EXPRESSION ANALYSIS BY QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION OF GENES FOR ASSOCIATION WITH RESISTANCE TO HIV-1 INFECTION IN HIGHLY EXPOSED UNINFECTED COMMERCIAL SEX WORKERS IN THE PUMWANI SEX WORKER COHORT

BY

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DEDICATION

This work is dedicated to my parents Paul and Pamela Apidi, my brothers Kevin and Rudyvoella and my grandmother Mama Susannah Apidi, the first scientist in my life.

ABSTRACT

Altered susceptibility to HIV-1 infection has been observed in multiple cohort studies especially in highly exposed commercial sex workers. Host genetic factors have a major impact on the pathology of infectious diseases and in humans, including HIV-1 infection. It is necessary to determine if genetic factors also contribute to the development of protective responses and escape from infection in HIV-1 exposed uninfected individuals. The search for genetic host factors that might affect the susceptibility of infection and subsequent clinical course of HIV infection is the basis of this study.

This study aimed at determining the differences in expression levels of differentially expressed genes identified in a microarray expression analysis screen of the glycolysis/pentose phosphate/insulin signaling pathways in HIV-1 resistant commercial sex workers in the Pumwani Sex Workers cohort.

mRNA expression levels of 9 genes: G6PD, GAPDH, GIPR, IRS1, PGM1, DPP4, PKM2, CDK2NB and PIK3CG in HIV-1 resistant, uninfected HIV-1 susceptible and HIV-1 infected women were measured by quantitative real-time PCR.

Data analysis was performed by the GraphPad Prism Software. The genes GAPDH and G6PD were found to be significantly down-regulated in HIV-1 resistant women ($p=0.0131$; $p=0.0298$ respectively) as compared to a lack of difference in expression levels in uninfected HIV-1 susceptible women ($p=0.347$; $p=0.5226$ respectively) and the HIV-1 infected women ($p=0.5824$; $p=0.9533$ respectively).

Overall, the expression levels of all the 9 genes studied were reduced in HIV-1 resistant women although statistical difference was observed in 2 genes only.

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made.

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CONTENTS

Dedication ii

Abstract iii

Acknowledgement iv

Declaration vi

List of figures x

List of tables xi

List of abbreviations xii

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction 1

1.2. Literature Review 2

1.2.1 Altered susceptibility to HIV-1 infection 3

1.2.2 Genomics in HIV infection 4

1.2.3 Expression analysis 6

1.2.4 Insulin signaling pathway

1.2.4.1 Insulin receptor substrate 8

1.2.4.2 Phosphoinositide 3-kinase 8

1.2.5. Glycolysis pathway 10

1.2.5.1 Glyceraldehyde-3-phosphate dehydrogenase 11

1.2.5.2 Cyclin-dependent kinase inhibitor 2B 12

1.2.5.3 Phosphoglucomutase 12

1.2.6 Pentose phosphate pathway 15

1.2.6.1 Glucose-6-phosphate dehydrogenase 15

1.2.6.2 Glucose-dependent insulinotropic polypeptide 17

1.2.6.3 Dipeptidyl-peptidase IV 19

1.2.7 Expression analysis techniques 21

1.2.7.1 Microarray technology 21

1.2.7.2 Real time PCR 24

1.2.8 Housekeeping genes 28

1.3 Statement of the problem 32

1.4 Justification 33

1.5 Hypothesis

1.5.1 Null hypothesis 33

1.5.2 Alternative hypothesis 34

1.6 Objectives

1.6.1 General objective 34

1.6.2 Specific objectives 34

CHAPTER 2

METHODOLOGY 35

2.1. Study population 35

2.1.1 Inclusion criteria 35

2.1.2 Exclusive criteria 36

2.2 Sample acquisition 36

2.3 Ethical considerations 36

2.4 Experimental procedures 37

2.4.1 RNA extraction 37

2.4.2 RNA quantitation 39

2.4.3 cDNA synthesis 41

2.4.4 Standards 42

2.4.5 Real time Polymerase Chain Reaction 42

2.4.6 Data management and anlaysis 45

CHAPTER 3

RESULTS

3.1 Quantitation of mRNA levels 47

3.2 Microarray vs Quantitative real time PCR 51

CHAPTER 4

DISCUSSION 59

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion 64

5.2 Recommendations 65

REFERENCES 67

APPENDICES 82

LIST OF FIGURES

Figure 1: Insulin signaling reference pathway

Figure 2: Glycolysis/Gluconeogenesis reference pathway

Figure 3: Pentose Phosphate Reference pathway

Figure 4: Typical cDNA microarray experiment

Figure 5: Real time PCR SYBR Green chemistry schematics

Figure 6: Relative expression of G6PD

Figure 7: Relative expression of GAPDH

Figure 8: Relative expression of GIPR

Figure 9: Relative expression of IRS1

Figure 10: Relative expression of PGM1

Figure 11: Relative expression of PIK3CG

Figure 12: relative expression of PKM2

Figure 13: Relative expression of CDK2NB

Figure 14: Relative expression of DPP4

Figure 15: Comparison of microarray and qRT-PCR expression of G6PD

Figure 16: Comparison of microarray and qRT-PCR expression of GAPDH

Figure 17: Comparison of microarray and qRT-PCR expression of CDK2NB

Figure 18: Comparison of microarray and qRT-PCR expression of GIPR

Figure 19: Comparison of microarray and qRT-PCR expression of IRS1

Figure 20: Comparison of microarray and qRT-PCR expression of PGM1

Figure 21: Comparison of microarray and qRT-PCR expression of PIK3CG

Figure 22: Comparison of microarray and qRT-PCR expression of PKM2

Figure 23: Comparison of microarray and qRT-PCR expression of DPP4

LIST OF TABLES

Table 1: Gene specific primers

Table 2: Thermocycling conditions used for qRT-PCR of all genes and samples

Table 3: Fold changes and fold decreases of gene expression

Table 4: Microarray Vs qPCR (HIV-Resistant women)

