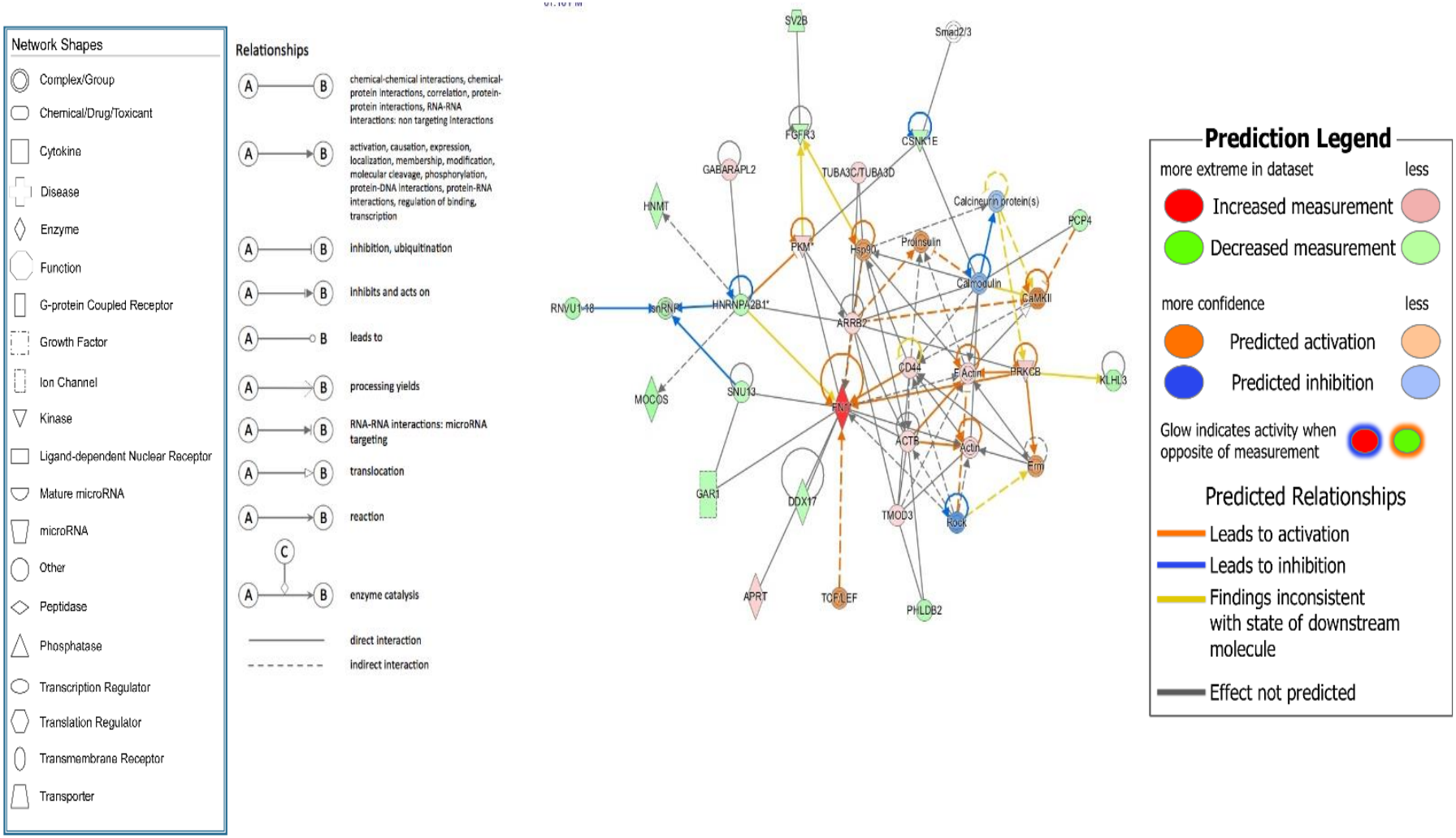

Figure 43: Network 6.

Legend: Malaria-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

Figure 42 represents network 6 has a score of 35 with overall 26 differentially expressed molecules by malaria. Amongst these 14 molecules were downregulated while 12 were upregulated. Activated in central positions of this interaction were; Proinsulin, Hsp 90, CaNKII, Erm, pathways predicted to be inhibited in this network are Rock, Calmodulin and calcineurin proteins. Most activities in this network lead to the overexpression of FN1-enzyme. Correlated to this network were the following biological functions: Cancer, organismal injury, and abnormalities, inflammatory disease.

3.11.2.3. Cellular movement, immune cell trafficking, hematological system development

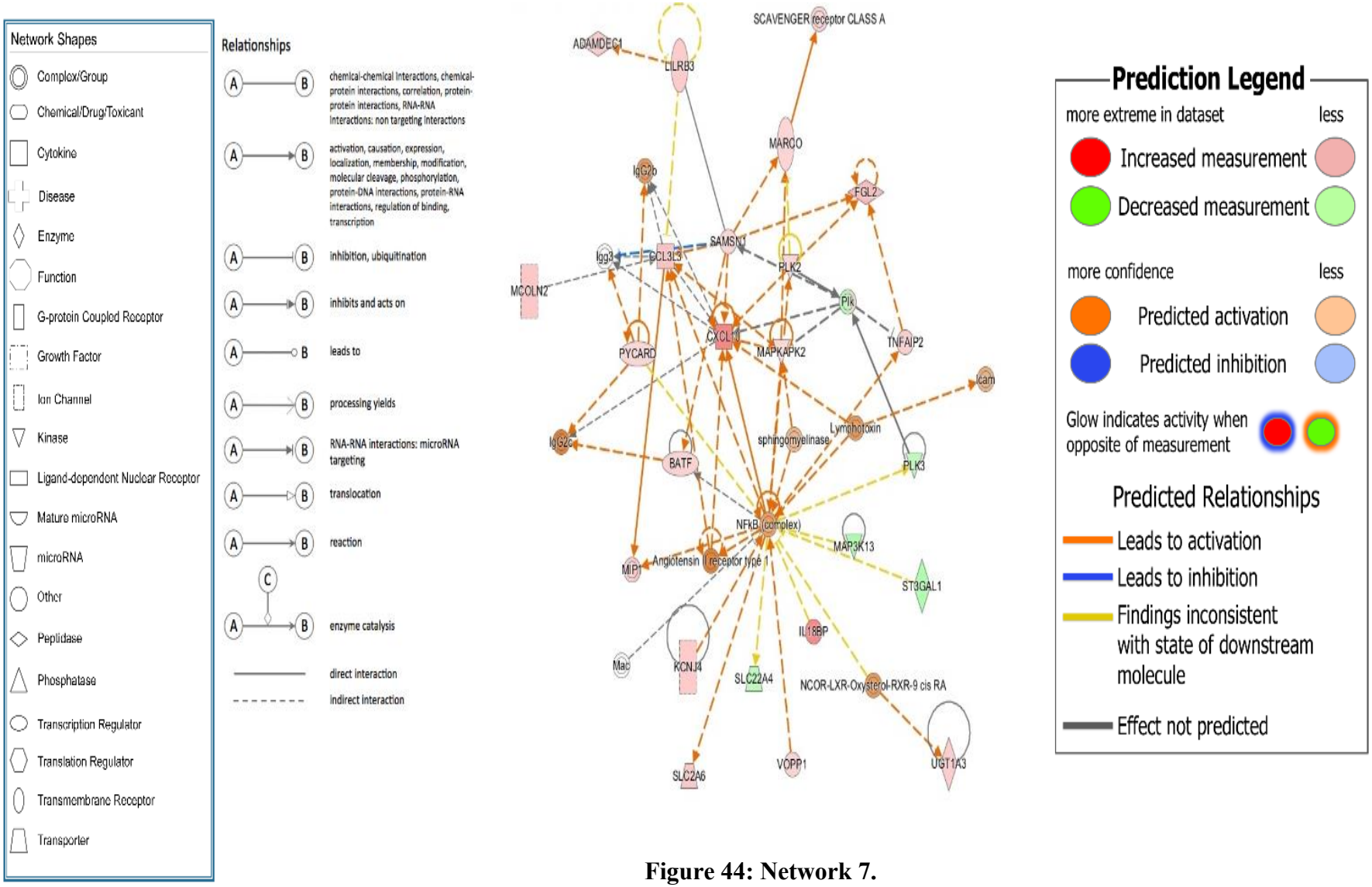

Figure 44: Network 7.

Legend: Malaria-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

A total of 26 molecules were affected by malaria in network 7 yielding a score of 32. In central position were NFkB complex, Angiotensin II receptor type I, sphingomylinase, lymphotoxin, IgG2b, IgG2c, Scavenger receptor type A, NCOR-LXR-Oxysterol-RXR-9 cis RA, and CXCL10. Most of these complex interactions predicted the activation of chemokine CXCL10 and overexpression of CCL3L3. The activation of the ICAM pathway and the pathways for the synthesis of IgG2b and IgG2c were also predicted by this network. Cellular movement, immune cell trafficking, hematological system development, and function are some biological functions associated with this network. This network is summarized on Figure 43.

3.11.2.4. Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking

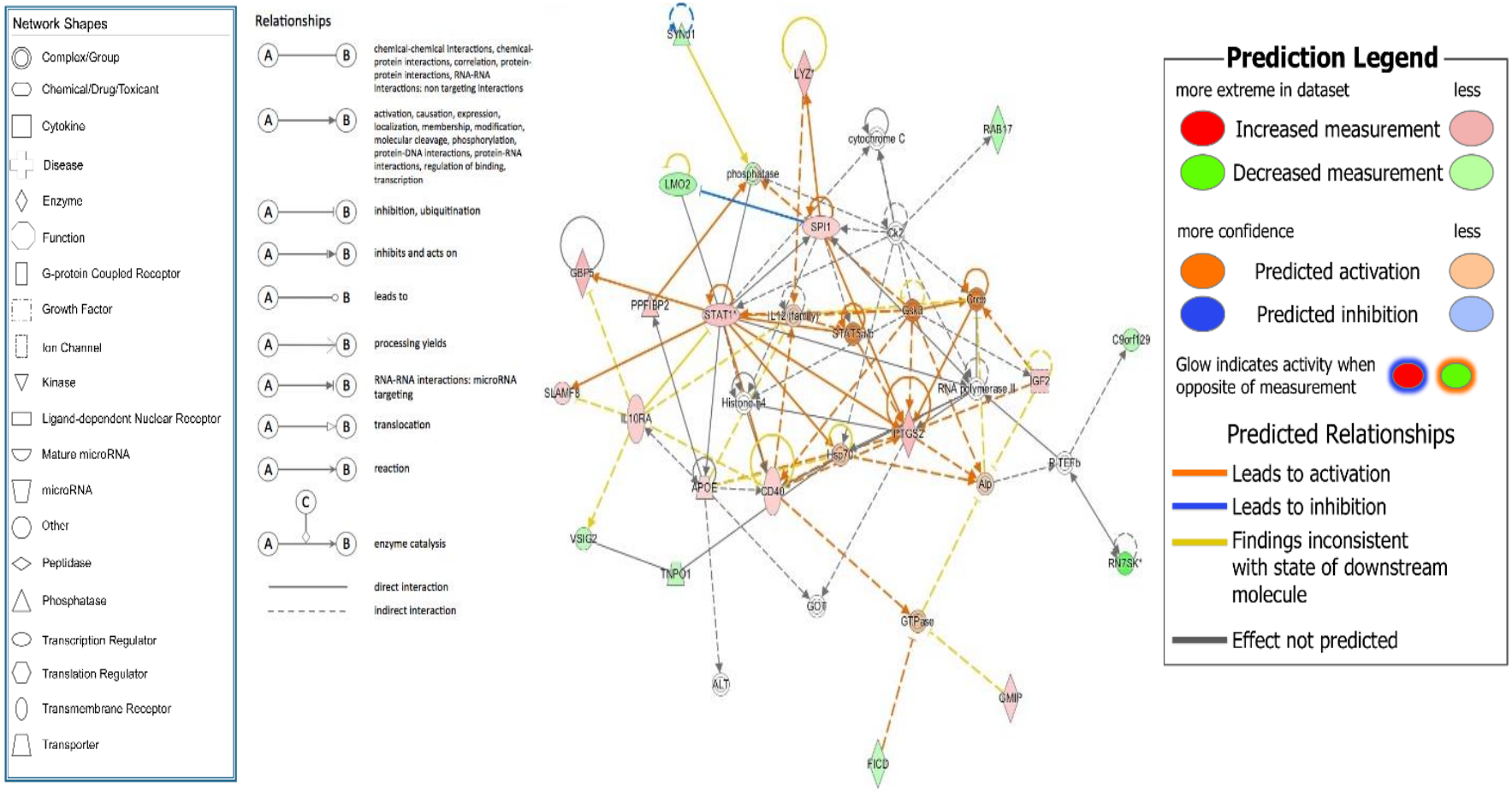

Figure 45: Network 8.

Legend: Malaria-induced specific genes in the placenta. Microarray results were normalized by a robust regression analysis and only genes with an expression above a conditional threshold of 0.05 (2 SD above background) were selected for analysis. Next, genes presenting a minimum of 1.5-fold ratio in regard to the control group were entered as focus genes in Ingenuity Pathway Analysis (IPA) for a core analysis which put the datasets in the context of biological processes, pathways, and molecular networks.

Network 8 on Figure 44 has a score of 28 with an overall 22 molecules affected by malaria. Molecules activated yielding a pathways activated in this interaction are Alp, IL12 family, Hsp70, STAT5a,b, GSK3, Creb, the small GTPase and phosphatase. Many of these pathways are predicted to be activated upstream by transcription factors STAT1, SPI1and STAT5a,b. This network matched with biological functions associated with cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking.

3.11.3. Selection of the most suitable reference genes for downstream confirmation assays

Reference genes were identified using Normfinder. After identifying DEGs, genes with the greatest p-values and fold change close to or equal to 1 were considered for this assay. Consequently, the expression values from 26 selected stable genes from the placenta of malaria positive women were entered into the software and the most stable genes were selected after computation. The results of this computation identified CECR1 and CDH12P (Table XV) genes as the most suitable pair to be used as reference genes when performing downstream confirmation of top dysregulated genes in the placenta by malaria.

Table XV: List of genes suitable for reference gene in qPCR to confirm malaria microarray data

Best gene	CDH12P
Stability value	0.017
The best combination of two genes	CECR1 and CDH12P
Stability value for the best combination of two genes	0.013

Chapter 4: DISCUSSION, CONCLUSION, SUGGESTIONS

Chapter 4: DISCUSSION, CONCLUSION, SUGGESTIONS

4.1. Discussion

- **HIV-1 does not significantly affect IGF axis and angiogenic factors in IVS blood**

The aim of our first objective was to determine whether maternal HIV is associated with the dysregulation of insulin-like growth factor (IGF) axis, angiogenic factors or other related biomarkers that regulate fetal growth. In this pilot study, a panel of biomarkers implicated in placental homeostasis and fetal growth were assessed in intervillous space plasma of HIV-1 positive normotensive women on antiretroviral therapy and their HIV-1 negative counterparts. This panel of biomarkers included those involved in angiogenesis, IGF axis, as well as the profile of MMPs and Gal-13. Angiogenic factors were not affected by maternal HIV-1 in our cohort of pregnant women receiving antiretroviral therapy. Angiopoietins 1 and 2 levels were not significantly different between HIV-1 positive and healthy women, even after adjusting for maternal factors. Studies have shown that persistent HIV infection contributes to the development of chronic arterial injury and subsequent endothelial damage, atherosclerosis, and thrombosis (Fourie et al., 2011). In addition, HIV-infected children have arterial stiffness and endothelial dysfunction in the absence of cardiovascular risk factors (Bonnet et al., 2004). Since most of the women in our study were on ART, it is likely that combination antiretroviral therapy (cART) prevents angiopoietin dysregulation. Graham et al.(Graham et al., 2013) reported that in non-pregnant Kenyan HIV-1 positive women with advanced HIV infection, initiation of cART significantly lowered ANG2 levels, while ANG1 was increased (Graham et al., 2013). In agreement with previous studies(Ataíde et al., 2015; Silver et al., 2010), we confirmed that HIV-1 negative women with PM had significantly lower ANG1 levels compared to uninfected pregnant women. The reason for this observation may stem from malaria parasite level in blood of PM+ women. Silver et al, (Silver et al., 2010) found an inverse association between parasitemia and ANG1 levels (Silver et al., 2010). With very low parasitemia in malaria positive women (0.13%) in this study, there is possibly no major alteration on the levels ANG1. This might explain the minimal changes observed between PM positive women when compared to HIV-1 negative, PM negative women. MMP2 and MMP9 levels in intervillous space plasma were not significantly different between HIV-1 positive and healthy pregnant women. MMPs are involved in vascular remodeling and vasculogenesis, especially in new blood vessel formation and angiogenesis (Chen et al., 2013). In line with findings for angiopoietins, these data support the fact that in HIV-1

infected pregnant women on antiretroviral therapy, angiopoietin pathway is not dysregulated. Placental malaria did not have any significant effect on MMP2 or MMP9 in this study. This is in line with a previous study, in which no significant changes in plasma levels of MMP9 were observed in children with malaria infection compared to malaria negative children (Dietmann et al., 2008).