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine triphosphate
CDK2NB	cyclin-dependent kinase inhibitor 2 beta
cDNA	complementary deoxyribonucleic acid
CSW	Commercial sex workers
CXCR4	HIV chemokine coreceptor X4
DNA	Deoxyribonucleic acid
DPPIV	Dipeptidyl peptidase type 4
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIP	Glucose-dependent insulinotropic (gastric inhibitory) polypeptide
GIPR	Glucose-dependent insulinotropic polypeptide receptor
GLP1	Glucagon-like peptide 1
Grb-2b	Growth factor receptor-bound protein 2
HIV-1	Human Immunodeficiency Virus type 1
HLA	Human Leukocyte Antigen
IgA	Immunoglobulin A
IL4	Interleukin 4
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
MCH	Mother-to-Child Health clinic attendees
mRNA	messenger ribonucleic acid

NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NF_kB	Nuclear factor kappa beta
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PGM1	Phosphoglucomutase type 1
PI3K	Phosphoinositide 3-kinases
PIK3G	posphatidylinositol-3-kinase Gamma
PK	pyruvate kinase
PKM2	pyruvate kinase muscle type 2
PPP	Pentose phosphate pathway
qRT-PCR	quantitative real time polymerase chain reaction
RBCs	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rRNA	ribosomal Ribonucleic acid
SH2	Src homology 2 domain
SHP2	Src homology 2-containing tyrosine phosphatase
STI	Sexually transmitted infections
TAR	Transactivation response element
Th1/Th2	T helper cell one/ T helper cell two
W.H.O.	World Health Organization

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

One characteristic of all populations is variability in susceptibility to infection and disease. Of those exposed to a pathogen, not all become infected, and not all of those infected develop disease.

Altered susceptibility to HIV-1 infection has been observed in multiple cohort studies especially in highly exposed commercial sex workers. One of the best characterized HIV-1 exposed but uninfected groups is a commercial sex worker cohort from Nairobi, Kenya, the Pumwani cohort, where some individuals can be epidemiologically defined as resistant to HIV (Plummer, *et al*, 1999; Ball, *et al*, 2007).

There has been an intense interest in understanding the mechanisms responsible for resistance to HIV-1 infection. Several studies have shown that susceptibility to HIV-1 infection is complex and resistance is likely polygenic in nature, involving more than a single factor. Apart from immune mediated factors associated with altered susceptibility to HIV-1 infection, genetic factors are also hypothesized to be associated with this altered susceptibility (Ball, *et al*, 2007).

The identification of genetic host factors that might affect the susceptibility of infection was the basis of this study.

Nine genes of the glycolysis, pentose phosphate and insulin signaling pathways from a preliminary microarray study showed differential expression in HIV resistant commercial sex workers compared to controls. This study aimed at confirming these results.

Quantitative real-time PCR is an important step in the validation of expression data generated by microarray analysis; and this technique was used to monitor expression levels of genes; to quantitate differences in mRNA expression and independently verify the microarray results.

1.2 LITERATURE REVIEW

Retroviruses and vertebrates have coexisted over several years and this has resulted in the evolution of multiple defense mechanisms to inhibit viral replication in vertebrate cells (Naghavi *et al*, 2005).

The interaction between viruses and host cells is complex and multifaceted. While viruses attempt to manipulate cellular functions to their advantage, the cell counteracts by mounting a variety of defensive responses including the induction of interferon, stress response, or apoptic pathways, all of which are accompanied by changes in gene expression (Guerra *et al*, 2004).

The ability to examine and characterize the expression patterns for as many genes as possible in a given cell population would provide valuable information regarding the biological potential of those cells. Recent advances have made it possible to measure simultaneously the expression of thousands of genes in cells or tissues of interest (DeRisi,*et al*, 1996; Schena, *et al*, 1996).

The search for genetic host factors that might affect the susceptibility to infection by HIV was based on initial observations that polymorphisms of chemokine receptors that are utilised as co-receptors along with the CD4 molecule were shown to protect against HIV-1 infection. These molecules are utilised as an entry portal for HIV-1 infection; and their ligands have been shown to modulate the efficiency of HIV-1 infection (Hogan and Hammer, 2001b).

The medical, social and economic impact of the HIV epidemic has underscored the need to quickly develop effective control strategies. Vigorous efforts to develop a vaccine and therapeutic agents have not yet succeeded in containing the spread of the virus. Studies of

persons who remain uninfected despite extensive exposure to HIV continue to provide valuable information on the mechanisms of natural protection, which can then be applied to vaccine design. Natural resistance to infection has been studied in multiple high risk cohorts, with resistance attributed to a combination of innate, genetic, and acquired immune-mediated mechanisms (Kulkarni, et al, 2003).

1.2.1 Altered susceptibility to HIV-1 infection

In the search for a successful vaccine to prevent HIV-1 infection, attention has been turned to people with documented HIV exposure who remain uninfected, in whom evidence of natural immunity could provide important clues for vaccine design. For such studies, it is particularly valuable to study people with repeated exposure to HIV, in whom persistent sero-negativity is more likely to indicate genuine resistance to infection (Fowke, et al, 1996).

The best model of resistance to infection is those individuals who remained uninfected even after repeated exposure to HIV type 1. The identification of correlates of protection could be critical in the development of a HIV vaccine (Heeney, et al, 1999).

Altered susceptibility to HIV infection has been observed in multiple cohort studies especially in highly exposed sex workers. One of the best characterized HIV-1 exposed yet uninfected groups is a commercial sex worker cohort from Nairobi, Kenya, where some individuals can be epidemiologically defined as HIV-Resistant. These women are repeatedly exposed to HIV through unprotected sex yet remain systematically seronegative and PCR-negative (Plummer, et al, 1999; Fowke, et al, 2000; Ball, *et al*, 2007). The cohort has been active since 1985 and was designed to study factors

associated with HIV-1 transmission. To date, 2985 women have been enrolled with 715 in active follow-up. Those classified as HIV-Resistant are 140. The women are resurveyed semi-annually including a physical exam; testing for bacterial S.T.I; collection of sexual behavior data such as frequency of condom use, number of partners etc. They participate in several studies and receive health care all year round. Those negative upon enrolment usually seroconvert within 5-7 years; but a small percentage remains HIV-1 negative, some subjects with more than 15 years of follow-up. These women are considered relatively resistant to HIV-1 infection if they are HIV-1 negative both by serology and PCR; active in commercial sex work and have been followed up in the cohort for more than 7 years (Fowke, *et al* 1996). This is based on analysis of infection pressure.