In linear regression models, maternal HIV-1 did not significantly associate with lower IGF1 in placental intervillous space plasma. Lower IGF1 levels were observed in HIV-infected Ugandan children (Mody et al., 2014). In non-pregnant adults, however, serum IGF1 has been shown to depend on level of immunodeficiency in HIV-infection and it was significantly higher in patients treated with protease inhibitors-based regimen compared to non-nucleoside reverse transcriptase inhibitors and healthy subjects (Parfieniuk-Kowerda et al., 2013). Similar findings were also reported by Matarazzo et al (year), who found an association between decreased IGF1 levels and diseases progression in HIV-1 positive individuals (Matarazzo et al., 1994). In this study, IGF1 was significantly higher in HIV-1 negative PM- positive women when compared to their healthy counterparts as previously described (Umbers et al., 2011). Further, in a small subset of HIV-1 positive/PM+ co-infected Cameroonian pregnant women in this study, IGF1 levels were significantly lower compared to healthy pregnant women, indicating that HIV-1 further exacerbates PM- associated dysregulation of IGF axis. Gal-13 is critical in trophoblast invasion during placentation and has also been reported to have angiogenic effects in the placenta (Gizurarson et al., 2013). Studies in animal models show that the expression of Gal-13 increases vasodilation (Gizurarson et al., 2013) and therefore placental perfusion. In addition, lack of expression of Gal-13 has been shown to impair syncytialization (Than et al., 2008, p. 200) and hence subsequent placental hormone production by the syncytiotrophoblast, which is vital in the development of the placenta. Gal-13 levels have not been studied in HIV-1 positive pregnant women, but they have been described for other pathological pregnancy conditions. No significant difference was found in Gal-13 intervillous space plasma levels between HIV-1 women under cART and their uninfected counterparts. Studies by Than et al. (N. G. Than et al., 2014; Than et al., 2008) showed that Gal-13 placental expression was lower in preterm preeclamptic placentas compared to preterm control placentas (Than et al., 2008, p. 200), while maternal peripheral serum Gal-13 concentration was higher in preterm preeclamptic women compared to preterm controls. In contrast, Sammar et al. (Sammar et al., 2011) did not observe any significant differences in maternal peripheral plasma Gal-13 levels in HIV-1 uninfected pregnant women with preeclampsia or hemolysis, elevated liver enzymes and low platelet count syndrome compared to HIV uninfected

(Sammar et al., 2011). Also, there was no difference in placental Gal-13 levels between HIV-1 negative PM-positive pregnant Cameroonian women and healthy pregnant women. This study has a number of limitations, including a limited number of LBW neonates in both HIV-positive and HIV-negative groups, inability to differentiate effects of HIV from those of ART because ART is the standard of care. Of course, an ideal study design would be to investigate these biomarkers with and without cART in a case-control study, it is not ethical to withhold cART from women. However, we observed that even though women have HIV, cART therapy was effective in maintaining their ANG levels close to that of HIV-negative women. A few women were not on cART or had a high viral load for some reason beyond our grasp. It is worth mentioning we did not have a viral load for all samples and thus could not perfectly do the analysis. Moreover, the findings from this pilot study will need to be confirmed in a larger study due to the small samples size. Within the sphere of our study, Cameroon was transitioning from PMTCT Option A to Option B+. Most studies have associated infant growth with Protease Inhibitors (Balogun et al., 2018; Papp et al., 2015) and very few studies have associated dysregulated fetal growth with prolonged cART. However, our sample size was a limiting factor in the assessment of this hypothesis (Balogun et al., 2018; Papp et al., 2015). The cross-sectional study design did not allow the monitoring of fetal growth rate and concomitant biomarker levels over time during pregnancy. Given that many of the aforementioned factors are important for placentation, vasculogenesis and placental perfusion and fetal growth, a longitudinal study design would be more effective. At the same time, it is well established that maternal peripheral plasma levels and placental plasma levels may not be the same (Ataíde et al., 2015). While a limitation of the study was the small number of cases, the study demonstrates that maternal HIV-1 infection might not have a dramatic influence on placental IGF1, IGFBP1, MMP2, MMP9, ANG1, ANG2 and Gal-13 levels in Cameroonian pregnant normotensive women with the majority receiving cART. It is not clear whether inflammatory cytokines in the placental environment of HIV-1 infected mothers (Lee et al., 1997; Moussa et al., 2001; Shearer et al., 1997; Stacey et al., 2009) or the direct effect of HIV infection on syncytial trophoblasts lead to subtle dysregulation of IGF1, IGFBP1, MMP2, MMP9, ANG1, ANG2 and Gal-13 expression in the placenta (Menu et al., 1999). Larger prospective longitudinal studies are required to determine, whether there is significant maternal HIV-associated dysregulation in the IGF1 axis and angiogenic factors during pregnancy, especially in women with low CD4 counts, and its effects on the neonate birth weight.

Our second research question aimed to investigate using a genome-wide study, changes in the gene expression and pathways, in the placenta of HIV-1 infected pregnant women, under ART at delivery. To achieve this, differentially expressed genes by HIV-1 and malaria were used to query IPA in order to construct pathways and molecular networks. Data generated indicate that some unique functions are altered in the placenta of HIV-1 positive and malaria positive women at delivery.

- **The placenta function is dysregulated by HIV-1 infection**

Analyses from IPA identified 292 dysregulated genes that overlapped with 36 pathways that were specifically affected by HIV-1. Amongst these pathways, only 3 pathways were flagged as activated by HIV-1 infection these pathways are; the Leucocyte extravasation pathway, G12/13 Signaling pathway and the LXR/RXR signaling pathway. The activation of leukocyte extravasation was modulated through the activation of genes responsible for cellular mobility, cellular polarity and tail retraction. These mechanisms are well established in regular leucocyte extravasation in other tissues (Sastry et al., 2006), however, the specificities of the process in the placenta has to be extensively described. Enhanced movement of immune cells within the placenta of HIV-1 positive women under ART could mean that even under treatment, the placenta of these women is host to increased immune activity generally skewed towards the Th1 type response. The cell types recruited into this tissue still need to be investigated. Nonetheless, chronic immune activation is common phenomenon in virally suppressed HIV positive individuals (Paiardini and Müller-Trutwin, 2013). Movement of immune cells in the placenta is a known process (Erkers et al., 2017; Pfeifer and Bunders, 2016). The implication of G12/13 signaling pathway in overall dysregulation of the placenta of HIV-1infected women is an interesting phenomenon. The activation of this pathway is modulated by the activation of transcription factors NFk-B, c-JUN and CTNN-b. They are responsible for cellular proliferation and differentiation by specifically causing the remodeling of the cytoskeleton (Lin et al., 2009). Although not well understood at this stage, this could result in conditions similar to hyperplasia in this tissue.

The LXR/RXR signaling pathway is an important pathway that regulates lipogenesis, cholesterol efflux and transport. Another key role played by this pathway is that of immune modulation. From our findings, HIV-1 causes an activation of lipogenesis, cholesterol transport and efflux in these women. Although the implication of these observations warrants further investigation, HIV-1 and ART consumption have both been associated with lipodystrophy and many metabolic abnormalities (Lana et al., 2014). LXR/RXR signaling pathway might provide leads to understanding metabolic abnormalities seen in these individuals.

Findings from this study indicate that some key functions are affected in the placenta of HIV positive women. This includes the hypothesis that HIV-1 activates the NFkB complex through the (RASSF5) through an indirect action (Network 1) which in turn increases the production of chemokine CCL5 whose expression has been shown to be significantly upregulated in the presence of HIV gp120 through the mediation of NFkB in astrocytes (Liu et al., 2014; Shah et al., 2011). This observed upregulation of CCL5 could have been triggered by the presence of gp120 from circulating viral particles in these women. Also of interest is the Hsp27 which we posit to be inhibited in this network has shown great potential in boosting anti-nef protein response (Milani et al., 2017; Vidyasagar et al., 2012). Downstream knock-on inhibition of CD163 and CD273 by Hsp27 is a proposed alteration caused by HIV-1.

From this network, we believe HIV-1 principally maintains chronic inflammation through the CCL5 and downregulation of anti-inflammatory Hsp27 associate molecules. Another interesting hypothesis proposed by this network is, HIV-1 hastens apoptosis by the mediation of the SOX4, IL24 and by inhibiting the activity of Hsp27 and its associated molecules. The overexpression of ISG15 indicates the placenta is fighting back viral infection (Sun et al., 2015).

The implication of genes relevant for immune response activation across several networks (Network 1, 2, 3 4) indicates HIV-1 sustains immune system activated through several pathways. Network 3 predicts the activation of genes of the interferon alpha which upregulates the production of IL-15 which has several downstream effects including anti-viral activity and a strong indicator of viremic setpoint(Paiardini and Müller-Trutwin, 2013). In Network 4 HIV is shown to activate TNF through the intermediate of several molecules such as RNASE2, IL1RAP, ILK, IL15RA and also through the inhibition of the glucocorticoid receptor NR3C1. Faye et al in their study investigating the cytokine profile in explanted placenta of HIV-1 positive women found lower levels of TNF-alpha and IL-8(Faye et al., 2007). There is a well-established link between placenta inflammation and adverse pregnancy outcomes (Ackerman and Kwiek, 2013; Mestan et al., 2010). Chronic inflammation observed in these women also increases their chances of MTCT of HIV (Pfeifer and Bunders, 2016).

Although in the setting of maternal HIV-1 seropositivity, the relationship between placental HIV-1 infection and adverse perinatal outcomes (Townsend et al., 2010) has been difficult to establish, our findings provide bouts of evidence as to which genes are affected in the placenta. In normal pregnancy, the placenta facilitates materno-fetal nutrient and gas exchange, serves as a tolerant immunological barrier, and produces hormones required to maintain pregnancy. Disruption in placental function is causally linked to obstetrical complications(Kramer, 2003).

- **Pregnancy-associated malaria dysregulates the function of the placenta**

The deleterious effect of placenta malaria on maternal and neonatal life is a little more documented. Our last objective whose purpose was to determine the extent to which gene expression and pathways are altered in placentas of women with placental malaria at delivery we found 372 genes expression which were matched to 100 pathways altered by this disease. Amongst 100 pathways dysregulated pathways, 14 were predicted to be activated meanwhile 4 pathways were predicted to be inhibited. These key pathways were: "Th1 Response", "Dendritic Response", "Acute Phase Response", "IL-8 Signaling", "TREM 1 Signaling", "Interferon Signaling", "Production of Nitric Oxide and Reactive Oxygen Species in Macrophage," On the other hand, 4 functional pathways in the placenta were flagged to be inhibited by malaria. The associated pathways involved with "Growth Signaling", "VEGF Family Ligand-receptor interaction" "Glioma Invasiveness Signaling" and "Prolactin Signaling."

Generalized inflammation modulated by the recruitment of monocytes, Th 1 cells and other inflammatory mediators is an established notion however, their implication in the pathogenesis or protection against placenta malaria remain conflicting(Cavaillon, 2001; R. Megnekou et al., 2015). The acute phase response pathway shows the implication of the innate component in response to placenta malaria (Saad et al., 2012). Our study has findings similar to a study carried out in Ethiopia, however, our study predicts a higher number of acute phase markers to be produced by the placenta during infection with *P. falciparum*. The IL-8, signaling pathway and the leukocyte extravasation pathway are well known mechanism implicated in the recruitment of immune cells into tissues and are responsible for localized inflammation that might impair sound fetal growth (Djontu et al., 2016; Rosette Megnekou et al., 2015; Sarr et al., 2017).

This study suggests that fetal growth might also be hampered by the inhibition of pathways involved in the production of growth hormone, vascularization and angiogenesis. This problem is further compounded by the inhibition of prolactin production in the placenta of these women. Our findings are conflicting to reports of Pearson (Pearson, 2007) who suggest higher levels of VEGF and prolactin in immune cells induced by Placenta malaria (Pearson, 2007). The reduction of vascular permeability during placenta malaria might be a protection mechanism by the host to minimize the recruitment of immune cells into the tissue.

Pathways associated with our findings confirm inflammation modulated through the activation of the NFkB complex by lymphotoxins, the KCNJ4 channel and sphingomyleniase. The activation of

NFkB complex leads to the activation of inflammatory cytokines involved in the pathogenesis of placenta as CXCL10, CCL3L3, MIP1. The activation of ICAM genes is also predicted by Network 7 and molecules expressed are similar to those of (Djontu et al., 2016; R. Megnekou et al., 2015). Network 5 shows overexpression of MHC class I gene family providing stronger discrimination by the immune system. Although this network also shows immunoglobulin activation in response to malaria, Network 7 interestingly identified IgG2 subclass to be specifically activated during placenta malaria upstream by the BATF and PYCARD transcription factors. Another important cytokine family activated in placenta malaria is IL12 family as depicted by Network 8. Interestingly, Hsp70 is activated to regulate the inflammatory response. This interplay between pathogenesis and protective mechanisms portray the complex interaction between molecules in this microenvironment.

The results here presented have to be taken in light of the choices made to provide a manageable focus for this paper. This study was carried out at a single time point. We understand that this time point may not identify preceding or proceeding events in the placenta. Therefore, the results of the regulatory network here presented should be viewed as a snapshot of the placenta at delivery. Nevertheless, genes identified as important at this time point can now be followed over time and at previous time points using other techniques. Also, it is not very clear from our study whether it was exposure to HIV-1 or ART that underlie the changes observed in these placentae. The data generated however is close to real life scenario which could identify useful molecules which could be used as correlates of disease across the level of compliance to treatment.

Literature is remarkably speculative on the mechanisms by which placenta malaria affects the placenta during pregnancy and is muter on those of HIV-1 infection. Our study provides interesting and more concrete leads to suspected routes by which these dual infections affect the placenta, and has the added advantage of providing a panoramic view on these pathways. It aids in providing a holistic view that would provide cross-cutting solutions from studies that viewed just a single aspect (Aaron et al., 2012; Ataíde et al., 2015; Blois and Barrientos, 2014; Conroy et al., 2017; Kramer, 2013, 2003; Matarazzo et al., 1994; Nissi et al., 2013; Rogers and Velten, 2011). Although it is difficult to visualize the direct impact of these findings on pregnant women infected by these diseases, it helps build evidence on the devastating consequence they might have on the placenta, maternal and fetal health. This would help policymakers to reinforce the measures geared towards the prevention and management of these diseases in these populations. Moreover, biomarkers to detect these alterations suggested by this study could be tested and confirmed.

Summarily, the present research approach identified genes affected by HIV-1 and malaria affected in the placenta and we successful confirmed that children born to these women undergo development stress due to their exposure to an inflamed environment. Since HIV is a chronic disease, we think these children might have been exposed for longer periods to stress associated to chronic immune inflammation. Although more acute in nature, placenta malaria mobilizes a both innate and adaptive components of the immune response to fight infection. On the other hand, there is visible effort by the placenta to minimize damages associated to the presence of secreting immune cells.

4.2. Conclusion

From this study we conclude that:

1. HIV-1 does not significantly affect IGF axis and angiogenic factors in pregnant women at delivery. Moreover, IGF1 levels were lowest in women co-infected with HIV and malaria.

2. There is some evidence that even under ART, the physiology of the placenta of HIV-1 infected women is altered compared to their uninfected counterparts. This is portrayed by the identification of 292 differentially expressed genes in the placenta of HIV infected women with 166 upregulated and 126 downregulated genes. These dysregulated genes matched 36 pathways of which 3 were significantly affected.

3. Pregnancy-associated malaria dysregulated 372 genes in the placenta. Amongst these 218 genes were upregulated and 154 were downregulated. These genes were matched to 100 pathways amongst which 18 were identified to be significantly altered with 14 pathways flagged to being activated whereas 4 were flagged to be inhibited.

4.3. Suggestions

- Larger prospective longitudinal studies will be required to provide stronger evidence on the dysregulation in the IGF1 axis and angiogenic factors during the pregnancy of HIV positive women, especially in women with low CD4 counts, and their effects on the neonate birth weight

- Significantly differentially expressed genes identified through microarray warrant confirmation using qPCR.

- Investigating the dynamics of the gene expression, pathways and protein expressions in pregnant women and infected with HIV and malaria and, children born to them would provide answers observed discrepancies in their health outcomes when compared to children born to uninfected women

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APPENDICES

APPENDICES

Appendix 1: Procedure for RNA extraction using the Qiagen Kit

Things to be done before experiment

- Dispense in a fume hood and wear appropriate protective clothing 10 µl β-ME per 1 ml Buffer RLT and store at –25°C.
- Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-Free DNase. Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase free water provided.

Procedure

1. Excise the tissue sample from the animal or remove it from storage. Remove RNA*later* stabilized tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg.
2. If using the entire tissue, place it directly into a suitably sized vessel for disruption and and 600uL of DDT/RLT buffer.
3. Perfectly disrupt the tissue and homogenize the lysate in Buffer RLT (do not use more than 30 mg tissue) using tissue ruptor for 30-40 seconds.

4. Centrifuge the lysate for 3 min at full speed(16000rpm). Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps.

5. Add 1 volume of 70% ethanol* to the cleared lysate, and mix immediately by pipetting. Do not centrifuge.

6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied).

7. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through. Reuse the collection tube in step 6. If the sample volume exceeds 700 µl. centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

8. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x *g* (10,000 rpm) to wash the spin column membrane. Discard the flow-through.

9. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. Buffer RDD is supplied with the RNase-Free DNase Set.

10. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.

11. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.

12. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm).

13. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm).

14. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.

15. If the expected RNA yield is >30 µg, repeat step 10 using another 30–50 µl RNase- free water, or using the eluate from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10. If using the eluate from step 10, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Appendix 2: Nucleic acid measurements (NanoDrop Lite)

Nucleic acid samples can easily be checked for concentration and purity using the Nanodrop lite spectrophotometer. To measure nucleic acid samples (dsDNA, ssDNA and RNA) select the appropriate Nucleic Acid application from the home screen

Making a Measurement

1. Select the appropriate application from the Home screen (DNA or RNA). For DNA measurements, select either the dsDNA or ssDNA assay
2. Following the on - screen instructions, establish a blank by pipetting 1-2 µl of the blanking buffer onto the bottom pedestal, lower arm and press Blank.
3. When measurement is complete, raise arm and wipe the buffer from the upper and lower pedestals using a dry laboratory wipe.
4. Confirm Blank by pipetting a fresh aliquot of blanking buffer onto the bottom pedestal, lower the arm and press Blank.
5. When measurement is complete, raise the arm and wipe the buffer from both the upper and lower pedestals using a dry laboratory wipe.
6. Measure sample by pipetting 1-2 µl of sample onto the bottom pedestal, lower arm and press measure
7. Wipe the upper and lower pedestals using a dry laboratory wipe and the instrument is ready for the next sample.

Appendix 3: RNA Bioanalyses
Bioanalyses of RNA quality

Maternal not supplied

16. RNaseZAP® recommended for electrode decontamination (Ambion, Inc. cat. no. 9780)

17. RNase-free water

18. Pipettes (10 µl and 1000 µl) with compatible tips (RNase-free, no filter tips, no autoclaved tips)

19. 1.5 ml microcentrifuge tubes (RNase-free)

20. Microcentrifuge (≥ 1300 g)

21. Heating block or water bath for ladder/sample denaturation

Preparing RNA ladder ladder from new stock
For proper handling of the ladder, following steps are necessary:

1. After reagent kit arrival, pipette the ladder in RNase-free vial. The ladder can be ordered separately (reorder number 5067-1529).
2. Heat denature it for 2 min at 70 °C.
3. Immediately cool down the vial on ice.
4. Prepare aliquots in RNase-free vials with the required amount for a typical daily use.
5. Store aliquots at -70°C.
6. Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process).

Preparing the Gel

1. Allow all reagents to equilibrate to room temperature for 30 minutes before use.
2. Place 550 µl of Agilent RNA 6000 Nano gel matrix (red) into the top receptacle of a spin filter.
3. Place the spin filter in a microcentrifuge and spin for 10 minutes at 1500 g ± 20 % (for Eppendorf microcentrifuge, this corresponds to 4000 rpm).
4. Aliquot 65 µl filtered gel into 0.5 ml RNase-free microfuge tubes that are included in the kit. Store the aliquots at 4 °C and use them within one month of preparation.

Preparing gel-Dye mix

5. Vortex RNA 6000 Nano dye concentrate (blue) for 10 seconds and spin down.

6. Add 1µl of RNA 6000 Nano dye concentrate (blue) to a 65 µl aliquot of filtered gel.

7. Cap the tube, vortex thoroughly and visually inspect proper mixing of gel and dye. Store the

dye concentrate at 4 °C in the dark again.

8. Spin tube for 10 minutes at room temperature at 13000 g (for Eppendorf microcentrifuge, this corresponds to 14000 rpm). Use prepared gel-dye mix within one day.

Loading Gel Dye Mix

9. Take a new RNA Nano chip out of its sealed bag.

10. Place the chip on the chip priming station.

11. Pipette 9.0 µl of the gel-dye mix at the bottom of the well marked (**G and circled in black**) and dispense the gel-dye mix.

12. Set the timer to 30 seconds, make sure that the plunger is positioned at 1 ml and then close the chip priming station. The lock of the latch will click when the Priming Station is closed correctly.

13. Press the plunger of the syringe down until it is held by the clip.

14. Wait for exactly 30 seconds and then release the plunger with the clip release mechanism.

15. Visually inspect that the plunger moves back at least to the 0.3 ml mark.

16. Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.

17. Open the chip priming station.

18. Pipette 9.0 µl of the gel-dye mix in each of the wells marked (**G and circled in white**).

19. Pipette 5 µl of the RNA 6000 Nano marker (green) into the well marked with the ladder symbol and each of the 12 sample wells.

20. Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process)

21. To minimize secondary structure, heat denature(70 °C, 2 minutes) the samples before loading on the chip.

22. Pipette 1 µl of the RNA ladder into the well marked with the ladder symbol .

23. Pipette 1 µl of each sample into each of the 12 sample wells.

24. Place the chip horizontally in the adapter of the IKA vortex mixer and make sure not to damage the buldge that fixes the chip during vortexing.

25. Vortex for 60 seconds at 2400 rpm.

26. Refer to the next topic on how to insert the chip in the Agilent 2100 bioanalyzer. Make sure that the run is started within 5 minutes.

Inserting a Chip in the Agilent 2100 Bioanalyzer

27. Open the lid of the Agilent 2100 bioanalyzer.

28. Check that the electrode cartridge is inserted properly and the chip selector is in position (1).

29. Place the chip carefully into the receptacle. The chip fits only one way.

30. Carefully close the lid. The electrodes in the cartridge fit into the wells of the chip.
31. The 2100 expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top left of the *Instrument* context.

Starting the Chip Run
32. In the *Instrument* context, select the appropriate assay from the *Assay* menu.
33. Accept the current *File Prefix* or modify it.

34. Click the *Start* button in the upper right of the window to start the chip run. The incoming raw signals are displayed in the *Instrument* context.

35. select the *Data File* link that is highlighted in blue or go to the *Assay* context and select the *Chip Summary* tab. To enter sample information like sample names and comments.

36. To review the raw signal trace, return to the *Instrument* context.

37. After the chip run is finished, remove the chip from the receptacle of the bioanalyzer and dispose it.

Appendix 4: In vitro transcription protocol

A. First-strand cDNA Synthesis

22. Anneal the T7-Oligo(dT) Primer to the RNA sample. If a "no template" control reaction is performed, substitute 2 µl of RNase-Free Water for the Total RNA sample.
1 µl RNase-Free Water
1 µl Total RNA sample (25-500 ng)
1 µl T7-Oligo(dT) Primer
=3 µl Total reaction volume
23. Incubate at 65°C for 5 minutes in a water bath or thermocycler.

24. Chill on ice for 1 minute. Centrifuge briefly in a microcentrifuge.

25. Prepare the 1st-Strand cDNA Synthesis Master Mix. ***Important!*** *Be sure to use SuperScript III Reverse Transcriptase. Do not use the SuperScript 5X Buffer or the DTT that is provided with the enzyme.*

26. For each 1st-strand cDNA synthesis reaction, combine on ice:

1.5ul 1st-Strand cDNA PreMix number of tubes

0.25ul DTT

0.25ul SuperScript III Reverse Transcriptase (200 U/µl)

2ul =Total reaction volume

27. Gently mix the 1st-Strand cDNA Synthesis Master Mix and then add 2 µl of it to each reaction.

28. Gently mix the reactions and then incubate each at 50°C for 30 minutes in a water bath or thermocycler. If the thermocycler has a heated lid function, heat the lid only if the temperature of the lid can be maintained at about 50°C.

B. Second-strand cDNA Synthesis

1. Prepare the 2nd-Strand cDNA Synthesis Master Mix.For each 2nd-strand cDNA synthesis reaction, combine on ice:

4.5ul 2nd-Strand cDNA PreMix
0.5ul 2nd-Strand DNA Polymerase
5ul Total reaction volume

2. Gently mix the 2nd-Strand cDNA Synthesis Master Mix and then add 5 µl of it to each reaction.

3. Gently mix the reactions and then incubate at 65°C for 10 minutes in a water bath or thermocycler. Centrifuge briefly in a microcentrifuge. ***Important!*** *Be sure to incubate the reactions at 65°C.*

4. Incubate the reactions at 80°C for 3 minutes. Centrifuge briefly in a microcentrifuge then chill on ice. ***Note:*** *If desired, the reactions can now be frozen and stored overnight at –20°C.*

C. *In Vitro* Transcription of Biotin-aRNA

1. Warm the T7 RNA Polymerase to room temperature. Thaw the remaining *in vitro* transcription reagents at room temperature. If a precipitate is visible in the thawed T7 Transcription Buffer, heat the buffer to 37°C until it dissolves. Keep the T7 Transcription Buffer at room temperature.
2. Thoroughly mix the thawed T7 Transcription Buffer and the NTP PreMix solutions. ***Important!*** *If a precipitate is visible in the thawed T7 Transcription Buffer, heat the Buffer to 37°C until it dissolves. Mix the Buffer thoroughly. Keep the Buffer at room temperature.*

3. Prepare the *In Vitro* Transcription Master Mix. For each *in vitro* transcription reaction,

2ul T7 Transcription Buffer
3ul UTP / Biotin-UTP
10ul NTP PreMix
3ul DTT
2ul T7 RNA polymerase
20ul Reaction volume
5. Gently but thoroughly mix the *In Vitro* Transcription Master Mix and then add 20 µl of it to each reaction.

6. Gently but thoroughly mix the reactions and then incubate at 42°C for 4 hours in a thermocycler. If the thermocycler has a heated lid function, heat the lid only if the temperature of the lid can be maintained at about 50°C. If the lid temperature cannot be maintained at about 50°C, then perform the incubations without heating the lid. ***Important!*** *Do not exceed 4hour incubation at 42°C. Optimal yield and length of Biotin-aRNA is achieved in 4 hours.*

7. Add 2 µl of RNase-Free DNase I to each reaction. Mix gently and then incubate each at 37°C for 15 minutes.

Appendix 5: Microarray procedure

Prepare RNA for Hybridization

1. Preheat the cRNA sample tube at 65°C for 5 minutes.
2. Vortex the cRNA sample tube, then pulse centrifuge the tube at 250 xg.
3. Allow the cRNA sample tube to cool to room temperature, then proceed as soon as the tube has cooled.
4. Using a single-channel precision pipette, add the appropriate volume from each cRNA sample tube into each hybridization tube (750 ngfor 12 for 12 samples)
5. Using a single-channel precision pipette, add the appropriate volume of RNase-free water into each cRNA sample tube.(5 ul for 12 samples)
6. Using a single-channel precision pipette, add the appropriate volume of HYB into each cRNA sample tube. (10 ul for 12 samples)

Assemble the Hyb Chambers

1. Place the following items on the bench top:

- BeadChip Hyb Chamber (1 per 4 BeadChips)
- BeadChip Hyb Chamber gasket (1 per Hyb Chamber)
- BeadChip Hyb Chamber inserts (4 per Hyb Chamber)

2.

- Place the Hyb Chamber Gasket into the Hyb Chamber as follows:
- a. Match the wider edge of the Hyb Chamber gasket to the barcode- ridge side of the Hyb Chamber.
- b. Lay the gasket into the Hyb Chamber, and then press it down all around.
- c. Make sure the Hyb Chamber gasket is properly seated.

3. Add 200 µl HCB into the eight humidifying buffer reservoirs in the Hyb Chamber. If you are hybridizing fewer than four BeadChips, only fill the reservoirs of sections that will contain BeadChips.
4. Close and lock the BeadChip Hyb Chamber lid.
   1. Seat the lid securely on the bottom plate.
   2. Snap two clamps shut, diagonally across from each other.
   3. Snap the other two clamps.
5. Leave the closed Hyb Chamber on the bench at room temperature until the BeadChips are loaded with the DNA sample.

Prepare BeadChips for Hybridization

1. Remove all the BeadChips from their packages.
2. Place each BeadChip in a Hyb Chamber Insert, orienting the barcode end so that it matches the barcode symbol on the Hyb Chamber Insert

Load Sample

3. Using a single-channel precision pipette, add the appropriate volume of DNA sample onto the center of each inlet port (15ul for 12 samples)
4. Visually inspect all sections. Ensure sample covers all of the sections of the stripe. Record any sections that are not covered. Some residual sample may still remain in the inlet port. This is normal.
5. Open the Hyb Chamber.
6. Load 4 Hyb Chamber Inserts containing sample-laden BeadChips into each Hyb Chamber.
7. Position the barcode end over the ridges indicated on the Hyb Chamber and ensure the inserts are securely seated.

Hybridize BeadChips

1. Close and lock the BeadChip Hyb Chamber lid.

1. Seat the lid securely on the bottom plate.
2. Snap two clamps shut, diagonally across from each other.
3. Snap the other two clamps.
4. Check to ensure that the Hyb Chamber is completely closed, as any gap in the seal will result in evaporation during hybridization and will compromise analytical data.

2. Place the Hyb Chamber into the 58°C Illumina Hybridization Oven so that the clamps face the left and right sides of the oven. The Illumina logo on top of the Hyb Chamber should face you.
3. (Optional) Start the rocker at speed 5. Turn on the switch just above the power switch.
4. Close the Illumina Hybridization Oven door.
5. Incubate the BeadChips for at least 14 hours but no more than 20 hours at 58°C.
6. Update the lab tracking form with the start and stop times.

Prepare High-Temp Wash Buffer

1. In preparation for the next day's washes, prepare 1X High-Temp Wash buffer from the 10X stock by adding 50 ml 10x High-Temp Wash buffer to 450 ml RNAse-free water.
2. Place the Hybex Waterbath insert into the Hybex Heating Base.
3. Add 500 ml prepared 1X High-Temp Wash buffer to the Hybex
4. Set the Hybex Heating Base temperature to 55°C.
5. Close the Hybex Heating Base lid and leave the High Temp Wash buffer to warm overnight.
6. Proceed to *Wash BeadChip* on page 53 the next day.

Wash BeadChip

In this process, prepare for the wash steps by removing the BeadChips from the overnight hybridization. Remove the BeadChip coverseals and then wash the BeadChips.

Preparation

- Add6mlE1BCbufferto2LRNase-freewatertomaketheWashE1BC solution.
- Place 1 L of diluted Wash E1BC buffer in a Pyrex No. 3140 beaker.
- Pour 250 ml of Wash E1BC buffer into a glass wash tray.
- Pour 250 ml of 100% EtOH into a separate glass wash tray.

Steps

- This process involves the following procedures:
- *Seal* *Removal*
High *Temp* *Wash*
First *Room-Temp* *Wash*
Ethanol *Wash*
Second Room-Temp Wash Block

Seal Removal

1. Remove the Hyb Chamber from the oven and place it on the lab bench. Disassemble the chamber.
2. Using powder-free gloved hands, remove all BeadChips from the Hyb Chamber and submerge them face up at the bottom of the beaker.
3. Using powder-free gloved hands, remove the coverseal from the first BeadChip under the buffer. This may require significant force, due to the strength of the adhesive. Ensure that the entire BeadChip remains submerged during removal.
4. Using tweezers or powder-free gloved hands, transfer the BeadChip to the slide rack submerged in the staining dish containing 250 ml Wash E1BC solution. This is the staging area to hold the BeadChips until all coverseals have been removed under the buffer.
5. Repeat steps 3 and 4 for all BeadChips from the same Hyb Chamber.

High Temp Wash

1. Using the slide rack handle, transfer the rack into the Hybex Waterbath insert containing High-Temp Wash buffer that was prepared the previous day.

2. Close the Hybex lid.

3. Incubate static for 10 minutes (Figure 33).

First Room-Temp Wash

1. After the 10-minute incubation in High-Temp Wash buffer is complete, immediately transfer the slide rack back into a staining dish containing 250 ml fresh Wash E1BC buffer .
2. Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.
3. Set the orbital shaker to medium-low.
4. Place the staining dish on the orbital shaker and shake at room temperature for 5 minutes.
5. Shake at as high a speed as possible without allowing the solution to splash out of the staining dish.

Ethanol Wash

1. Transfer the rack to a new staining dish containing 250 ml fresh 100% Ethanol.
2. Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.
3. Place the staining dish on the orbital shaker and shake at room temperature for 10 minutes.

Second Room-Temp Wash

1. Transfer the rack to the same staining dish containing 250 ml Wash E1BC buffer.
2. Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.
3. Place the staining dish on the orbital shaker and shake at room temperature for 2 minutes.

Block

1. Place the BeadChip wash tray on the rocker mixer.
2. Add 4 ml Block E1 buffer to the Wash Tray.
3. Using tweezers, transfer the BeadChip face up into the BeadChip wash tray. The barcode should be at the well end. Use the well at the end of the wash tray to grip the BeadChip.
4. Pick the wash tray up and gently tilt it manually to ensure the BeadChip is completely covered with buffer.
5. Place the wash tray back onto the rocker platform and rock at medium speed for 10 minutes.
6. Clean the Hyb Chambers:
    1. Remove the rubber gaskets from the Hyb Chambers.
    2. Rinse all Hyb Chamber components with DI water.
    3. Thoroughly rinse the eight humidifying buffer reservoirs.
7. Discard unused reagents in accordance with facility standards.
8. Proceed to *Detect*

Detect Signal

In this process, Cy3-SA is introduced to bind to the analytical probes that have been hybridized to the BeadChip. This allows for differential detection of signals when the BeadChips are scanned.

Preparation

RemovetheCy3-Streptavidinfromcoldstorage.Leaveitonthe benchtop for at least 10 minutes at room temperature.

Prepare 2 ml Block E1 buffer with a 1:1,000 dilution of Cy3-Streptavidin (stock of 1 mg/ml) for *each* BeadChip in a glass wash tray.

Add 2 ml Block E1 buffer + streptavidin-Cy3 into a new BeadChip wash tray.

Steps

Prepare BeadChip

1. Using tweezers, grasp the BeadChip at the barcode end via the well in the blocker wash tray.
2. Transfer the BeadChip to the wash tray containing Cy3-Streptavidin. Place it flat with the barcode near the tweezer well.

3. Pick the wash tray up and gently tilt it manually to ensure the BeadChip is completely covered with buffer.
4. Cover the wash tray with the flat lid provided.

5. Place the tray on the rocker mixer.
6. Rock the BeadChip on medium for 10 minutes.

Third Room-Temp Wash

1. Add 250 ml Wash E1BC into a clean staining dish with a slide rack.
2. Using tweezers, grasp the BeadChip at the barcode end and remove it

from the wash tray.

3. Transfer the BeadChip into the slide rack submerged in the staining dish. Immediately submerge the BeadChip into the Wash E1BC.
4. Using the slide rack handle, plunge the rack in and out of the solution 5 times.
5. Set the orbital shaker to medium-low.
6. Ensure the BeadChip is completely submerged in the Wash E1BC.
7. Place the staining dish on the orbital shaker and shake at room temperature for 5 minutes.

Dry BeadChips

It is important to centrifuge the BeadChips immediately after removal from the wash to prevent surface evaporation.

1. Set the centrifuge to 1,400 rpm at room temperature for 4 minutes.
2. Place clean paper towels on the centrifuge microtiter plate holders to absorb excess solution.
3. Fill the staining dish balance slide rack with an equivalent number of standard glass microscope slides.
4. Using powder-free gloved hands, quickly pull the slide holder out of the Wash E1BC.
5. Transfer the rack of BeadChips from the staining dish to the centrifuge, close the door, and press Start.
6. Transfer the rack of BeadChips from the staining dish to the centrifuge. Centrifuge at 1,400 rpm at room temperature for 4 minutes.
7. Once the BeadChips are dry, store them in a dark, ozone-free environment until ready to scan.
8. Discard unused reagents in accordance with facility standards.