Immunologically, resistance correlates with systemic HIV-1-specific T-helper responses, systemic and mucosal cytotoxic T-lymphocyte responses, HIV-1 specific IgA levels (Plummer, *et al*, 1999; Alimonti, *et al*, 2005) and a global IL-4 hyporesponsiveness to HIV-1 and other antigens (Trivedi, *et al*, 2001). Those findings suggest that HIV-1 resistant individuals exhibit a biased cellular or T-helper type 1 (Th1) immune responses.

1.2.2 Genomics in HIV-1 infection

Host genetic factors have a major impact on the pathology of infectious diseases; and in humans, these interactions have been intensely studied in the context of the HIV infection. By characterizing genetic variation among individuals and among populations, a better understanding may be gained of differential susceptibility to disease (Collins, *et al*, 2003).

The interaction of viral and host factors is believed to determine not only the risk for the HIV-1 acquisition but also the course of infection (Hogan and Hammer, 2001a). Association of resistance to infection with the chemokine receptor mutation CCR5 D32 which blocks HIV-1 infection of $CD4^+$ lymphocytes is clear cut. A deletion in the chemokine receptor CCR5 may account for HIV-1 resistance. However, this genetic mutation is very uncommon in African populations, and neither this nor their receptor defects inhibiting HIV-1 cell entry are likely to account for their resistance in the Pumwani cohort (Fowke, *et al*, 1998).

Polymorphisms in genes influencing the immune response to HIV-1 may account for the variation in susceptibility to HIV-1 infection between individuals with similar risk of exposure (Hardie, *et al*, 2008).

Genetically, it is suggested that specific human leukocyte antigen (HLA) genotypes correlate with HIV-1 resistance (Dunand, *et al*, 1997; Fowke, *et al*, 1998; MacDonald, *et al*, 2000) The HLA complex, located on the short arm of chromosome 6, is the most polymorphic system in the human genome. It is fundamental in the immune system's response to infectious agents, as HLA class I and class II molecules restrict presentation of antigenic peptides to $CD8^+$ and $CD4^+$ T cells, respectively (Germain, 1994). Previous studies have shown that individuals may differ in their immune responses to HIV-1 due to differences in their HLA alleles' ability to present antigen to CD4+ T cells (MacDonanld, *et al*, 2000; Bird, *et al*, 2002).

It is possible that genetic differences between populations studied could be responsible for some of the variability in the results obtained by different groups studied (Mcllroy, *et al*, 2006).

Several genes and pathways have been implicated in HIV-1 pathogenesis and disease progression (Geiss, *et al*, 2000; Corbeil, *et al*, 2001; Woelk, *et al*, 2004).

1.2.3. Expression analysis

Selected genes with distinct patterns as identified by microarray analysis have been chosen for verification of transcriptional changes. By analyzing the data for genes associated with each other on the basis of temporal expression profiles, it is possible to make connections between genes not previously associated with each other at the functional level and gain an insight into the extended interactions between different cellular processes (DeRisi, *et al*, 1997).

Potential host mechanisms in HIV-1 resistant subjects may each play some degree of protection, and unknown protective host factors are yet to be discovered (Jin, *et al*, 2007). As part of an ongoing study, some genes from a preliminary microarray study showed high association values with the HIV resistant ML cohort members. These have been filtered based on their biological pathway i.e. only those that are predicted to functionally cluster together by a software cell pathway architect. Most, but not all, of the genes to be studied have no prior association with HIV.

Glycolysis/Pentose Phosphate/Insulin pathways

1.2.4 Insulin signaling pathway

Insulin is a hormone released by pancreatic beta cells in response to elevated levels of nutrients in the blood. Insulin triggers the uptake of glucose, fatty acids and amino acids into adipose tissue, muscle and the liver and promotes the storage of these nutrients in the form of glycogen, lipids and protein respectively. Failure to uptake and store nutrients

results in diabetes (White, 1997). Insulin activates various signaling molecules in its target cells, the signaling pathways mediated by Insulin receptor substrate (IRS) and phosphatidylinositol-3-kinase PIK3 are thought to play a central role in the metabolic actions of insulin suggesting that disturbance of these pathways (insulin-stimulated signal transduction pathways) may contribute to the development of insulin resistance. Indeed, disruption of the genes for IRS1 or IRS2, both of which are expressed in many tissues and cells has been demonstrated (Ogawa, *et al*, 1998; White, 1998).

The insulin signaling system has emerged as a flexible network of interacting proteins. The insulin receptor is a transmembrane receptor that is inactivated by insulin and belongs to a large class of tyrosine kinase receptors. These all mediate their activity byn phosphorylation of tyrosines on certain proteins within the cell. The insulin receptor phosphorylates the insulin receptor substrate proteins (IRS). By utilizing the IRS proteins, the insulin signal can be amplified or attenuated independently of insulin binding and tyrosine kinase activity, providing an extensible mechanism for signal transmission in multiple cellular backgrounds. IRS proteins are employed by the insulin receptor (IR) to engage various signaling proteins eventually leading to an increase in the high affinity glucose transporter molecules and thus an increase in glucose uptake into tissues (White, 1997; Duckworth, *et al*, 1998).

Insulin receptor substrates (IRS), the major intracellular substrates of the insulin receptor (IR), are adaptor proteins that transduce signals from the IR to downstream effectors that are important for the biological effect of insulin. After insulin stimulation, IRS proteins are rapidly phosphorylated on multiple tyrosine residues. Once phosphorylated, IRS proteins bind and activate Grb-2, SHP2 and the PI3-K p85 subunit (Ogawa, *et al*, 1998

White, 1998). IRS-1 functions as one of the key regulators of IR and the insulin-like growth factor-1 receptor (White *et al*, 1997). Diabetes mellitus results either from a lack of insulin or the failure to compensate for a diminished insulin response at various target tissues. No link has been found between HIV and the genes of this pathway.

1.2.4.1. Insulin receptor substrate-1

The insulin receptor substrate-1 (IRS1) is a critical element in the insulin-signaling pathway. Mutations in the IRS gene have been reported to have a role in determining susceptibility to traits related to type 2 Diabetes (Kovacs, *et al*, 2003).

Insulin receptor substrate 1 mRNA is of low abundance in human tissues (6.9kb and 6kb species) Most, if not all insulin signals are produced or modulated through tyrosine phosphorylation of IRS1 or its homologue IRS2, or other scaffold proteins. IRS1 controls body growth and peripheral insulin action (White, 2003). IRS1 is important in transmitting the metabolic actions of insulin and is strongly expressed in insulin sensitive tissues.

Insulin receptor substrate 1 is an intracellular substrate after phosphotyrosines on the IRS proteins bind the p85 regulatory subunits of phosphatidylinositol 3' kinase (PI3 kinase). IRS1 signaling may be more closely linked to the regulation of genes involved in glucose homoestasis, which is impaired by liver specific down-regulation of IRS proteins (Taniguchi, *et al*, 2005).

1.2.4.2. Phosphoinositide 3-kinases are a family of related enzymes that are capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns). They are also known as phosphatidylinositol-3-kinases. PI3Ks interact with the IRS (Insulin receptor substrate) in order to regulate glucose uptake through a series of

phosphorylation events (Vanhaesebroeck, *et al*, 1999). PI3K enzyme is activated during association with IRS proteins. (White, 2003) Class 1 Phosphoinositide 3-kinase (PI3K) signaling pathways regulate several important cellular functions, including cellular growth, division, survival and movement (Hawkins and Stephens, 2007). Class 1 PI3Ks are well established signal transduction enzymes that drive extensive signaling networks downstream of cell surface receptor activation (Hawkins, *et al*, 2006).

PI3K-Gamma allows fast acting, heterotrimeric G-Protein-coupled receptors to access PI3K signaling networks. Most of the ligands established to activate PI3KG are involved in the regulation of multiple cell types in the immune system and vascular lining (Ruckle, *et al*, 2006; Hirsch, *et al*, 2006). It was originally characterized as a heterodimer of P101 regulatory and P110 catalytic subunits and its activation in response to insulin results primarily through its association with the IRS proteins (Stoyanov, *et al*, 1995; Stephens, *et al*, 1997; White, 1997). PI3KG is a ubiquitous lipid kinase that catalyses the conversion of phoshatidylinositol-4, 5-triphosphate in the inner leaflet of the plasma membrane and functions downstream of cell-surface receptors and in constitutive intracellular membrane and protein-trafficking pathways (Walker, *et al*, 1999; Suire, *et al*, 2006). This gene has not been linked with HIV yet.