Image BeadChip on the iScan System

The iScan Reader uses a laser to excite the fluor of the single-base extension product on the beads of the BeadChip sections. Light emissions from these fluors are then recorded in high-resolution images of the BeadChip sections. Data from these images are analyzed to determine SNP genotypes using Illumina's GenomeStudio Gene Expression Module.

Preparation On the lab tracking form,record the following for each BeadChip:

- Scanner ID
- Scan date
- For more information about the iScan System, iScan Control Software, or AutoLoader2, see the *iScan and AutoLoader2 System Guide*.
- For scanning instructions using the HiScan System, see the *HiScan System User Guide*.

Steps Overview

The iScan Control Software leads you through the BeadChip scanning process, which is as follows: Turn on the iScan Reader, boot up the iScan PC, and start the iScan Control Software application.

- Let the iScan Reader warm up for at least 5 minutes before beginning a scan. It is fine to use the iScan Control Software during this time.
- Load the BeadChips to be scanned, and copy their decode data into the Input Path.
- Load the BeadChips to be scanned, and copy their decode data into the Input Path.
- Check the scan settings and input/output paths, making modifications if necessary.
- If you wish, remove BeadChip sections or entire BeadChips from the scan.
- Start the scan and monitor its progress.
- Review the scan metrics.

Starting Up the iScan System

1. For each BeadChip, download the decode content from iCom or copy the contents of the DVD provided with the BeadChip (if purchased) into the Decode folder. The folder name should be the BeadChip barcode (for example, 4264011131).

If there is no decode folder, follow the instructions in *Setting Up Input and Output Paths* on page 64.

2. Double-click the iScan Control Software icon on the desktop.

The Welcome window appears. The iScan Control Software automatically connects to the iScan Reader and initializes it. When the reader is initialized, the red dot in the status bar turns green, and the sta- tus changes to Initialized.

3. Set the LIMS dropdown list to None and enter your Windows user name.
4. 4. Click Start.

The iScan Reader tray opens.

Loading BeadChips and Starting the Scan

1. Load the BeadChips into their carrier and place the carrier into the iScan Reader tray. Click Next.

The tray closes and the iScan Reader begins scanning the barcodes.

When the iScan Reader has read all of the barcodes, the Setup window displays the barcode, description, and scan setting for each BeadChip in the position corresponding to its location in the tray. You can click any barcode to view an image of the corresponding BeadChip.

2. The Type column should say “BeadChip 8x1” and the Scan Setting should say “Direct Hyb”.
3. If the Scan Setting field beside each BeadChip does not say "Direct Hyb", click Settings. The Scan Settings File window appears.
4. Select Direct Hyb and click Open.
5. If you want to change the image format (*.jpg or *.tif), click the Menu

button and select Tools | Options. The Options dialog box appears.

- JPEG files let you review the image of the scanned array sections, but you cannot extract bead intensity data.
- TIFF files let you review the scanned images and extract bead intensity data. The file size is much larger than *.jpg.

6. Click the Scan Settings tab.
7. Select Direct Hyb in the left pane. The scan settings appear in the right pane.
8. Click the down arrow beside Image Format, and select Tiff. Click OK.
9. Make sure that the input and output paths are correct.
10. If you do not want to scan certain sections of a BeadChip, click the barcode to display an image of the corresponding BeadChip in the Setup window. Click any BeadChip section to remove it from the scan. The section is longer highlighted blue.
11. If you want to remove an entire BeadChip from the scan, delete the barcode from the Setup window.
12. To begin scanning the BeadChips, click Scan. Scanning should take 24 minutes per BeadChip. As the scan progresses, status icons and messages are displayed in the bottom left corner of window. For more information about what happens during the scan.
13. At the end of the scan, a Review window appears. The Scan Metrics table at the top shows the intensity values, registration, and focus metrics for each stripe on the BeadChip. You can also review scan metrics for any BeadChip in the Output Path folder. Scan metrics are in a document titled Barcode_qc.txt, where “Bar- code” represents the barcode number for a single BeadChip. The focus metric ranges between 0 and 1. High focus scores indicate a sharp, well defined image, leading to high bead intensity values.

14. If any stripes failed registration, cclick Rescan to automatically rescan all failed areas on the BeadChips in the carrier.
15. When you finish reviewing the data, click Done to return to the Start window. If you click Done on the Review window without rescanning failed sec- tions, the *.idat files will be saved for those sections but not accessible. The entire sample section will have to be rescanned to generate *.idat files.

When you return to the Start window, images from the scan are no longer available to be viewed in the iScan Control Software. Use another pro- gram such as Illumina’s GenomeStudio to view images from the scan.

Setting Up Input and Output Paths

This step should only occur once, when you install the iScan Control Software on the iScan PC. After that, all scans use these paths.

1. Create a folder on the iScan PC D: drive to contain the decode (*.dmap) and Sentrix

Ministry of Health, Cameroon IRB approval: No 2013/11/366/L/CNERSH/SP University of Hawaii IRB approval: CHS# 21370			
Investigators:	LEKE, ROSE, PhD	University of Yaounde	237 99 95 73 29
	BABAKHANYAN, ANNA, PhD	University of Hawaii	annab8@hawaii.edu
Protocol Title: **Infectious Diseases Affecting Pregnant Cameroonian Women & Newborns**			

descriptor (*.sdf) file that were downloaded from iCom or came on each BeadChip DVD (for example, D:\Decode). The iScan Control Software refers to this folder as the Input Path.

2. Create another folder on the iScan PC D:_drive where you want the iScan Control Software to store the image data from the scan (for example, D:\ImageData).

3. During the scan, the iScan Control Software automatically creates sub- folders named with each BeadChip's barcode number. The folder will be populated with image files (*.jpg or *.tif), scan metrics (*.txt), and inten- sity data files (*.idat) for each BeadChip. If the images are in *.tif format, the output path will also contain bead location files (*.locs).

4. During the Scan

5. *Calibration*

6. The iScan System begins with a calibration step, which may take several minutes to complete. The BeadChips are automatically tilted and aligned to ensure that they are in the optimal position for the scan.

7. Tilt—The iScan Reader autofocus feature records the Z-position (height) of three corners of the BeadChip to determine its current tilt, and adjusts the BeadChip until it is flat.

8. Align—The iScan Reader identifies the X-Y position (lateral location) of the fiducials (focus points) on the BeadChip edges, and adjusts the optics.

9. If there are defective or dirty sections at any of the three alignment corners, the software attempts to use alternate sections until satisfactory calibration is achieved. If no alternate sections are available, calibration fails and an error message is displayed.

10. *Hard Drive Space*

11. Before beginning a scan, the iScan Control Software checks the hard drive to ensure sufficient space is available. If sufficient disk space is not available, an error message is displayed, and the arrays will not be scanned.

12. *Monitoring the Scan*

13. After calibration, the iScan Reader begins scanning. You can view the progress of the scan in the Scan window.

Appendix 6: Informed consent form
UNIVERSITY OF YAOUNDE I & UNIVERSITY OF HAWAII
RESEARCH INFORMED CONSENT

Participant ID:

PURPOSE OF RESEARCH

Dear Madam, you are invited to participate in a research study called: **"Infectious Diseases Affecting Pregnant Women and Newborns".** Malaria is a huge problem in Cameroon. Malaria is especially serious in pregnant women and leads to complications that threaten the health of the mother and the baby. Women who have HIV during pregnancy are two times more likely to have malaria and have more complications from HIV and malaria. In this small research project we hope to learn how HIV and malaria infections affect pregnant women and their babies.

INFORMED CONSENT

You are asked to participate in this study because you are pregnant and in the third trimester of pregnancy. It is important for you to understand why we are doing the research study and what we will ask you to do if you decide to participate. This is a consent form that provides information about our research study. Please, read it carefully. One of our staff members will also explain it to you. You should ask any questions you have about this research study. You may talk to your family or friends before deciding to participate. If you want to take part in this research study, you will be asked to sign this consent form or give your oral consent. This research study is looking to enroll 242 pregnant women from prenatal clinics in Yaounde I and nearby villages.

VOLUNTARY PARTICIPATION

Your participation in this study is entirely voluntary. Your decision not to participate will not have any negative effect on you or your medical care. You can decide to participate now, but withdraw your consent later and stop being in the study without any loss of medical care to which you are entitled.

DURATION OF STUDY INVOLVEMENT

This research study is expected to take approximately one year. You will meet with our study team two times and your participation in this study will take total of 1 hour:

- **Visit 1.** During the initial visit and enrollment into the study, information about your health from your medical record book will be recorded (about 30 min).
- **Visit 2.** Second visit will take place soon **after** delivery, when small blood sample from your arm, cord and placental samples will be collected. In addition, medical information about pregnancy outcome, maternal and baby health will be collected (about 30 min).

PROCEDURES

If you agree to participate:

Visit 1.

- During first visit you will be asked to provide information including age, current and past pregnancies, date of your last menstrual period or estimated date of delivery, if you had malaria, HIV or other infections during pregnancy, about medications you have been taking and other health related information from your medical record book.
- Note, in order to be eligible for this study, you will have to provide information about your HIV status, HIV test results and medication used. If you have not done so already, you should be tested for HIV at a facility approved by the Ministry of Health and provide the results to the

research project. The information will be kept confidential and used by researchers to accurately interpret results of this research study. HIV testing and treatment in Cameroon is FREE. Knowing if you have HIV will also help your doctor provide you with treatment for HIV and prevent transmission of HIV to the baby.

- At this visit you have to agree to deliver at the clinic where you have been recruited. This will help the doctors during delivery, who will know your medical history, as well as our study to collect samples. If your doctor advises you to deliver at a different clinic, you will have to let our study team know ahead of time.

Visit 2.

- When it is time for you to deliver, you or your family will inform a member of the project about the impending delivery. You must deliver at the clinic we agreed on when you enrolled in the study, unless your doctor advised you otherwise.
- A certified medical technician or hospital nurse will collect few drops of blood from your finger to test for malaria and anemia before delivery.
- Soon after delivery, medical information about the pregnancy outcome, your and your baby's health (baby weight, weight of the placenta, etc.) and any new information since last visit on HIV or malaria test results and medications will be collected.
- After the baby is born and the placenta comes out, we will obtain 50cc (3.5 table spoons) cord and placental blood, as well as a very small piece of the placenta (size of a box of matches ~ 5cm x 5cm x 5cm) immediately after delivery. Then, the rest of the placenta will be returned to the hospital staff. These procedures will pose **NO** health risk to you or your baby.
- If there are no complications after delivery and you do not have anemia, a certified medical technician will collect 10cc (less than 1 table spoon) of blood from your arm. This procedure has minimal health risk. If determined that we cannot safely collect blood sample from your arm without endangering your health, we will not collect blood samples from you and only collect cord and placental samples.

THE RESEARCH STUDY

- We will take your blood samples and piece of placenta to the University of Yaounde I Biotechnology Centre in Nkolbisson. There we will examine the blood to see if you have malaria. We will also examine blood from the placenta to see if there are any malaria parasites. A piece of the placenta will be also examined using a microscope for parasites and for changes they might have caused. Your blood and blood from the placenta and umbilical cord will be tested for substances that might affect malaria parasites or HIV. Additional studies on effect of malaria and HIV on your blood cells will be conducted at the Chantal Biya International Research Center.
- Test results will be kept confidential.
- If you test positive for malaria or anemia and have agreed to receive this information, you will be notified of test results. The study will pay for the anti-malarial drugs, folic acid and iron supplements that would be prescribed by your doctor according to the government policy.

PARTICIPANT RESPONSIBILITIES

As a participant, your responsibilities include:

- Follow the instructions of the Protocol Director and study staff.
- Keep your study appointments. If it is necessary to miss an appointment, please contact us as soon as possible to reschedule. Protocol Director: Rose Leke (Tel: 237 99 95 73 29), Anna Babakhanyan [Tel: 237 22225955].
- Provide information about your HIV status

- Tell the Protocol Director or research study staff about any side effects, doctor visits, or hospitalizations that you may have.
- Deliver the baby at the clinic that was agreed on during the first visit, unless your doctor recommends you to deliver in other clinic. In this case, you have to notify our study team.
- Ask questions as you think of them.
- Tell the Protocol Director or research staff if you change your mind about staying in the study.

WITHDRAWAL FROM STUDY

If you first agree to participate and then you change your mind, you are free to withdraw your consent and discontinue your participation at any time. Your decision will not affect your ability to receive medical care for your disease and you will not lose any benefits to which you would otherwise be entitled. If you decide to withdraw your consent to participate in this study, you should notify Rose Leke (Tel #: 237 99 95 73 29).

The Protocol Director may also withdraw you from the study without your consent for one or more of the following reasons:

- Failure to follow the instructions of the study staff
- Failure to notify staff if you cannot come to the scheduled appointment
- Failure to provide HIV test results
- The Protocol Director decides that continuing your participation could be harmful to you
- The study is cancelled
- Unanticipated circumstances

POSSIBLE RISKS, DISCOMFORTS, AND INCONVENIENCES

- There are minimal risks associated with this study. You should talk with the Protocol Director or study personnel, if you have any questions.
- Risk is associated with *collection of blood samples*, including mild pain and bruising, slight risk of infection at the collection site. The risk of infection is minimal, since only sterile equipment will be used to collect blood. Our clinical team has been collecting blood since 1994 without any adverse effects. If an adverse effect occurs (i.e. bruising or infection), the study will pay for attending doctor to provide necessary remedies per local governmental policies.
- Since no interventions are going to be used, there are no potential risks associated with vulnerable pregnant women.
- We will test for anemia and if you have anemia blood sample from your arm will not be collected.
- Risk associated with loss of confidentiality. See section below.

POTENTIAL BENEFITS

Women participating in this study will receive the following benefits:

- Test for anemia and malaria for the mother
- Reimbursement for antimalarial drugs if parasites are detected in the blood
- Reimbursement for iron tablets if prescribed by physician

CONFIDENTIALITY

Your identity and test results (including HIV test results) will be kept as confidential as possible as required by law. Your personal health information related to this study may be disclosed as authorized by you. Your research records may be disclosed, but in this case, you will be identified

only by a unique code number. Information about the code will be kept in a secure location and access limited to research study personnel.
The results of this research study may be presented at scientific or medical meetings or published in scientific journals. However, your identity will not be disclosed**.**
Patient information may be provided to US or Cameroonian government agencies, such as Ministry of Health, University of Yaounde, University of Hawaii, National Institutes of Health, study staff and site monitors.

FINANCIAL CONSIDERATIONS

You will not be paid to participate in this research study. There is no cost to you for participating in this study. A small token of appreciation will be provided for the study participants, such as baby clothing, soap or baby blanket.
Sponsor: Fogarty International Center, National Institutes of Health, USA is providing financial support and materials for this study.

COMPENSATION for RESEARCH-RELATED INJURY

In the case of infection results from collection of blood, the project will pay for drug and other appropriate treatment.

ALTERNATIVES

You may decide not to participate in this research study. If you decide not to participate, you will receive the normal care provided by the clinic.

CONTACT INFORMATION

Questions, Concerns, or Complaints: You should also contact study director listed below at any time if you feel you have been hurt by being a part of this study. If you have any questions, concerns or complaints about this research study, its procedures, risks and benefits, or alternative courses of treatment, you should call
Study director: Dr. Rose Leke 237 99 95 73 29, Dr. Anna Babakhanyan 237 2222 5955
Dr. Kaptue, Head, Ethical Committee, Ministry of Health 237 22 23 1 52
You are welcome to visit us at the Biotechnology Center to learn more about our research.

PARTICIPANT'S BILL OF RIGHTS

As a research participant you have the following rights. These rights include but are not limited to the participant's right to:

- be told why the research is being done
- be given an explanation of the procedures performed
- be given a description of any discomforts and risks that you may experience
- be given an explanation of any benefits to you
- be given a disclosure of any other choices you have for your health care
- be told of the avenues of medical treatment if you will have complications
- be given an opportunity to ask questions concerning the research or the procedures involved
- be told that you may withdraw from the study at any time and still receive your regular medical care provided by the government clinics
- be given a copy of the signed and dated consent form
- be told that you are free to decide if you want or do not want to be in this research project.

CONSENT TO PROVIDE INFORMATION ON HIV STATUS

The Ministry of Health recommends that all pregnant women should be tested for HIV/AIDS. If you enroll in this project, you must be tested for HIV at a facility approved by the MOH and provide the results to the project. Providing information on your HIV status is required for participation in this research study. If you do not provide information on your HIV status, you will be ineligible and cannot participate. The information will be maintained in strict confidentiality and used only for research purposes.

I agree to provide information on my HIV/AIDS status ☐YES ☐NO

_____

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

Do you wish to be told the results of malaria and anemia tests? ☐ YES ☐ NO

Statement of Consent

I have read the above information or it has been read to me. I have had the opportunity to discuss this research study with the staff, and I have had my questions answered by them in language I understand. I take part in this research study of my own free will. I understand that I may withdraw from participation at any time. This will not affect my medical care. A copy of this consent form has been given to me.

_____

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

OR

_____

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

_____

_____

Name of person reading the consent form Signature Date (dd/mm/yy)

_____

_____

Name of person witnessing the consent process Signature Date (dd/mm/yy)

Statement of Consent for Unmarried Minors

If the subject is less than 18 years of age and is unmarried, a parent must sign on behalf of the subject in the space provided below AND the subject must sign this Assent Form.

_____

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

_____

_____

Name of person reading the consent form Signature Date (dd/mm/yy)

Relationship:
Mother ☐ Father ☐ Guardian ☐ specify relationship_____

AND

If the subject is less than 18 years of age and is unmarried, she must sign attesting that she understands the purpose of this research study, she understands what is expected of her if she participates, and that she willingly agrees to participate.

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

_____

Name of person witnessing (PRINT) Signature Date (dd/mm/yy)

Consent for Storing Samples

Research using samples from people is an important way to try to understand human disease. The investigators want to include your samples in future research projects. There are several things you should know before allowing your samples to be studied. Your blood and placental samples will be stored in the freezer under your participant identification number. After your samples are stored, you cannot withdraw your consent to the use of the samples in future research. Coded samples will be used for future research by the current researcher and other researchers from Cameroon and US institutions. Your name, address and other personal identification will not be stored with the samples for future research.