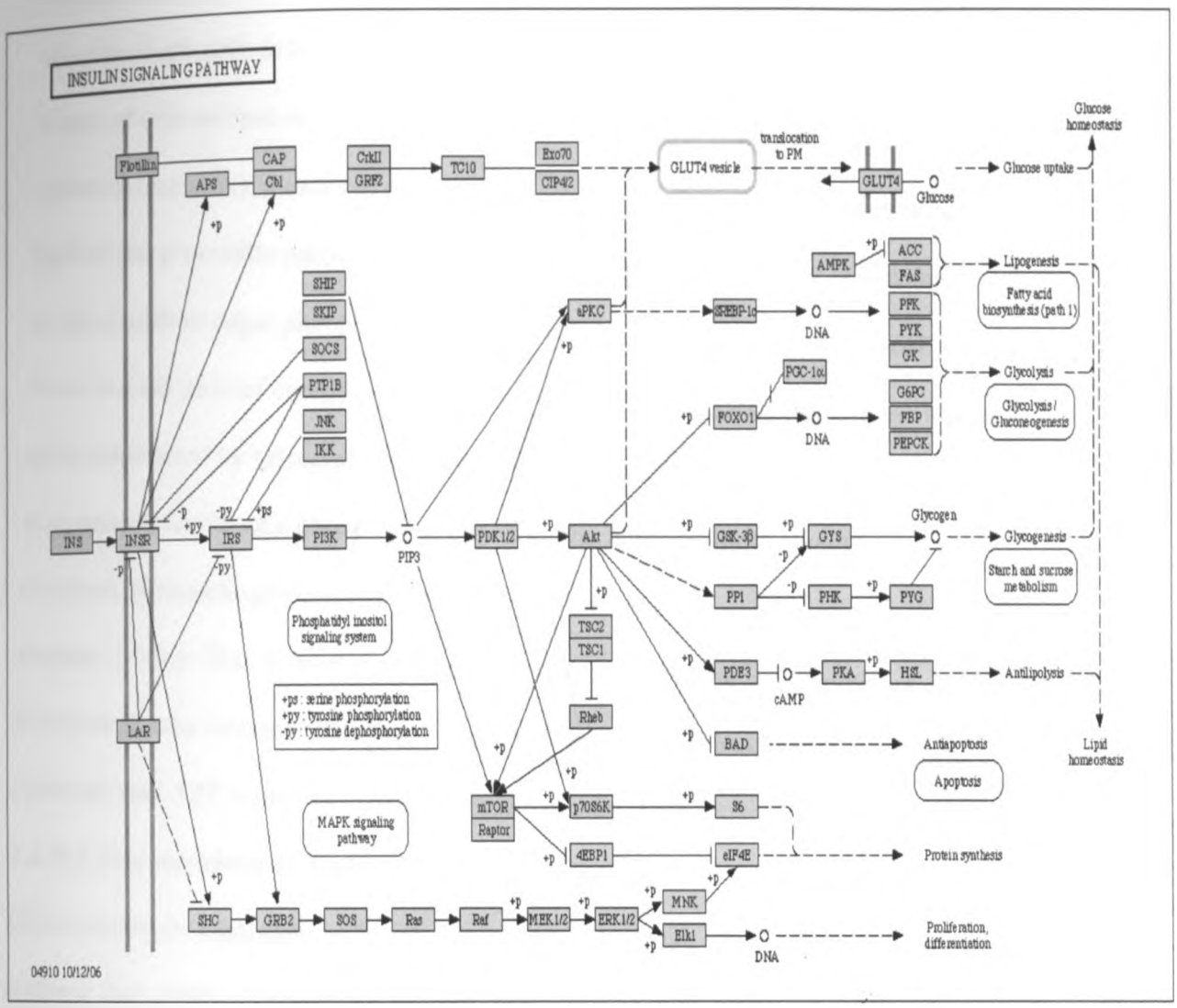


Figure 1: Insulin Signaling Pathway, Bevan P, 2006

Adapted from Kegg Insulin Signaling Pathway

www.genome.ad.jp/**kegg/pathway**/hsa/hsa04910.html

1.2.5 Glycolysis pathway

Glycolysis is the sequence of reactions that converts glucose into pyruvate with the concomitant production of a relatively small amount of adenosine triphosphate (ATP). It is the initial process of most carbohydrate catabolism (Romano and Conway, 1996).

In glycolysis, as glucose enters a cell, it is immediately phosphorylated by ATP to glucose-6-phosphate in an irreversible first step prevent the glucose leaving the cell. In times of excess lipid or protein energy sources, glycolysis may run in reverse (gluconeogenesis) in order to produce glucose-6-phosphate for storage or starch. The first half of the glycolytic pathway is the preparatory phase since energy is consumed into two to three carbon sugar phosphates. The second half of glycolysis is the payoff phase where there is a net gain of energy rich molecules of ATP and NADH. Here, triose sugars are dehydrogenated by Glyceraldehyde-3-phosphate dehydrogenase. (GAPDH) There is an enzymatic transfer of a phosphate group from the biphosphoglycerate kinase. The resultant 3-phosphoglycerate is converted to 2-phosphoglycerate by phosphoglycerate mutase (PGM). This is then converted to phosphoenlpyruvate by the enzyme enolase. Pyruvate kinase enzyme (PK) then conducts a final level phosphorylation to form pyruvate and ATP molecules (Romano and Conway, 1996).

1.2.5.1. Glyceraldehyde 3-phosphate dehydrogenase

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a major NADH-generating enzyme that catalyzes the conversion of glyceraldehydes 3-phosphate to 1,3-diphosphoglycerate in red blood cells (Yan, *et al*, 1999). This is the sixth step of glycolysis and GAPDH thus serves to break down glucose for energy and carbon molecules. Glycolysis is an important pathway of energy and carbon molecule supply in the cytosol of eukaryotic cells. Although GAPDH was originally thought of as an enzyme important only for glycolysis, and as a housekeeping gene appropriate for use as a control in the measurement of equal loading in experiments, over the last decade, every succeeding year has brought to light a new function for this protein. The many and

diverse roles for this multifunctional protein include among others: DNA replication and repair, nuclear membrane fusion, apoptosis, microtubule bundling, vesicular secretory transport, and maintenance of telomere structure, with its roles in part related to subcellular localization. GAPDH has been found in nuclear, cytoplasmic, and membrane localizations, and can shuttle between these compartments (Sirover, 2005; Tarze, *et al*, 2007; Zhou, *et al*, 2008;). GAPDH has been shown to be ROS in mammalian cells (Ravichandran, *et al*, 1994). Thus, ROS-induced inactivation of GAPDH may cause an accumulation of methaemoglobin in RBCs because NADH is essentially required as a cofactor for methaemoglobin reductase (Yan, *et al*, 1999).

1.2.5.2. Cyclin-dependent kinase inhibitor 2Beta

Cyclin-dependent kinase inhibitor 2Beta (CDNK2B) is a cyclin dependent kinase inhibitor which is an important negative regulator of cell division (Martignetti, *et al*, 1999). This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, cyclin-dependent kinases, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression. CDKs are serines/threonines that require binding on a cycline in order to become activated (Bird, 2003). CDK activity is regulated by several processes, including phosphorylation on threonine and tyrosine residues, some of these phosphorylation steps being stimulatory others inhibitory.

1.2.5.3. Phosphoglucomutase 1

Phosphoglucomutase 1 (PGM1) is an enzyme that facilitates the inter-conversion of glucose 1-phosphate and glucose 6-phosphate. The subsequent products can then travel down the glycolysis or pentose phosphate pathway. PGM is an enzyme widely distributed

in nature that catalyzes the redox reaction glucose-1-phosphate to glucose-6-phosphate. This is an essential step in carbohydrate metabolism (Gloria-Bottini, *et al*, 2001).

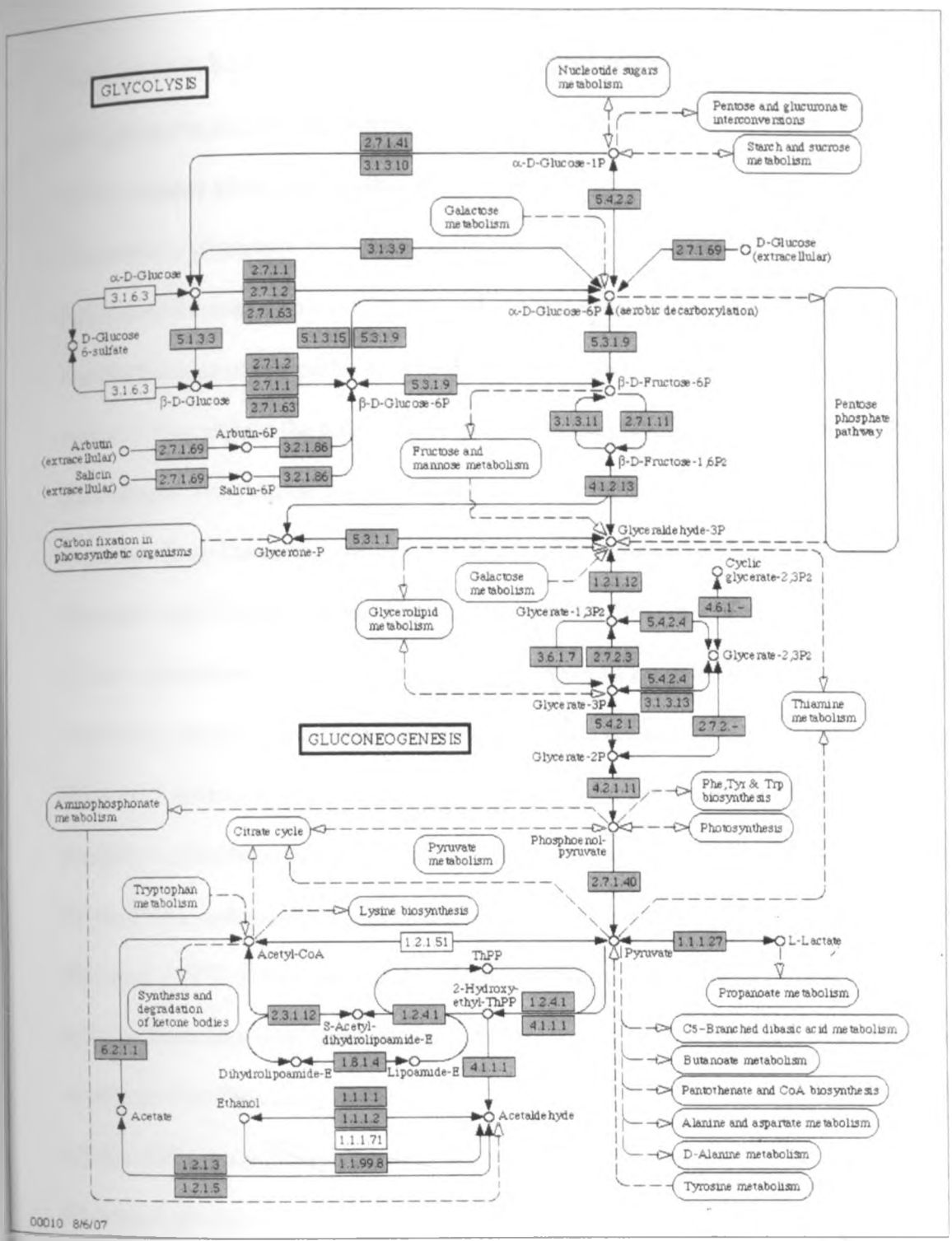


Figure 2: Glycolysis/Gluconeogenesis pathway, Michal G, 2007

Adapted from KEGGS glycolysis/ gluconeogenesis reference pathway.

www.genome.ad.jp/**kegg**/**pathway**/ot/ot00010.html

1.2.6. Pentose phosphate pathway

The pentose phosphate pathway is also known as the phosphogluconate pathway or hexomonophosphate shunt. Glucose 6-phosphate dehydrogenase (G6PD) is the committed step of this pathway. The pentose phosphate pathway is primarily an anabolic pathway that utilizes the 6 carbons of glucose to generate 5 carbon sugars and reducing equivalents. The pentose phosphate pathway is strongly reliant on glycolysis as a source of metabolites. However, this pathway does oxidize glucose under certain conditions (Romano and Conway, 1996). Thus it may be an alternative to glycolysis.

Pentose phosphate pathway is a process that serves to generate NADPH and the synthesis of pentose sugars. In its oxidative phase, $NADP^+$ is reduced to NADPH using energy from the conversion of glucose-6-phosphate into ribulose 5-phosphate by glucose 6-phosphate dehydrogenase. (Dehydrogenation step) Hydrolysis then takes place followed by oxidative carboxylation and isomerization.

The gene G6PD of this pathway has been linked to viruses whereby cellular host G6PD activity modulates viral gene expression thus modulating the cellular susceptibility to viral infection (Wu, *et al*, 2008).

1.2.6.1. Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step in the hexose monophosphate pathway of glucose metabolism, and it produces glutathione in its reduced form (Beutler, 1994). Glutathione protects red blood cells from oxidative damage. G6PD deficiency is clinically manifested as acute hemolytic anaemia, chronic

non-spherocytic hemolytic anaemia and neonatal hyperbilirubinemia, which is of hemolytic origin by agents causing destruction of the red blood cells (Dennery, *et al*, 2001). G6PD, the first and rate-limiting enzyme of the pentose phosphate pathway is a major intracellular source of NADPH (Park, *et al*, 2006). It is highly expressed in the adipocytes in cases of obesity. This over-expression provokes dysregulation of lipid metabolism and adipocytokine expression, resulting in insulin resistance (Park, *et al*, 2005). Over-expression has been shown to promote oxidative stress, and NF-$_k$B signaling, which are the key causative factors of chronic inflammation and insulin resistance in obesity and its related metabolic disorders (Park, *et al*, 2006).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzyme deficiency involving more than 400 million people worldwide (WHO, 1989). It still reigns as the most common of all clinically significant enzyme defects.

The protective role of erythrocyte (E) Glucose-6-phosphate dehydrogenase (G6PD) deficiency against infection with *Plasmodium falciparum* malaria is supported by case control studies performed in Africa (WHO, 1989; Denney, *et al*, 2001). In these studies, it has been established that inherited deficiency of erythrocyte G6PD confers protection against *Plasmodium falciparum*.

Glucose-6-phosphate dehydrogenase enzyme activity has an important role in the cell defense against oxidative damage produced by hydrogen peroxide, a compound known to activate HIV-1 expression through the intermediate of NF-kappa $_B$ (Cappadoro, *et al*, 1998; Shreck, *et al*, 1998).