* Any of your samples which are used in research may result in new discoveries. However, investigators will NOT use your samples to develop any products and will NOT obtain any commercial value.

I consent to my samples being saved for future research on

A.	HIV and malaria	☐ YES	☐ NO
B.	infectious diseases affecting pregnant women and babies	☐ YES	☐ NO
C.	any other medical research	☐ YES	☐ NO

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

OR

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

_____

Name of person reading the consent form Signature Date (dd/mm/yy)

_____

Name of person witnessing the consent process Signature Date (dd/mm/yy)

Statement of Consent for Unmarried Minors

If the subject is less than 18 years of age and is unmarried, a parent must sign on behalf of the subject in the space provided below AND the subject must sign this Assent Form.

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

_____

Name of person reading the consent form Signature Date (dd/mm/yy)

Relationship:
Mother ☐ Father ☐ Guardian specify relationship_____

AND

If the subject is less than 18 years of age and is unmarried, she must sign attesting that she understands the purpose of this research study, she understands what is expected of her if she participates, and that she willingly agrees to participate.

_____

Subject Name (PRINT) Signature Date (dd/mm/yy)

_____

Name of person witnessing (PRINT) Signature Date (dd/mm/yy)

Appendix 7: Case Report Form

Woman's Identification Number Woman's Initials

W		1							

CASE REPORT FORM

HPM STUDY: Infectious Diseases
Affecting Pregnant Women & Newborns

FOR

PREGNANT WOMEN ENROLLED
AT DELIVERY

IN CENTRAL HOSPITAL

Version 1.0
Prepared by
Anna Babakhanyan

Woman's Identification Number | Woman's Initials

H IV		W	1							

Procedures for Pregnant Women

At the Hospital

Before Delivery	Informed Consent Form Signed, copy given to the woman	
	Contact Information, Demographics, Pregnancy information & Medical History Collected	
	5ml of Peripheral Blood Collected & make dry blood spots	
	RDT for Malaria and HemoCue for Anemia	
After Delivery	Delivery Data & Information on baby collected	
	Samples of placental biopsies, impression smears, dry blood spots, placental and cord blood collected	
	Gave woman baby clothing, if anemic or with malaria noted to reimburse for medications	
	Record information into the Logs	

At the Laboratory

		Peripheral	IVS	Cord
EDTA	Complete blood cell count		n/a	n/a
	Blood smear for malaria, parasitemia determination			
	Differential counts			
Heparinized	RBC and plasma stored			
	PBMCs cryopreserved			
	PBMC put in culture for B cells & cytokines			
	PBMC stored in RNA later			
Other	Dry Blood spots stored in –80C			
	Impression smears read & stored in slide boxes			

Woman's Identification Number Woman's Initials

W		1								

CONTACT INFORMATION

Date of enrollment

				2	0		

Day Month Year

First Name	
Last Name	
Place of Residence	
Phone Number	
Emergency contact name	
Emergency contact place of residence	
Emergency contact Phone Number	
Page Completed Date:	Page Completed Initials:

Woman's Identification Number Woman's Initials

W		1				

DEMOGRAPHIC INFORMATION

1. Date of Birth: ☐☐/☐☐☐/1 9☐☐ (DD/MMM/YYYY)

2. Ethnic group: ☐ Beti ☐ Bamileke ☐ Hausa / Fulani ☐ Northwest / Southwest ☐ Other

3. Marital status: ☐ Currently Married ☐ Widowed ☐ Separated ☐ Never married ☐ Divorced ☐ Other

4. Years of education: ☐☐ (Woman self) ☐☐ (Husband if any)

5. Occupation ☐ Farmer ☐ Housewife ☐ Professional ☐ Other

5a. Husband if any ☐ Farmer ☐ House worker ☐ Professional ☐ Other

6. Do you smoke cigarettes now? ☐ Yes ☐ No

7. Do you drink alcohol now? ☐ Yes ☐ No

PREVIOUS PREGNANCY

Number of:

Previous pregnancies ☐☐ Full Term ☐☐ Premature Delivery ☐☐

Still Birth ☐☐ Spontaneous Abortion ☐☐ Voluntary Abortion ☐☐

Low Birth Weight (<2.5kg) ☐☐ C-section ☐☐ Living Children ☐☐

Time since last pregnancy (in months)	
Number of pregnancies on IPT-SP	
IPT SP and bednet used during last pregnancy	
Number of IPT-SP doses during last pregnancy	

Woman's Identification Number

Woman's Initials

W		1							

CURRENT PREGNANCY

Date of last menstrual period					2	0		
	Day		Month		Year			
Expected delivery due date					2	0		
	Day		Month		Year			

Length of gestation	Weeks:	Days:

Preeclampsia		Renal insufficiency	
Hypertension		Severe liver insufficiency	
Diabetes		History of TB	
Overbleeding		AIDS	
Cutaneous allergies		None	
Other, specify			

Number of SP doses received during current pregnancy	
Did the woman use a bednet? (Y/N)	
Number of malaria episodes during current pregnancy	
Antimalarial Drug 1:	Start Date For how many days?
Antimalarial Drug 2:	Start Date For how many days?

Date of last Malaria episode					2	0	
	Day		Month		Year		

Woman's Identification Number							Woman's Initials		
W	1								

CURRENT PREGNANCY

Is the woman HIV positive? (Y/N)	

Date of HIV diagnosis								
				2	0			
Day		Month		Year				

Is the woman HIV positive? (Y/N)	
CD 4 counts & name of clinical lab	
Is the woman on ART? (Y/N)	
ART Drug name(s)	
Did the woman adhere to ART? (Y/N)	

ART start date								
				2	0			
Day		Month		Year				

PHYSICAL EXAMINATION

General condition (normal, chronically ill, acutely ill, mildly ill)	
Weight (kg)	
Height (cm)	
Axillary temperature (Celsius)	
Heart rate (pulse/min)	

Systolic/Diastolic blood pressure (mm Hg)	
Hb level by HemoCue (g/dL)	
Malaria status at delivery by RDT (finger prick)	

Woman's Identification Number Woman's Initials

W		1							

DELIVERY DATA

Outcome of delivery (for singleton only):	☐ Full term ☐ Premature ☐ Still birth ☐ Spontaneous abortion		
Number of infants:	☐ **Singleton**	☐ **Twin**	☐ **Triplet**
. Date of birth (dd/mmm/yyyy):	☐☐/☐☐☐/☐☐☐☐	☐☐/☐☐☐/☐☐☐☐	☐☐/☐☐☐/☐☐☐☐
. Time of birth (hh:mm):	☐☐:☐☐	☐☐:☐☐	☐☐:☐☐
Type of delivery:	☐ Vaginal ☐ C-section	☐ Vaginal ☐ C-section	☐ Vaginal ☐ C-section
Infant's gender:	☐ M ☐ F	☐ M ☐ F	☐ M ☐ F
Placental weight (gm):	☐☐☐☐	☐☐☐☐	☐☐☐☐
Infant birth weight (gm):	☐☐☐☐	☐☐☐☐	☐☐☐☐
Head circumference (cm):	☐☐.☐	☐☐.☐	☐☐.☐
Infant Length (cm):	☐☐.☐	☐☐.☐	☐☐.☐
APGAR Score: 1 minute	☐☐	☐☐	☐☐
5 minute	☐☐	☐☐	☐☐
. Bilirubin done by Bilicheck?	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
If yes, date examined:	☐☐/☐☐☐/☐☐☐☐	☐☐/☐☐☐/☐☐☐☐	☐☐/☐☐☐/☐☐☐☐
Total serum bilirubin (mg/dL)	☐☐.☐☐	☐☐.☐☐	☐☐.☐☐
Reason determined	☐ Routine ☐ Jaundiced ☐ Other	☐ Routine ☐ Jaundiced ☐ Other	☐ Routine ☐ Jaundiced ☐ Other
Received photo treatment?	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No

Comments on baby health:	
Abnormal placenta: (placenta accreta, privia, abruption,	
Cord abnormalities (true knot, velamentous, etc.)	

Woman's Identification Number Woman's Initials

W | | 1 | | | | | | | |

LABORATORY TEST RESULTS

Maternal Peripheral Blood

PCV: ☐☐.☐☐ (%)

WBC Count (per uL) ☐☐☐☐☐

RBC Count (10^6/per uL) ☐☐.☐☐

HgB (Coulter) ☐☐.☐☐ g/dL

MCV ☐☐☐.☐☐ fl

34. MCH ☐☐ pg

MCHC ☐☐☐ g/dL

36. Ptl (per uL) ☐☐☐☐☐☐

LY Percentage ☐☐.☐☐ %

38. LY Number (per uL) ☐☐☐☐

Differential WBC count (%)

a. Monocytes & macrophages ☐☐☐

b. Lymphocytes ☐☐.☐

c. Neutrophils ☐☐☐

d. Others ☐☐.☐

Maternal Peripheral Blood Smear Read Out

Measure	Technician 1 Initials ☐☐☐	Technician 2 Initials ☐☐☐	Technician 3 Initials ☐☐☐
a. Malaria Parasites:	☐ Pos ☐ Neg	☐ Pos ☐ Neg	☐ Pos ☐ Neg
b. If malaria positive, Plasmodium species (check all found):	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*
c. Number of parasites / 200 WBC:	☐☐☐☐	☐☐☐☐	☐☐☐☐
d. Number of parasites / mm^3:	☐☐☐☐☐	☐☐☐☐☐	☐☐☐☐☐

Maternal IVS Blood Smear Read Out

Measure	Technician 1 Initials ☐☐☐	Technician 2 Initials ☐☐☐	Technician 3 Initials ☐☐☐
a. Malaria Parasites:	☐ Pos ☐ Neg	☐ Pos ☐ Neg	☐ Pos ☐ Neg
b. If malaria positive, Plasmodium species (check all found):	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*
c. Number of parasites / 200 WBC:	☐☐☐☐	☐☐☐☐	☐☐☐☐
d. Number of parasites / mm^3:	☐☐☐☐☐	☐☐☐☐☐	☐☐☐☐☐

Woman's Identification Number Woman's Initials

W | | 1 | | | | | | | |

LABORATORY TEST RESULTS Placental Impression Smear Read Out

Measure	Technician 1 Initials ☐☐☐	Technician 2 Initials ☐☐☐	Technician 3 Initials ☐☐☐
a. Malaria Parasites:	☐ Pos ☐ Neg	☐ Pos ☐ Neg	☐ Pos ☐ Neg
b. If malaria positive, Plasmodium species (check all found):	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*
c. Number of infected RBCs:	☐☐☐☐	☐☐☐☐	☐☐☐☐
d. Number of RBCs counted:	☐☐☐☐	☐☐☐☐	☐☐☐☐
e. % parasitemia	☐☐.☐☐☐	☐☐.☐☐☐	☐☐.☐☐☐
f. Differential WBC count (%) Monocytes & macrophages Lymphocytes Neutrophils Others	☐☐.☐ ☐☐.☐ ☐☐.☐ ☐☐.☐	☐☐.☐ ☐☐.☐ ☐☐.☐ ☐☐.☐	☐☐.☐ ☐☐.☐ ☐☐.☐ ☐☐.☐

Cord Blood Smear Read Out

Measure	Technician 1 Initials ☐☐☐	Technician 2 Initials ☐☐☐	Technician 3 Initials ☐☐☐
a. Malaria Parasites:	☐ Pos ☐ Neg	☐ Pos ☐ Neg	☐ Pos ☐ Neg
b. If malaria positive, Plasmodium species (check all found):	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*	☐ *P. falciparum* ☐ *P. ovale* ☐ *P. malarie*
c. Number of infected RBCs:	☐☐☐☐	☐☐☐☐	☐☐☐☐
d. Number of RBCs counted:	☐☐☐☐	☐☐☐☐	☐☐☐☐
e. % parasitemia	☐☐.☐☐☐	☐☐.☐☐☐	☐☐.☐☐☐
f. Differential WBC count (%) Monocytes & macrophages Lymphocytes Neutrophils Others	☐☐.☐ ☐☐.☐ ☐☐.☐ ☐☐.☐	☐☐.☐ ☐☐.☐ ☐☐.☐ ☐☐.☐	☐☐.☐ ☐☐.☐ ☐☐.☐ ☐☐.☐

Appendix 8: Ethical clearance

COMITE NATIONAL D'ETHIQUE DE LA RECHERCHE POUR LA SANTE HUMAINE

Arrêté N° 0977/A/MINSANTE/SESP/SG/DROS/ du 18 avril 2012 portant création, organisation et fonctionnement des comités d'éthique de la recherche pour la santé humaine au sein des structures relevant du Ministère en charge de la santé publique

N° 2013/11/366/L/CNERSH/SP Yaoundé, le 21 Novembre 2013

Cnethique_minsante@yahoo.fr

CLAIRANCE ETHIQUE

Le Comité National d'Ethique de la Recherche pour la Santé Humaine (CNERSH), en sa session du 21 novembre 2013, a examiné le dossier de demande de clairance éthique pour le projet de recherche intitulé **«Infectious diseases affecting pregnant cameroonian women and newborns»** soumis par le Docteur **Anna BABAKHANYAN**, Investigateur principal, Department of Tropical Medecine/University of Hawaii at Manoa.

Le projet est d'un grand intérêt scientifique et social. La procédure de l'étude est bien documentée et claire. Les risques liés à l'étude ainsi que la prise en charge des personnes exposées ont été présentés. La notice d'information et le formulaire de consentement éclairé, en français et en anglais, sont bien élaborés et simples à comprendre. Les mesures prises pour garantir la confidentialité des données collectées sont incluses dans le document. Les CVs des Investigateurs les décrivent comme des personnes compétentes, capables de mener à bien cette étude. Pour toutes ces raisons, le Comité National d'Ethique approuve pour une durée de deux ans, la mise en œuvre de la présente version du protocole.

Les investigateurs sont responsables du respect scrupuleux du protocole approuvé et ne devraient y apporter aucun amendement aussi mineur soit-il, sans avis favorable du CNERSH. Les investigateurs sont appelés à collaborer pour toute descente du CNERSH pour le suivi de la mise en œuvre du protocole approuvé. Le rapport final du projet devra être soumis au CNERSH et aux autorités sanitaires du Cameroun.

La présente clairance peut être retirée en cas de non respect de la réglementation en vigueur et des recommandations sus-mentionnées.

En foi de quoi, la présente clairance éthique est délivrée pour servir et valoir ce que de droit.

Ampliations

- MINSANTE

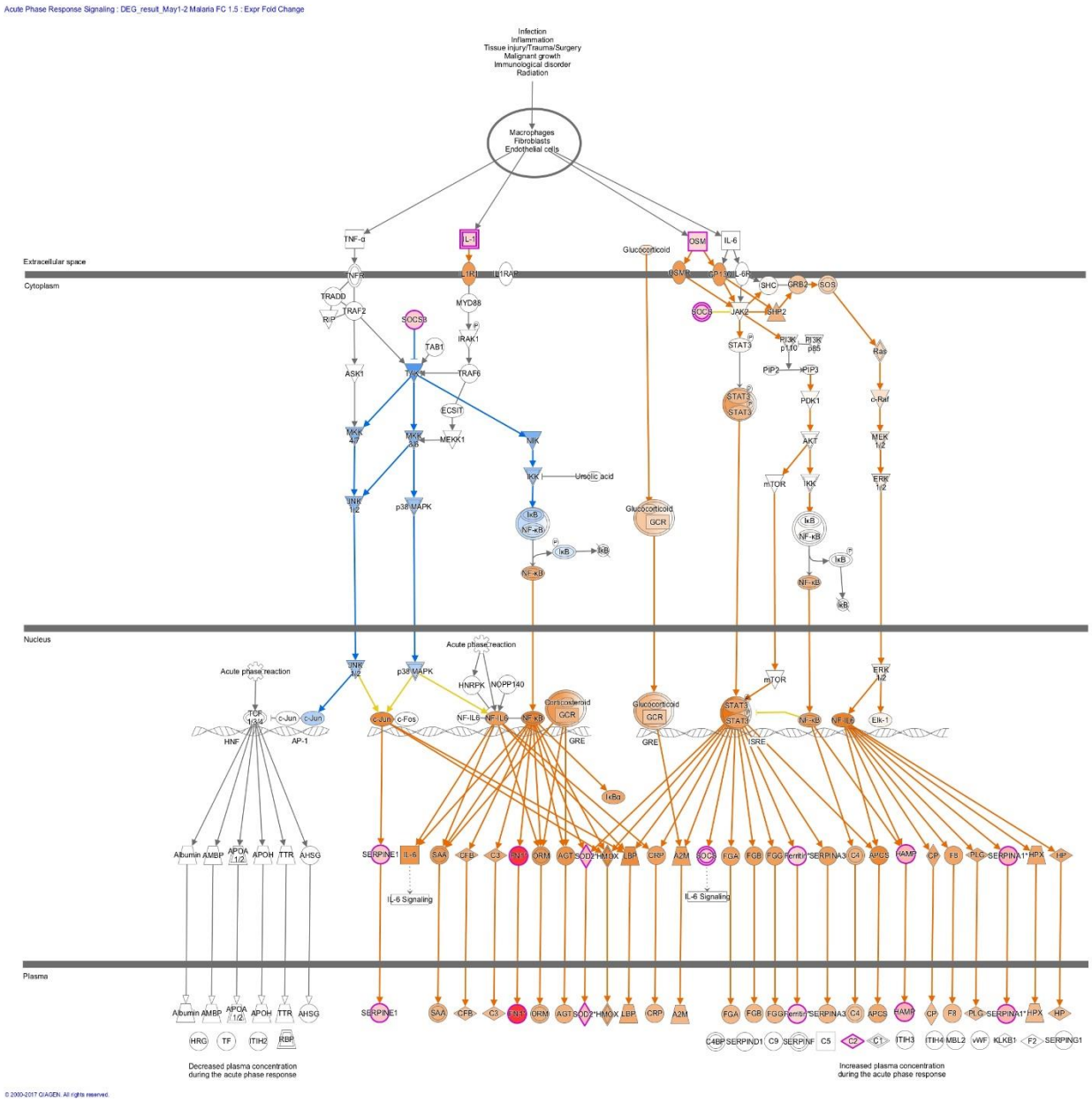
LE PRESIDENT

Pr Lazare KAPTUE

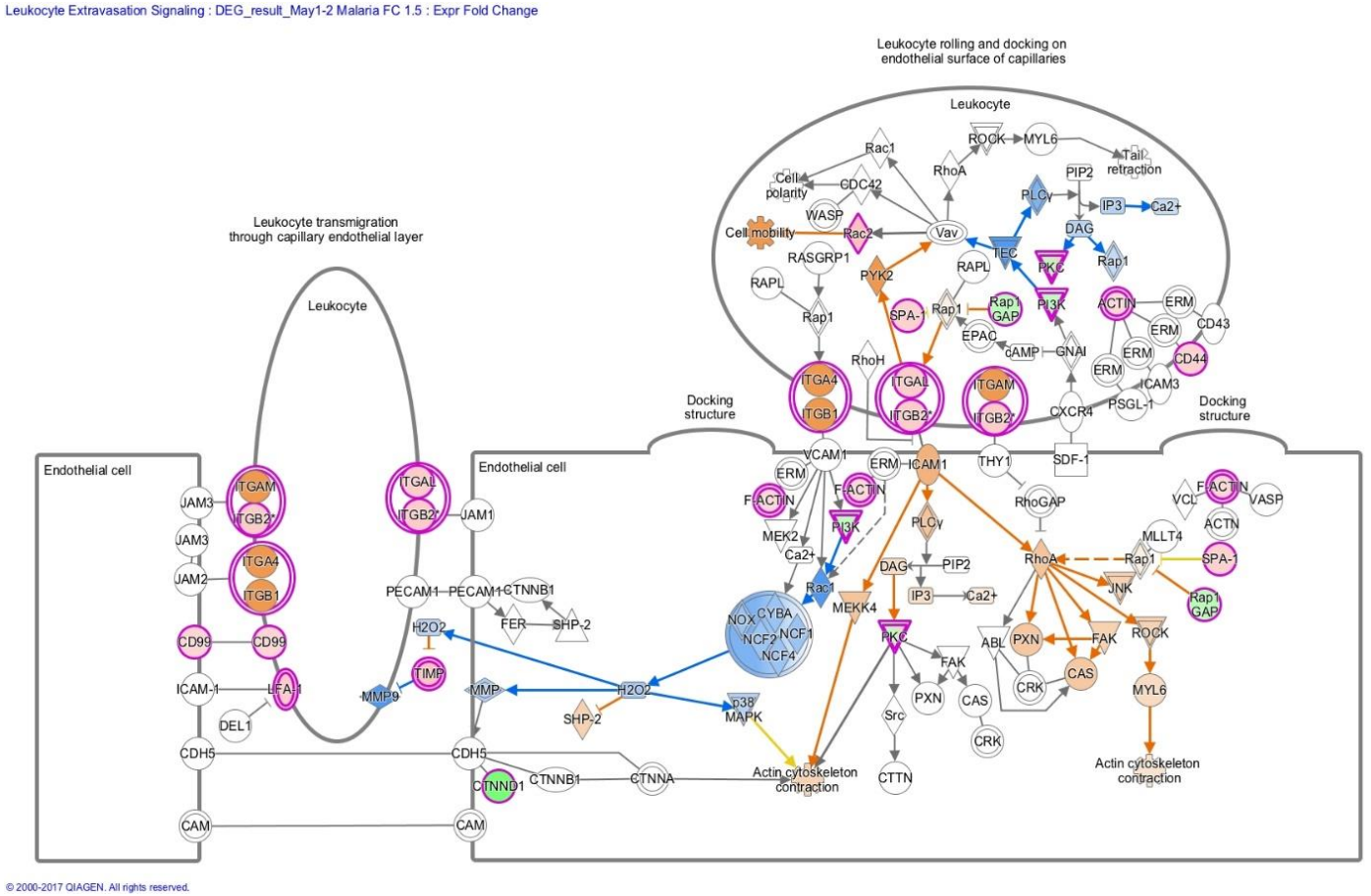
N.B : cette clairance éthique ne vous dispense pas de l'autorisation administrative de recherche (AAR), exigée pour mener cette étude sur le territoire camerounais. Cette dernière vous sera délivrée par le Ministère de la Santé Publique.

Appendix 9: Other Pathways activated by malaria

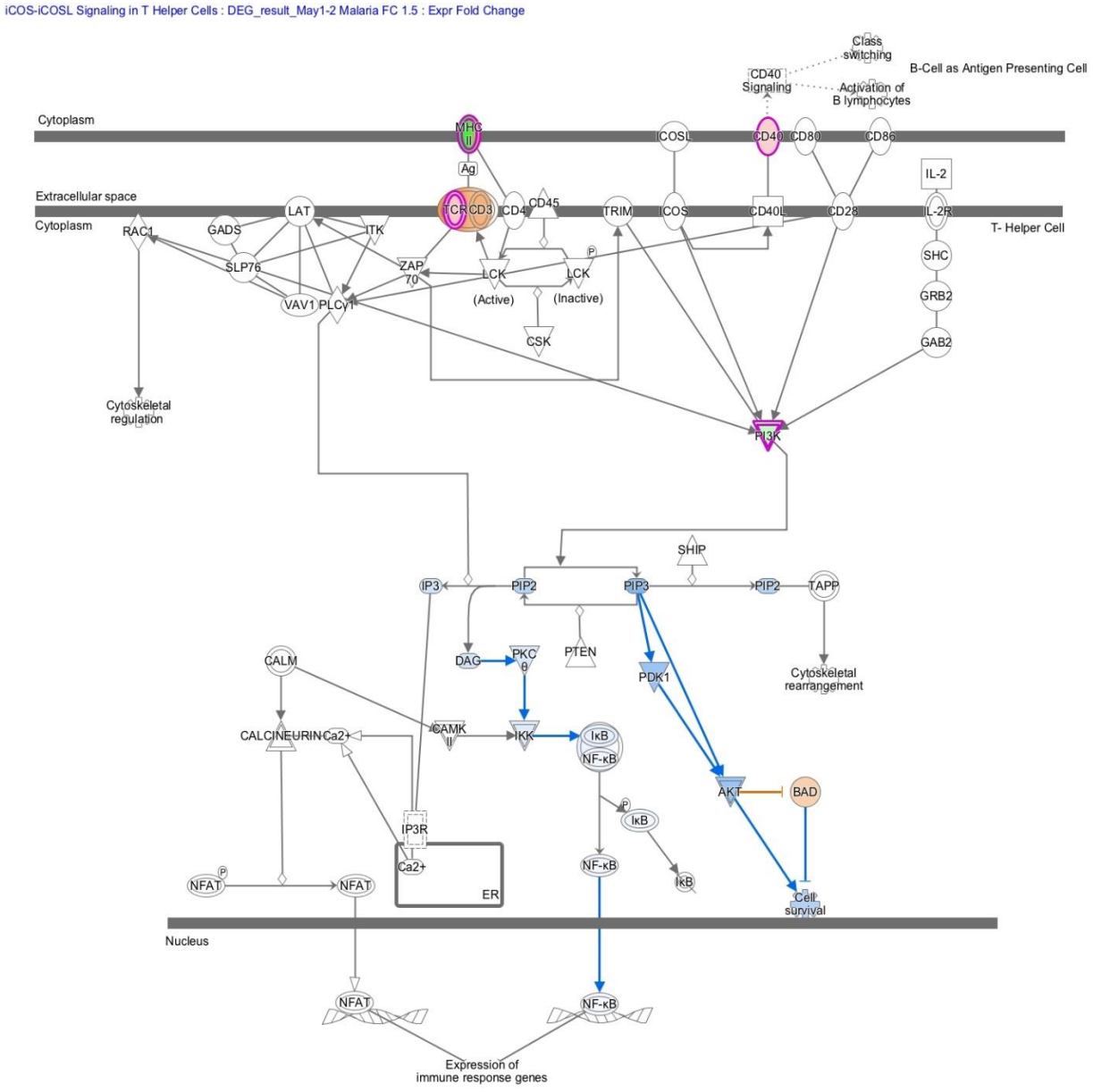
1. Acute phase response pathway



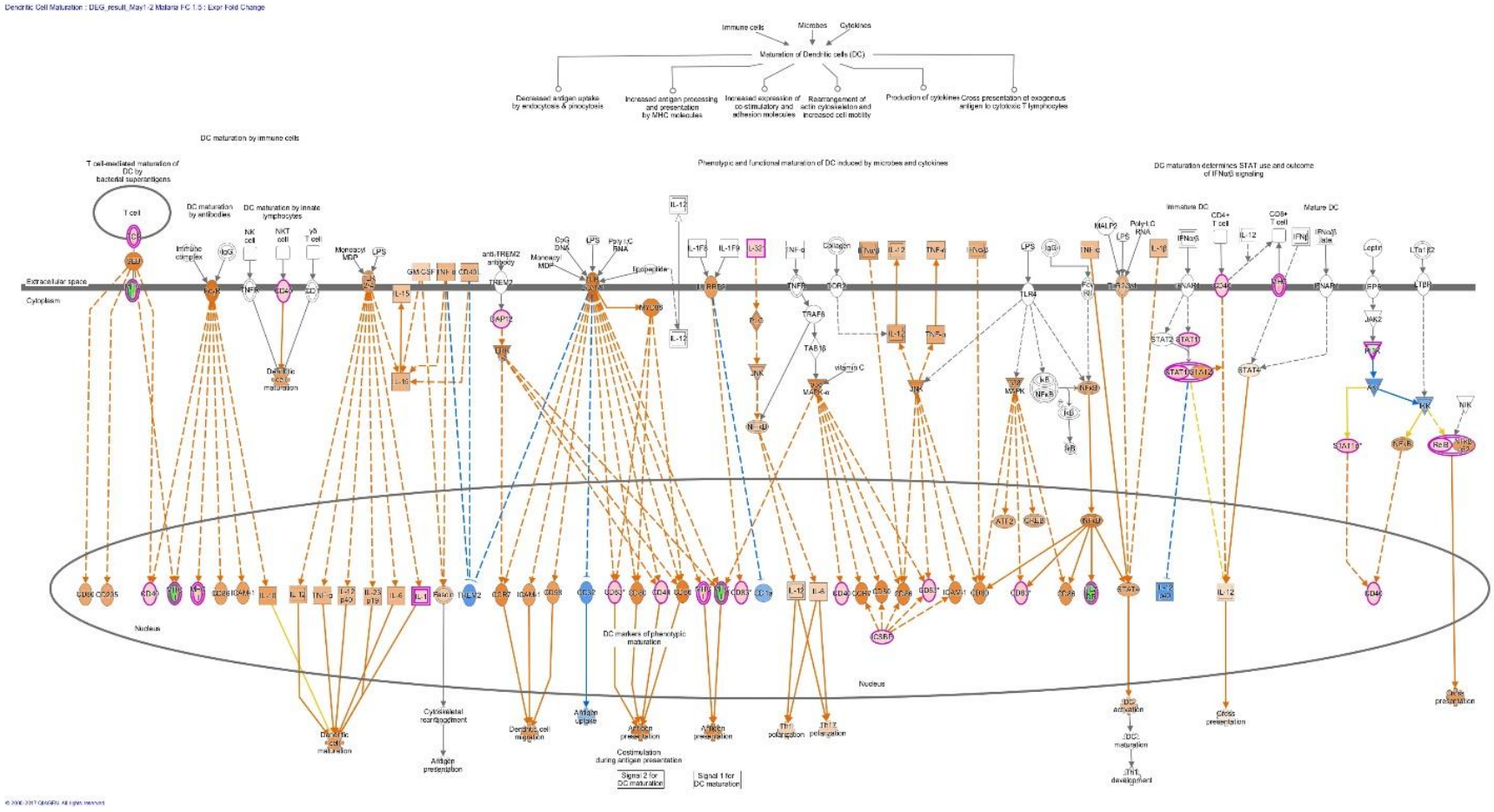
2. Leucocyte extravasation pathway



3. ICOS-ICOSL interaction



4. Dendritic cell maturation



LIST OF PUBLICATIONS

PUBLICATION 1

PLOS ONE

RESEARCH ARTICLE

Impact of HIV-1 infection on the IGF-1 axis and angiogenic factors in pregnant Cameroonian women receiving antiretroviral therapy

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Citation: Esemu LF, Yuosembom EK, Fang R, Rasay S, Fodjo BAY, Nguasong JT, et al. (2019) Impact of HIV-1 infection on the IGF-1 axis and angiogenic factors in pregnant Cameroonian women receiving antiretroviral therapy. PLoS ONE 14(5): e0215825. https://doi.org/10.1371/journal.pone.0215825

Editor: Daniel Gemechu Datiko, Management Sciences for Health, ETHIOPIA

Received: October 9, 2018

Accepted: April 9, 2019

Published: May 1, 2019

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This project was supported by NIH Research Training Grant #R25 TW009345 awarded to the Northern Pacific Global Health Fellows Program by the Fogarty International Center, the University of Hawaii at Mānoa Minority Health International Research Training (MHIRT) grant #

Abstract

Although mother-to-child transmission of HIV has dramatically declined, the number of *in utero* HIV-exposed, uninfected infants is on the increase. HIV-exposed infants are at an increased risk of mortality, morbidity and slower early growth than their non-HIV exposed counterparts. Maternal HIV increases the risk of having preterm deliveries, intrauterine growth restriction and low birth weight babies. However, the mechanism underlying dysregulation of fetal growth in HIV-infected pregnant women is unknown. We sought to determine whether maternal HIV is associated with dysregulation of the insulin-like growth factor (IGF) axis, some angiogenic factors or other related biomarkers that regulate fetal growth. A total of 102 normotensive pregnant women were enrolled in a small cross-sectional study. Amongst these were thirty-one HIV-1 positive women receiving combination antiretroviral therapy (cART) (Mean age: 30.0 ± 5.1 years; % on ART: 83.9%; median plasma viral load: 683 copies/ml; median CD4 count: 350 cells/ul) and 71 HIV uninfected women (mean age: 27.3 ± 5.8) recruited at delivery. A panel of biomarkers including IGF1 and IGF binding proteins (IGFBP1, IGFBP3), angiopoietins (ANG) 1 and 2, matrix metalloproteinases (MMP) 2 and 9, and galectin 13, was measured in plasma collected from the placental intervillous space. The levels of IGF1, IGFBP1, ANG1, ANG2, MMP2, MMP9 and Gal-13 were not affected by maternal HIV, even when adjusted for maternal factors in linear regression models (all p>0.05). It was observed that HIV-infection in pregnancy did not significantly affect key markers of the IGF axis and angiogenic factors. If anything, it did not affect women. These findings highlight the importance of the use of ART during pregnancy, which maintains factors necessary for fetal development closer to those of healthy women. However, decrease in IGF1 levels might be exacerbated in women con-infected with HIV and malaria.

T37MD08636-01. LFE was supported by NIH Fogarty training grant 5D43 TWO1264.

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

Introduction

In sub-Saharan Africa, women disproportionately bear the burden of the HIV epidemic [1,2]. Each year, 1.4 million HIV-infected women become pregnant [1], with up to 5.3% of those pregnant being HIV positive in many African countries [3]. In Cameroon, the national HIV prevalence in 2011 was 5.6% in women and 2.9% in men, but the prevalence of HIV among pregnant women was 7.8% [3,4]. Maternal HIV-1 infection increases the risk of pre-term birth (<37 weeks of gestation), small-for-gestational age babies, and fetal intrauterine growth restriction [5–9], resulting in low birth weight (LBW) infants (<2500g) [10–12]. Low birth weight occurs in over 20 million children and 95% of this condition is observed in developing countries [13,14]. Approximately 10% of children born to HIV positive Cameroonian women under prolonged HAART were born with LBW[15]. LBW is a significant cause of infant morbidity and increases the risk of mortality during the first year of life by 40-fold [16].

Mechanisms underlying LBW among HIV-exposed infants remains unknown. In term deliveries, HIV-associated LBW is likely to be caused by several factors, but dysregulated vasculogenesis in the placenta is likely to be an important component [17]. Early events such as implantation and development of the placenta are critical for successful pregnancy outcomes [18]. Placental vascular development is tightly regulated by pro-angiogenic angiopoietin 1 (ANG1) and anti-angionetic angiopoietin 2 (ANG2) [19]. During the first trimester, angiogenesis is important for remodeling of uterine spiral arteries into low resistance, high capacity vessels [17,20], which continues until mid-second trimester [19–23]. Dysfunctional remodeling of uterine spiral arteries is associated with complications of pregnancy, such as preeclampsia [24], gestational diabetes mellitus[25], Intra Uterine Growth Restriction[26], and Small for Gestational Age in the neonate [27].

Another important regulator of placental and fetal growth is the insulin like growth factor (IGF1)[28]. During pregnancy, IGF1 and its regulatory proteins are produced by placental trophoblasts and fetal cells, with the fetal liver being the main source of IGF after birth [29]. IGF1 plays a role in trophoblast migration, invasion, differentiation as well as proliferation. It also functions to influence placental angiogenesis and therefore transplacental transfer of nutrients such as amino acids and glucose. IGF receptors are found on placental cells that mediate IGF activity[30,31]. However, placental bioavailability of IGF1 is modulated by the IGF binding proteins: IGFBP1 and IGFBP3 [32,33]. Changes in IGF1 levels in maternal, placental or fetal compartments during the first trimester have been implicated in fetal growth restriction and LBW and would likely remain altered throughout pregnancy[28,29,34,35].

In addition, galectins are expressed at the maternal-fetal interface of the placenta and play key roles in placental formation and vascularization[36]. Among the 19 galectins known, placental galectin 13 (or placental protein 13, Gal-13) has been shown to be expressed by the syncytiotrophoblast, endovascular trophoblast and decidual spiral arteries and is important in trophoblast invasion and vascular remodeling during placentation [37]. Gal-13 is also regarded as an endogenous danger/damage signal, as its secretion from the syncytiotrophoblast is dramatically upregulated at the onset of preeclampsia and the hemolysis, elevated liver enzymes and low platelet count syndrome [36]. This lectin likely also plays an important role in feto-maternal tolerance, as it has been shown to promote apoptosis of activated T cells and macrophages[36].