Replication of HIV depends on efficient expression of the viral regulatory Tat gene. Tat codes for a protein that binds a cis-acting stem loop structure (TAR) present at the 5'end

of all viral transctripts. Binding of TAR is essential for the Tat function, required early in the viral life to increase viral gene expression (Rosen, 1991). During the activation of latent HIV-1 infection by H_2O_2, Tat further stimulates G6PD to ensure cell survival and therefore virus production (Ursini, *et al*, 1993).

1.2.6.2. Glucose-dependent insulinotropic polypeptide

Glucose-dependent insulinotropic polypeptide (GIP) also called gastric inhibitory polypeptide, is an incretin hormone released from the upper small bowel in response to luminal nutrients that amplifies insulin release from pancreatic beta cells (Pederson, 1994).

Glucose-dependent insulinotropic polypeptide and stimulates proximal and distal steps of the exocytotic cascade by acting on a seven-transmembrane spanning G-protein-coupled receptor coupled to stimulation of adenyl cyclase, activation of phospholipase A_2, and increases in intracellular calcium (McIntosh, *et al*, 1996; Ding, *et al*, 1997; Gromada, *et al*, 1998; Ehses, *et al*, 2001). In addition to enhancing insulin release, GIP further acts as an insulinotropic agent by stimulating pro-insulin gene transcription and translation (Fehman and Burkhard, 1995). Glucose-dependent insulinotropic polypeptide also upregulates plasmalemmal glucose transported and hexokinase in the beta cell (Wang, *et al*, 1996).

Glucose-dependent insulinotropic polypeptide is a 42-amino acid peptide hormone secreted by endocrine K cells in the proximal small intestine (duodenum and jejunum) in response to the ingestion of nutrients, especially fats. It exerts its effects in the stomach and pancreas through binding to specific G-protein coupled to gastric inhibitory receptors (GIPR) of the glucagons secreting family of peptides (Krarup, *et al*, 1985; Elliot, et al,

1993; Baggio and Drucker, 2007). Together with the structurally related peptide, glucagons-like peptide 1 (GLP1) released from the intestinal L-cell, GIP is considered an incretin hormone. The incretin effect denominates the phenomenon that oral glucose elicits a higher insulin response than does intravenous glucose. GIP hormone is responsible for the incretin effect and is secreted after oral glucose loads, and augments insulin secretion in response to hyperglycemia. The insulinotropic response to GIP is secreted normally or hypersecreted in type 2 diabetes (Nauck, *et al*, 1993). The gut hormone GIP is released from the duodenum and upper jejunal endocrine K cells and modulates insulin secretion after carbohydrate or fat consumption in humans and rodents (Isken, *et al*, 2008). GIP receptors (GIPR) are expressed in pancreatic beta cells but are also found in other tissues such as the brain and adipose tissue (Yip and Wolfe, 2000). Exposure to high blood glucose concentrations in vivo and in vitro causes decrease in the GIPR gene expression in the islets (Xu, *et al*, 2007).

Glucose-dependent insulinotropic polypeptide is known to be degraded by DPP4, forming an inactive metabolite, but the extent of the enzyme's role in regulating the activity of Glucose-dependent insulinotropic polypeptide in vivo is still largely unknown (Deacon, *et al*, 1999). *In vitro* studies have shown Glucose-dependent insulinotropic polypeptide to be a substrate for the enzyme DPP4, resulting in the formation of a truncated inactive metabolite, (GIP-3-42). Inhibition of DPPIV reduces the clearance of GIP and results in increased levels of intact biologically active GIP. This is accompanied by markedly increased insulin secretion and improves glucose tolerance (Ahren, *et al*, 2000). This is associated with enhancement of its insulinotropic activity and results in a reduction in the glucose excursion after an intravenous glucose load. Studies have shown

that Dipeptidyl-peptidase IV inhibitors enhance glucose-induced insulin secretion and inhibition of glucagons secretion (Mari, *et al*, 2005).

1.2.6.3. Dipeptidyl-peptidase IV

Dipeptidyl-peptidase IV is the founding member of a family of DPPIV activity and/or structural homologue (DASH) proteins, enzymes that are unified by their common post-proline cleaving serine peptidylpeptidase mechanism (Sedo and Malik, 2001). Dipeptidyl-peptidase IV is identical to CD26, a marker for activated T-cells (De Meester, *et al*, 1999).

Dipeptidyl-peptidase IV is the key enzyme responsible for inactivating both GLP-1 and GIP, and genetic disruption or pharmacological inhibition of DPPIV lowers blood glucose in rodent and human studies (Marguet, *et al*, 2000; Ahren, *et al*, 2002). DPPIV inhibitors modify the activity of multiple peptide substrates with glucose-lowering effects in addition to GIP and GLP1 (Henriksen, *et al*, 1998; Zhu, *et al*, 2003). Either inhibiting the enzymatic activity of DPPIV on various animal models or knocking out DPPIV in mice and rats prolongs the half lives of these two insulin-sensing hormones, increases insulin secretion and improves glucose tolerance (Ahren, *et al*, 2000; Deacon, *et al*, 2001 ; Popsilik *et al*, 2002; Sudre, *et al*, 2002 ; Villhauer, *et al*, 2002).

CD26/ Dipeptidyl-peptidase IV protein plays a major role in immune response. Abnormal expression is found in the case of autoimmune diseases, HIV-related diseases and cancer (Boonacker and Van Noorden, 2002). HIV gp120 protein inhibits binding between adenosine deaminase and DPPIV, and thus inhibits adenosine breakdown. In this way, it may contribute to the pathogenesis of HIV-related diseases (Valenzula, *et al*, 1997).

CD26 colocalizes with CXCR4, which is the receptor for one of the natural substrates of CD26, SDF-alpha. This binding induces chemotaxis and antiviral activities of Th2 cells (Herrera, *et al*, 2001). There is a selective decrease in CD26/DPPIV positive T cells in HIV-1 infected individuals prior to a general reduction in the number of $CD4^+$ cells (Blazquez, *et al*, 1992). This indicates the importance of the immunomodulating role of DPPIV which is supported by the finding that antigen response in HIV-1 infected individuals can be restored by the addition of CD26/DPPIV in vitro (Schmitz, *et al*, 1996). It has been shown that DPPIV protease activity of plasma DPPIV was both decreased in HIV-infected individuals and inversely correlated with HIV-1 RNA levels in cells.