Finally, placental matrix metalloproteinases (MMP) are proteolytic enzymes that have been shown to have a vital role in trophoblast invasion, regulation of vascular endothelial cell functions and placental angiogenesis[38,39]. The MMP function by essentially degrading the extracellular matrix (ECM), releasing growth factors that allow the activation of signals that are important for angiogenesis [40]. For instance, MMP2 and MMP9 are secreted by placental

trophoblasts and are critical in trophoblast invasion, vascular endothelial cell migration, attachment, proliferation and survival; therefore, supporting angiogenesis[39,41]. These enzymes have been described as potential candidates in the pathogenesis of preeclampsia [42]. The impact of HIV on levels of angiopoietins, MMPs, IGF1 and Gal-13 has not been investigated. Based on the importance of these factors in the homeostasis of pregnancy, it is plausible that HIV-associated immune activation[43] may dysregulate levels of angiopoietins, MMPs, IGF1 and gal-13. Thus, in this pilot study, the impact of maternal HIV infection on angiopoietins, IGF1 and IGFBPs, MMPs and gal-13 in normotensive pregnant women receiving antiretroviral therapy was examined.

Materials and methods

Ethical considerations

The archival, coded samples used in the current study were exempt from human subject research by the Committee on Human Studies, University of Hawaii, Manoa (CHS 22572). The original study protocol was reviewed and approved by the National Ethics Committee Cameroon (No 2013/11/366/L/CNERSH/SP) and the Institutional Review Board of the University of Hawaii (CHS 21370). Written informed consent was obtained from each woman specifying their sample would be used for further studies, prior to enrollment into the study.

Study site and population

In this pilot cross sectional study, archival plasma samples, obtained from a study carried out between January 2014 and September 2015 at the Yaoundé Central Hospital Maternity, a referral teaching hospital in Yaoundé, Cameroon were used. The prevalence of HIV in the city of Yaoundé is 4.4% [44]. The goal of the mother study was to investigate underlying mechanisms to poorer health observed in children born to HIV-positive women. A total of 102 mother-neonate pairs were recruited in the study at delivery. Women with pre-existing health conditions [e.g. diabetes, preeclampsia and Hemolysis, Elevated Liver enzymes, Low platelet count (HELLP) syndrome] and/or had spontaneous abortions were excluded from the study. Information on each woman's demographic and clinical history including HIV status, ART intake, use of the intermittent preventive treatment (IPT) with sulphadoxine pyrimethamine (SP) and insecticide treated bednets (ITN) during pregnancy was available. The birth weight, length and APGAR score of newborn were also available. Gestational age was estimated based on date of last menstrual period or ultrasound scan data when available. Women with axillary temperature greater than 37.5˚C were considered as having fever. Neonates born between 28 and 37 weeks were classified as premature. Singletons weighing less than 2,500 grams were considered LBW babies. Women were tested for HIV during pregnancy and vaccinated with tetanus vaccine according to national guidelines. All HIV positive women were placed on cART following national guidelines.

Sample collection

Maternal venous blood and cord blood were collected in EDTA and sodium heparin tubes while blood from intervillous space (IVS) was obtained using the pool-biopsy method[45], processed and preserved at -20˚C until analyses. In addition, impression smears of placental tissue were prepared and a piece of placental biopsy was stored in 10% buffered formalin for histological studies.

HIV RNA levels. HIV diagnostic data was available from the medical records at Yaoundé Central Hospital. HIV copy number was determined for all HIV positive women when

sufficient amount of plasma was available (n = 14). Testing was conducted at the Chantal Biya International HIV Reference Center, Yaoundé, Cameroon using the using Abbott RealTime PCR HIV-1 kit (Abbott Park, Illinois, USA). Lower detection limit of the assay was less than 150 copies/ml; upper detection limit was 10,000,000 copies/ml.

Diagnosis of peripheral malaria, placental malaria and anemia. *P. falciparum* infections in peripheral, IVS and cord blood were detected by microscopy as described previously[46]. Peripheral blood smears were microscopically examined for presence of *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax*. Placental biopsies were also fixed in buffered formalin, embedded, stained with hemotoxylin-eosin, and examined for parasites. A woman was considered to have placental malaria (PM) if infected erythrocytes were detected in blood smears of IVS, impression smears of villous tissue, or histological sections of the placenta [47]. Maternal peripheral blood was used to determine the hemoglobin levels (Hb) using HemoCue Hb 201 (HemoCue, Sweden). Anemia was defined as Hb less than 11 g/dl [48].

Detection of fetal blood contamination in placental blood. In order to confirm that IVS was collected without fetal blood contamination, the degree of purity of maternal blood was assessed using Fetal Cell Stain Kit (Simmler Inc, High Ridge, MO, United States, SKU: S0412-100) per manufacturer instructions. Positive control cord blood was used as reference.

Measurement of MMP2, MMP9, ANG1, ANG2, IGFBP1 and IGFBP3 levels in placental intervillous space plasma. These biomarkers were measured using Luminex Screening Assay kits (R&D Systems, MN). A four-plex cocktail containing ANG1, ANG2, IGFBP1 and IGFBP3 (R&D Systems, MN, Cat. LXSAHM-04) was used to screen at 1:2 dilution IVS plasma, MMP2 and MMP9 containing 2-plex cocktail (R&D Systems, MN, Cat. LXSAHM-02) was used to screen at 1:50 dilution IVS plasma. The assay was carried out according to the manufacturer's instructions. Plates were washed using magnetic plate separator (Luminex, Austin, Texas, Cat# CN-0269-01) and a MAGPIX instrument (EMD Millipore, Billerica, MA) was used to read plates. The results were expressed as median fluorescence intensity (MFI). A standard curve was generated for each analyte to convert MFI into corresponding protein concentration. Protein concentrations were adjusted for dilution factors used for each analyte.

Measurement of IGF1 and Gal-13 levels in placental IVS plasma. IGF1 levels in IVS plasma were measured using Human IGF-I Immunoassay Quantikine ELISA kit (R&D Systems, MN, Cat. DG100) according to the manufacturer's instruction. Gal-13 levels in IVS plasma were measured using Human placenta protein13 (PP13) ELISA Kit (My Biosource, CA, Cat. MBS293460). The plates were read using microplate reader (ELISA iMARK BioRad, S#13738, JAPAN) set at 450 nm with wavelength. Results were expressed in optical density (OD) and standard curves were used to calculated protein concentrations. For IGF1, the values were multiplied by 100 (dilution factor from plasma pretreatment step). The detectable concentration range of IGF-I was 0.007 ng/ml—0.056 ng/mL and galectin 13 was 5pg/ml - 2000pg/ml.

Statistical analysis. Biomarker levels, demographic and clinical variables were summarized using descriptive statistics: means and standard deviations or median and interquartile range (IQR), for continuous variables such as age or parity; and frequencies and percentages for categorical variables, e.g., maternal anemia status (yes or no) and HIV-1 infection status (yes or no). Two-sample t-tests or Mann-Whitney U-tests for continuous variables, and Chi-square tests or Fisher's exact tests for the categorical variables were used to compare women with and without HIV-1. The biomarker values were log transformed into natural logarithm scales. The effects of maternal HIV-1 infection on levels of each of biomarkers were evaluated through linear regression models, controlling for the selected demographic and clinical variables. All p values less than 0.05 were considered significant. All statistical analysis was performed using SAS 9.4 and GraphPad Prism 7.0.

Table 1. Demographic and clinical characteristics of mothers.

Characteristic	HIV-1 (-)	HIV-1 (+)	p-value
Number of enrolled participants, n	71	31	-
Age in years, mean ± SD$^{\theta}$	27.3 ± 5.8	30.0 ± 5.1	**0.027**
Maternal fever, n (%)$^{\Phi}$	18 (25.4)	6 (19.4)	0.35
Maternal weight in kg, mean ± SD$^{\theta}$	75.7 ± 12.5	73.9 ± 12.9	0.64
Maternal BMI in kg/m^2, mean ± SD$^{\theta}$	29.1 ± 4.3	28.6 ± 3.7	0.72
Maternal hemoglobin level in g/dL, mean ± SD$^{\theta}$	12.1 ± 1.6	11.7 ± 1.7	0.41
Maternal anemia, n (%)$^{\Phi}$	13 (18.3)	6 (19.4)	0.73
ART use by pregnant women, n (%)	0	26 (83.9)	-
Maternal viral load, median, (25th, 75th)	0	683 (0, 130,680)	-
Maternal CD4 Count median, (25th, 75th)	N/A	350 (248,675)	-
Maternal IPT use, n (%)$^{\Phi}$	60 (84.5)	30 (96.8)	0.18
Number of SP doses pregnant women took, median, (25th, 75th)$^{\pi}$	2 (1, 3)	2 (2, 2)	0.92
Maternal bednet use, n (%)$^{\Phi}$	52 (73.2)	26 (83.9)	0.41
Maternal heart rate in beats per minute, mean ± SD$^{\theta}$	84.7 ± 13.9	88.5 ± 15.9	0.39
Maternal blood pressure in mmHg, mean ± SD$^{\theta}$			
Systolic	120.9 ± 17.6	119.7 ± 8.8	0.70
Diastolic	75.2 ± 13.3	76.1 ± 8.7	0.74
Maternal peripheral malaria by blood smears, n (%)$^{\Phi}$	11 (15.5)	4 (12.9)	0.75
Maternal parasite density in peripheral blood$^{£}$ in parasites/uL, median (25th, 75th)$^{\pi}$	1,880 (400, 15,940)	1,080 (440, 12,490)	0.61
Malaria by RDT on maternal peripheral blood$^{\theta}$	14 (19.7)	4 (12.9)	0.44
Placental malaria, n (%)$^{\Phi}$	10 (14.1)	3 (9.7)	0.33
Parasitemia$^{£}$ in %, median (25th, 75th)$^{\pi}$	5.35 (0.06, 26.0)	0.23 (0.03, 0.61)	0.11
Parity including current child, median (25th,75th)$^{\pi}$	2 (1, 3)	3 (1, 4)	0.40
Primigravidae, n (%)$^{\Phi}$	12 (16.9)	4 (12.9)	0.46
Multigravidae, n (%)$^{\Phi}$	41 (57.8)	22 (71.0)	0.46
Length of gestation in weeks, mean ± SD$^{\theta}$	39.2 ± 3.0	38.9 ± 2.6	0.66
Preterm deliveries, n (%)$^{\Phi}$	10 (14.1)	6 (19.4)	0.59
C-section, n (%)$^{\Phi}$	6 (8.5)	5 (16.1)	0.28

The data were summarized based on the non-missing values. The total % is not 100 due to missing values or values rounded. £ Calculated for only smear positive individuals. P-values were based on

$^{\theta}$ two-sample T-tests

$^{\pi}$ Mann Whitney's tests

$^{\Phi}$ Fisher's exact tests.

https://doi.org/10.1371/journal.pone.0215825.t001

Results

Participant characteristics

Demographic and clinical characteristics of study participants at delivery are summarized in Table 1 and S1 Table. Overall, 102 women were enrolled in the study, 31 HIV-1 positive and 71 HIV-1 negative. HIV-1 positive and negative women were similar with respect to maternal factors: IPT use, hemoglobin level, temperature, blood pressure, peripheral malaria status, parity and pregnancy outcomes: length of gestation, proportion of singleton deliveries and C-section, neonate sex, neonate weight and prevalence of LBW babies (all p-values>0.05). However, HIV-1 positive women were older compared to their healthy counterparts (p = 0.027) with average age of 30.0 ± 5.1 vs. 27.3 ± 5.8 years, respectively. Majority (83.9%) of the HIV-1 positive pregnant women were receiving ART, and most of the women were on Tenofovir Lamivudine and Efavirenz tritherapy. HIV viral load was available for 14(47%) HIV-1 positive women

Table 2. Demographic and clinical characteristics of neonates.

Characteristic	HIV-1 (-)	HIV-1 (+)	p-value
Singleton deliveries, n (%)[Φ]	67 (94.4)	29 (93.6)	0.59
Male neonates, n (%)[Φ]	38 (53.5)	20 (64.5)	0.38
Placental weight in g, mean ± SD[θ]	616 ± 155	609 ± 177	0.85
Neonate weight in g, mean ± SD[θ]	3169 ± 587	3127 ± 497	0.74
Low birth weight, n (%)[Φ]	6 (8.5)	2 (6.5)	1.00
APGAR at 1min, mean ± SD[θ]	7.9 ± 1.5	8.4 ± 1.0	0.17
APGAR at 5min, mean ± SD[θ]	8.8 ± 1.4	8.9 ± 1.0	0.81
Cord malaria infection by blood smears, n (%)	0	0	-

The data were summarized based on the non-missing values. The total % is not 100 due to missing values or values rounded. £ Calculated for only smear positive individuals. P-values were based on

[θ] two-sample T-tests

[π] Mann Whitney tests

[Φ] Fisher's exact tests.

https://doi.org/10.1371/journal.pone.0215825.t002

with median (25th, 75th) of 683 (0, 130,680) copies/μl. CD4 counts were available for 9 (30%) HIV-1 positive women; median (25th, 75th) of 350 (248, 675) cells/μl. Four (13%) HIV-1 positive pregnant women were also infected with placental malaria.

Validation of intervillous space blood collection and placental histopathology

A total of 9 random intervillous blood samples were tested for fetal blood contamination. The average proportion of fetal erythrocytes in intervillous space blood was 1.7 ± 0.3%, which shows that the level of contamination was extremely low (S1 Fig). Thus, the sample collection methodology was validated and the experiment results are reflective of what occurs on the maternal side of the placenta.

Placental weight was not significantly different between HIV-1 positive and HIV negative women (p = 0.85, Table 2). In placentas from HIV-1 positive mothers, lesions and syncytial knots were occasionally observed; placentas from HIV-1 and PM co-infected women had lesions, fibrinoid tissue (Fig 1). Prevalence of placental malaria was not significantly different between HIV-1 positive (9.7%) and uninfected (14.1%) women (p = 0.33, Table 1). Except in women coinfected with malaria and HIV, women with PM did not have placental inflammation (Fig 1).

Angiopoetin 1 and 2 are not dysregulated in HIV-1 positive women on antiretroviral therapy

The placental levels of ANG1 and ANG2 biomarkers in natural logarithm scales by HIV-1 status are presented in Table 3. There was no significant difference between HIV-1 positive and HIV-1 negative women in ANG1 (p = 0.68) and ANG2 (p = 0.20) as depicted on Table 3. In general linear regression models adjusted for maternal age and malaria status (Table 4), HIV-1 infection did not have significant impact on ANG1 (p = 0.93) and ANG2 (p = 0.33).

In order to determine whether angiopoetins are dysregulated during HIV-1 infection, ANG1, ANG2, as well as ANG2/ANG1 ratio were measured in placental intervillous space plasma from HIV-1 positive PM-negative and HIV-1 negative PM-negative women. No significant differences in ANG1 or ANG2 (all $p>0.05$) were observed between women with HIV-1 and their healthy counterparts (Fig 2). No significant differences between HIV-1 negative PM-

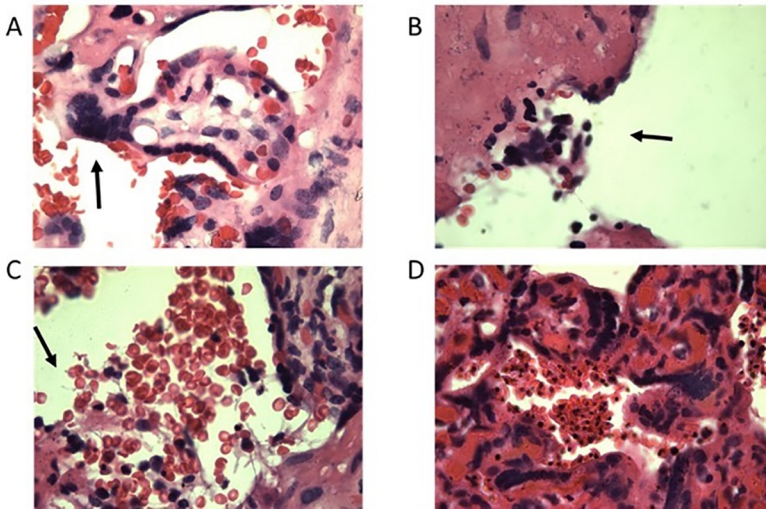

Fig 1. Placental histology. (A) HIV-1 infected woman. Arrow points to a syncytial knot. (B) HIV-1 and placenta malaria co-infected woman. Arrow points to a lesion. (C) HIV-1 and PM co-infected woman. Arrow points to fibrinoid tissue. (D) HIV-1 negative placenta malaria-positive woman. Infected erythrocytes are present in large numbers, no monocytes. 400x magnification.

https://doi.org/10.1371/journal.pone.0215825.g001

positive women and their healthy counterparts were observed for either ANG1 or ANG2 (all $p>0.05$, Fig 2). ANG1 was lower in 3 co-infected pregnant women compared to healthy women, but the difference was not statistically significant ($p = 0.08$, Fig 2).

IGF axis is not dysregulated in HIV-1 infected women receiving antiretroviral therapy

The placental biomarkers levels of IGF axis in natural logarithm scales by HIV-1 status are presented in Table 3. There was no significant difference between HIV-1 positive and HIV-1 negative women in IGF1 ($p = 0.76$) and IFGBP1 ($p = 0.92$). In linear regression models adjusted for maternal age and malaria status (Table 4), IGF-1 was not significantly reduced as a result of HIV-1 ($p = 0.12$) but due to malaria status ($p = 0.0038$), while no effect of HIV-1 on IFGBP1 was identified ($p = 0.84$).

The impact of HIV-1 on IGF axis was evaluated by probing placental intervillous space plasma obtained from HIV-1 positive PM-negative, HIV-1 negative PM-negative and HIV-1 negative PM-positive women for IGF-1 and IGFBP1 and IGFBP3. Lower but not significant

Table 3. Placental biomarker levels by HIV-1 status.

*Biomarker	HIV-1 (-) n = 71	HIV-1 (+) n = 31	p-value
ANG1(pg/ul)	10.64 ± 0.53	10.6 ± 0.52	0.68
ANG2(pg/ul)	9.22 ± 0.42	9.38 ± 0.54	0.20
IGF1 (ng/ul)	4.31 ± 0.19	4.29 ± 0.24	0.76
IGFBP1(ug/ul)	12.02 ± 0.36	12.01 ± 0.36	0.92
MMP2(ug/ul)	12.45 ± 0.30	12.60 ± 0.36	**0.066**
MMP9(ug/ul)	13.18 ± 1.05	13.15 ± 0.87	0.91
Gal-13(ug/ul)	5.70 ± 0.47	5.45 ± 0.31	**0.06**

*Biomarker levels were log transformed and the data were summarized by mean ± SD, based on non-missing values. P-values were based on two-sample T-tests.

https://doi.org/10.1371/journal.pone.0215825.t003

Table 4. Placental biomarker level reduction due to HIV-1.

		HIV-1 (+) vs. HIV-1 (-)		Malaria (+) vs. Malaria (-)		Age	
Biomarker	**R^2**	**Estimate (95% CI)**	**p-value**	**Estimate (95% CI)**	**p-value**	**Estimate (95% CI)**	**p-value**
ANG1(pg/ul)	0.081	0.012 (-0.26, 0.29)	0.93	-0.22 (-0.55, 0.11)	0.19	-0.020 (-0.043, 0.004)	0.11
ANG2(pg/ul)	0.036	0.14 (-0.14, 0.41)	0.33	-0.038 (-0.37, 0.29)	0.82	0.011 (-0.015, 0.031)	0.49
IGF1(ng/ul)	0.27	-0.086 (-0.19, 0.022)	0.12	-0.19 (-0.32, -0.067)	**0.0038**	-0.004 (-0.014, 0.006)	0.44
IGFBP1(ug/ul)	0.0038	0.10 (-0.23, 0.19)	0.84	0.12 (-0.29, 0.21)	0.75	-0.002 (-0.019, 0.016)	0.84
MMP2(ug/ul)	0.039	0.12 (-0.042, 0.28)	0.15	0.092 (-0.21, 0.15)	0.74	0.007 (-0.012, 0.016)	0.79
MMP9(ug/ul)	0.029	0.018 (-0.48, 0.52)	0.95	0.28 (-0.19, 0.93)	0.20	0.021 (-0.055, 0.028)	0.53
Gal-13(ug/ul)	0.089	-0.25 (-0.55, 0.042)	0.090	0.17 (-0.33, 0.30)	0.82	0.013 (-0.031, 0.023)	0.79

The Placental biomarker levels were in natural logarithm scales and the model was adjusted for mat maternal age and malaria status. The malaria status was confirmed by either placental malaria or maternal peripheral blood RDT. P-values were based on linear regression analyses.

https://doi.org/10.1371/journal.pone.0215825.t004

levels (p = 0.3) of IGF-1 were observed in HIV-1 positive PM-negative women compared to healthy women (Fig 3). No significant differences in IGFBP1 were observed between HIV-1 infected and healthy women. In linear regression models adjusted for maternal age and anemia status no significant effect of HIV-1 on IGFBP1 was identified (Table 4). IGFBP3 was not detected in any of the samples and thus excluded from analysis.

HIV-1 is not associated with decreased levels of MMP2, MMP9 and Gal-13

The placental levels of MMP2, MMP9 and Gal-13 biomarkers in natural logarithm scales by HIV-1 status are also presented in Table 3. There was no significant difference between HIV-1 positive and HIV-1 negative women in MMP9 (p = 0.91), but marginally significant in MMP2 (p = 0.066) and Gal-13 (p = 0.060). After adjusting for maternal age and malaria status, HIV-1 status had no significant impact on MMP2, MMP9 and Gal-13 (all p>0.05, Table 4).

The impact of HIV-1 on additional biomarkers of placental formation and vascularization were also explored, including MMP2, MMP9 and Gal-13. No significant differences were

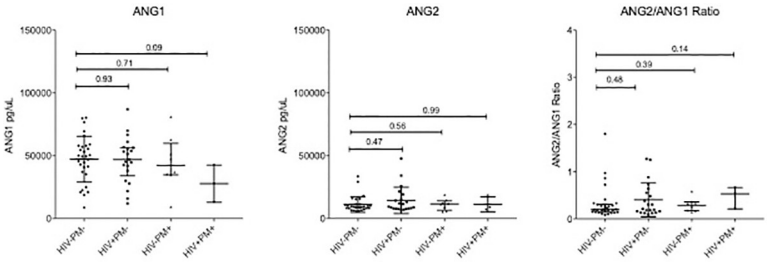

Fig 2. Angiopoietin levels in placental intervillous space. ANG1 and ANG2 levels, as well as ANG2/ANG1 ratio was measured in placental intervillous space in healthy (HIV-&PM-, n = 30), HIV-infected (HIV+&PM-, n = 21), PM-positive (HIV&PM+, n = 8) and co-infected (HIV+&PM+, n = 3) women. Median and interquartile ranges (IQR) are plotted; differences between the healthy and infected women were assessed using Mann-Whitney test. HIV: Human Immunodeficiency Virus; ANG1: Angiopoetin 1; ANG2: Angiopoetin 2; PM: Placenta Malaria positive mothers.

https://doi.org/10.1371/journal.pone.0215825.g002

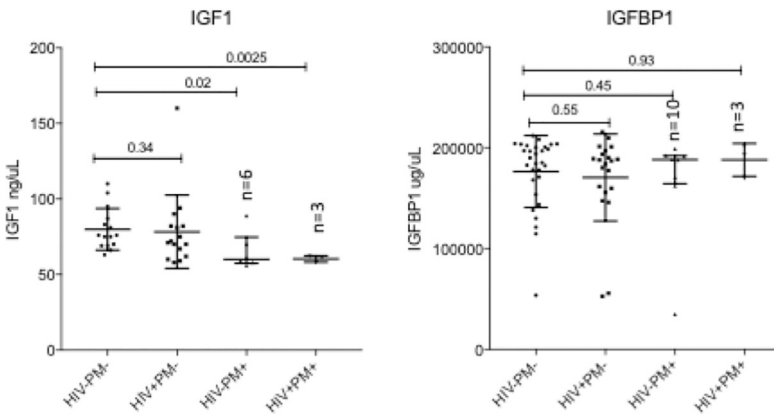

Fig 3. IGF1 and IGFBP1 levels in placental intervillous space. IGF1 levels were measured in placental intervillous space plasma of healthy (HIV-&PM-, n = 15), HIV-infected (HIV+&PM-, n = 16), PM-positive (HIV-&PM+, n = 6) and co-infected (HIV+&PM+, n = 3) women. IGFBP1 and IGFBP3 levels were also measured in healthy (HIV-&PM-, n = 30), HIV-infected (HIV+&PM-, n = 21), PM-positive (HIV-&PM+, n = 8) and co-infected (HIV+&PM+, n = 3) women. Median and interquartile ranges (IQR) are plotted; differences between the healthy and infected women were assessed using Mann-Whitney test. HIV: Human Immunodeficiency Virus; IGF1: Insulin Growth Factor 1; IGFBP1: IGF Binding Protein 1; PM: Placenta Malaria positive mothers.

https://doi.org/10.1371/journal.pone.0215825.g003

observed between HIV-1 positive PM-negative and healthy women for MMP2 and MMP9 levels in intervillous space plasma (all $p>0.05$, Fig 4 and Table 3). No significant differences in MMP2 and MMP9 levels were observed between HIV-1 negative PM-positive women and their healthy counterparts (all $p>0.05$, Fig 4). Intervillous space plasma Gal-13 levels were not significantly different between HIV-1 positive and healthy women (Fig 4), and linear regression model showed HIV-1 had no significant impact on Gal-13 levels (Table 4).

Discussion

The goal of our study was to determine whether maternal HIV is associated with the dysregulation of insulin-like growth factor (IGF) axis, angiogenic factors—or other related biomarkers that regulate fetal growth. In this pilot study, a panel of biomarkers implicated in placental homeostasis and fetal growth were assessed in intervillous space plasma of HIV-1 positive

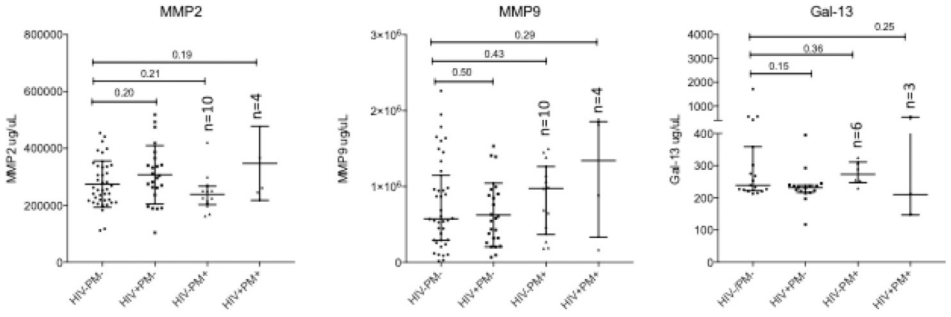

Fig 4. MMP and Gal 13 levels in plasma from placental intervillous space plasma. MMP2 and MMP9 levels were measured in placental intervillous space in uninfected (HIV-&PM-, n = 40), HIV-infected (HIV+&PM-, n = 24), PM-positive (HIV-&PM+, n = 12) women and co-infected (HIV+&PM+, n = 4) women. Gal-13 were measured in intervillous space plasma of healthy (HIV-&PM-, n = 17), HIV-infected (HIV+&PM-, n = 16), PM-positive (HIV-&PM+, n = 6) and co-infected (HIV+&PM+, n = 3) women. Median and interquartile ranges (IQR) are plotted; differences between the healthy and infected women were assessed using Mann-Whitney test. HIV: Human Immunodeficiency Virus; MMP: Matrix Metalloproteinase; Gal-13: Galectin-13; PM: Placenta Malaria positive mothers.

https://doi.org/10.1371/journal.pone.0215825.g004

normotensive women on antiretroviral therapy and their HIV-1 negative counterparts. This panel of biomarkers included those involved in angiogenesis, IGF axis, as well as profile of MMPs and Gal-13. Angiogenic factors were not affected by maternal HIV-1 in our cohort of pregnant women receiving antiretroviral therapy. Angiopoetins 1 and 2 levels were not significantly different between HIV-1 positive and healthy women, even after adjusting for maternal factors.

Studies have shown that persistent HIV infection contributes to the development of chronic arterial injury and subsequent endothelial damage, atherosclerosis and thrombosis [49]. In addition, HIV-infected children have arterial stiffness and endothelial dysfunction in the absence of cardiovascular risk factors [50]. Since most of the women in our study were on ART, it is likely that combination antiretroviral therapy (cART) prevents angiopoietin dysregulation. Graham et al. reported that in non-pregnant Kenyan HIV-1 positive women with advanced HIV infection, initiation of cART significantly lowered ANG2 levels, while ANG1 was increased [51]. In agreement with previous studies, we confirmed that HIV-1 negative women with PM had significantly lower ANG1 levels compared to uninfected pregnant women [52,53]. The reason for this observation may stem from malaria parasite level in blood of PM+ women. Silver et al, (2010) found an inverse association between parasitemia and ANG1 levels [54]. With very low parasitemia in malaria positive women (0.13%) in this study, there is possibly no major alteration on the levels ANG1. This might explain the minimal changes observed between PM positive women when compared to HIV-1 negative, PM negative women.

MMP2 and MMP9 levels in intervillous space plasma were not significantly different between HIV-1 positive and healthy pregnant women. MMPs are involved in vascular remodeling and vasculogenesis, especially in new blood vessel formation and angiogenesis [39]. In line with findings for angiopoietins, these data support the fact that in HIV-1 infected pregnant women on antiretroviral therapy, angiopoietin pathway is not dysregulated. Placental malaria did not have any significant effect on MMP2 or MMP9 in this study. This is in line with a previous study, in which no significant changes in plasma levels of MMP9 were observed in children with malaria infection compared to malaria negative children[55].

In linear regression models, maternal HIV-1 did not significantly associate with lower IGF1 in placental intervillous space plasma. Lower IGF1 levels were observed in HIV-infected Ugandan children [56]. In non-pregnant adults, however, serum IGF1 has been shown to depend on level of immunodeficiency in HIV-infection and it was significantly higher in patients treated with protease inhibitors-based regimen compared to non-nucleoside reverse transcriptase inhibitors and healthy subjects [57]. Similar findings were also reported by Matarazzo et al, who found an association between decreased IGF1 levels and diseases progression in HIV-1 positive individuals [58]. In this study, IGF1 was significantly higher in HIV-1 negative PM- positive women when compared to their healthy counterparts as previously described [59]. Further, in a small subset of HIV-1 positive/PM+ co-infected Cameroonian pregnant women in this study, IGF1 levels were significantly lower compared to healthy pregnant women, indicating that HIV-1 further exacerbates PM- associated dysregulation of IGF axis.

Gal-13 is critical in trophoblast invasion during placentation and has also been reported to have angiogenic effects in the placenta [60]. Studies in animal model show that the expression of Gal-13 increases vasodilation [60] and therefore placental perfusion. In addition, lack of expression of Gal-13 has been shown to impair syncytialization [61] and hence subsequent placental hormone production by syncytiotrophoblast, which is vital in the development of the placenta. Gal-13 levels have not been studied in HIV-1 positive pregnant women, but they have been described for other pathological pregnancy conditions. No significant difference was found in Gal-13 intervillous space plasma levels between HIV-1 women under cART and

their uninfected counterparts. Studies by Than et al. showed that Gal-13 placental expression was lower in preterm preeclamptic placentas compared to preterm control placentas [62], while maternal peripheral serum Gal-13 concentration was higher in preterm preeclamtic women compared to preterm controls. In contrast, Sammar et al. did not observe any significant differences in maternal peripheral plasma Gal-13 levels in HIV-1 uninfected pregnant women with preeclampsia or hemolysis, elevated liver enzymes and low platelet count syndrome compared to HIV uninfected [63]. Also, there was no difference in placental Gal-13 levels between HIV-1 negative PM-positive pregnant Cameroonian women and healthy pregnant women.

This study has a number of limitations, including limited number of LBW neonates in both HIV-positive and HIV-negative groups, inability to differentiate effects of HIV from those of ART because ART is standard of care. Of course, an ideal study design would be to investigate these biomarkers with and without cART in a case-control study, it is not ethical to withhold cART from women. However, we observed that even though women have HIV, cART therapy was effective in maintaining their ANG levels close to that of HIV-negative women. A few women were not on cART or had high viral load for some reason beyond our grasp. It is worth mentioning we did not have viral load for all samples and thus could not perfectly do the analysis. Moreover, the findings from this pilot study will need to be confirmed in a larger study due to small samples size. Within the sphere of our study, Cameroon was transitioning from PMTCT Option A to Option B+. Most studies have associated infant growth with Protease Inhibitors [64,65] and very few studies have associated dysregulated fetal growth with prolonged cART. However, our sample size was a limiting factor in the assessment of this hypothesis [64,65]. The cross-sectional study design did not allow monitoring of fetal growth rate and concomitant biomarker levels over time during pregnancy. Given that many of the aforementioned factors are important for placentation, vasculogenesis and placental perfusion and fetal growth, a longitudinal study design would be more effective. At the same time, it is well established that maternal peripheral plasma levels and placental plasma levels may not be the same [53].

While a limitation of the study were the small number of cases, the study demonstrates that maternal HIV-1 infection might not have a dramatic influence on placental IGF1, IGFBP1, MMP2, MMP9, ANG1, ANG2 and Gal-13 levels in Cameroonian pregnant normotensive women with majority receiving cART. It is not clear whether inflammatory cytokines in the placental environment of HIV-1 infected mothers [66–69] or direct effect of HIV infection on syncytial trophoblasts lead to subtle dysregulation of IGF1, IGFBP1, MMP2, MMP9, ANG1, ANG2 and Gal-13 expression in the placenta [70]. Larger prospective longitudinal studies are required to determine, whether there is significant maternal HIV-associated dysregulation in the IGF1 axis and angiogenic factors during pregnancy, especially in women with low CD4 counts, and its effects on the neonate birth weight.

Supporting information

S1 Fig. Fetal blood contamination of intervillous space blood. Nine randomly selected maternal intervillous blood samples were screened for presence of fetal erythrocytes (experimental). In addition, known amount of cord blood was mixed with corresponding maternal intervillous space blood as a positive control. Percentage of fetal erythrocytes in each intervillous blood sample was determined; mean and standard deviation for the samples are presented in the figure.
(TIF)

S1 Table. Other demographic and clinical characteristics of mothers. The data were summarized based on the non-missing values. The total % is not 100 due to missing values or values rounded. £ Calculated for only smear positive individuals. P-values were based on $^{\theta}$ two-sample T-tests, $^{\Phi}$ Fisher's exact tests.
(DOCX)

S1 Database. Database of IDCPC_Rui_Livo v3.
(XLSX)

Acknowledgments

We would like to thank all the mothers and their neonates who have participated in our study. We are grateful to Dr. Robert Leke, doctors and nurses at the maternity ward of the Yaoundé Central Hospital for their assistance with this study. We are grateful to Dr. Claude Djontu, Mrs. Grace Sama, Mrs. Philomina Gwamensia, Mr. Joshua Manjo Sahfe, Prof. Wilfred Mbacham, Dr. Olivia Achonduh at the Biotechnology Center, Univ. of Yaounde I; Nicole Hobbs, Drs. Vivek Nerurkar and Joe Zunt from the Northern Pacific Global Health Fellows Program. We are thankful to SIMMLER Inc, High Ridge, MO, USA for providing fetal stain kit for the study. Finally, we are thankful to Valerie Johnson at Luminex Corp. for her support with MagPix instrument. R&D systems.

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