Dipeptidyl-peptidase IV has been considered to be a unique peptidase that cleaves dipeptides and proteins containing praline in the penultimate position. It can also cleave dipeptides with alanine in that position (proteolysis) (Meintlein, 1999; Ejiiri, 2002). It is also involved in signal transduction and can bind to a variety of proteins. Due to its multifunctional character and its widespread expression, the exact functions of DPPIV in vivo have not yet been elucidated (Jeffrey, 1999).

Dipeptidyl-peptidase IV is a well documented drug target for the treatment of Type II Diabetes (Pauly, *et al*, 1996; Mentlein, 1999).

The enzyme Dipeptidyl peptidase IV (DPP4), a serine protease that preferentially hydrolyses peptides after a penultimate NH_2 terminal praline (Xaa-Pro) or alanine (Xaa-Ala) has been shown to rapidly metabolise GIP and GLP-1 in vitro (Demulth and Heins, 1995; Mentlien, 1999). Dipeptidyl peptidase IV encodes a plasma membrane

exopeptidase that is highly expressed in several tissues including the small intestine, lung and kidney (Bohm, *et al*, 1995).

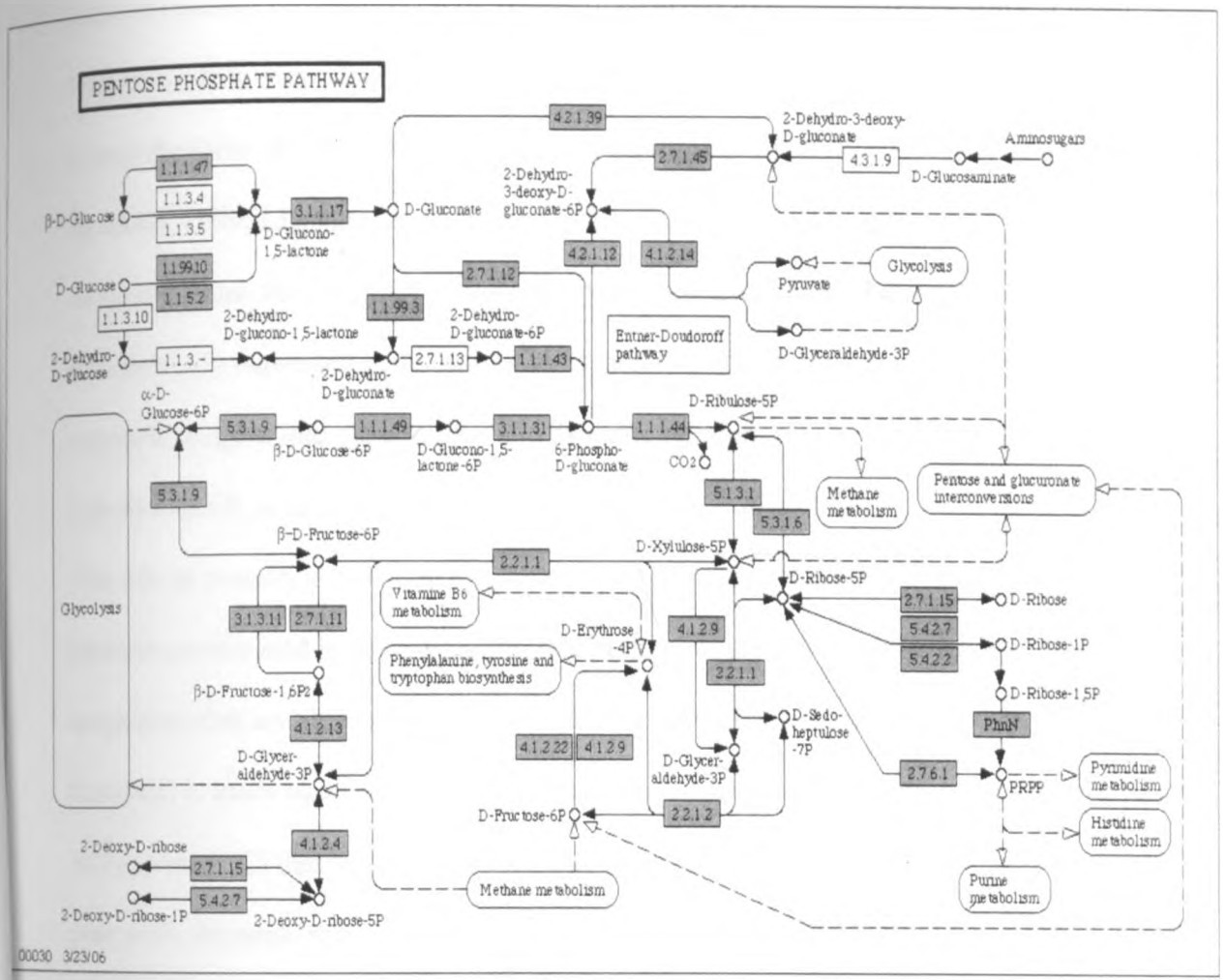


Figure 3: Pentose Phosphate Pathway, Michal G, 2006

Adapted from KEGGS Pentose Phosphate Pathway-Reference Pathway.

www.genome.ad.jp/dbget-bin/show_**pathway**?hsa00030+5238

2.7. Expression analysis techniques

2.7.1. Microarray technology

With the advent of large scale gene expression monitoring, new information on genes is obtained more easily through the use of microarrays. Microarray technology has been used to measure the expression of thousands of genes independently and simultaneously. It is possible to view changes in gene expression and cellular function in a much more comprehensive manner than was preciously possible (Giri, *et al*, 2006; Brown and Botstein, 1999; Lipshutz, *et al*, 1999).

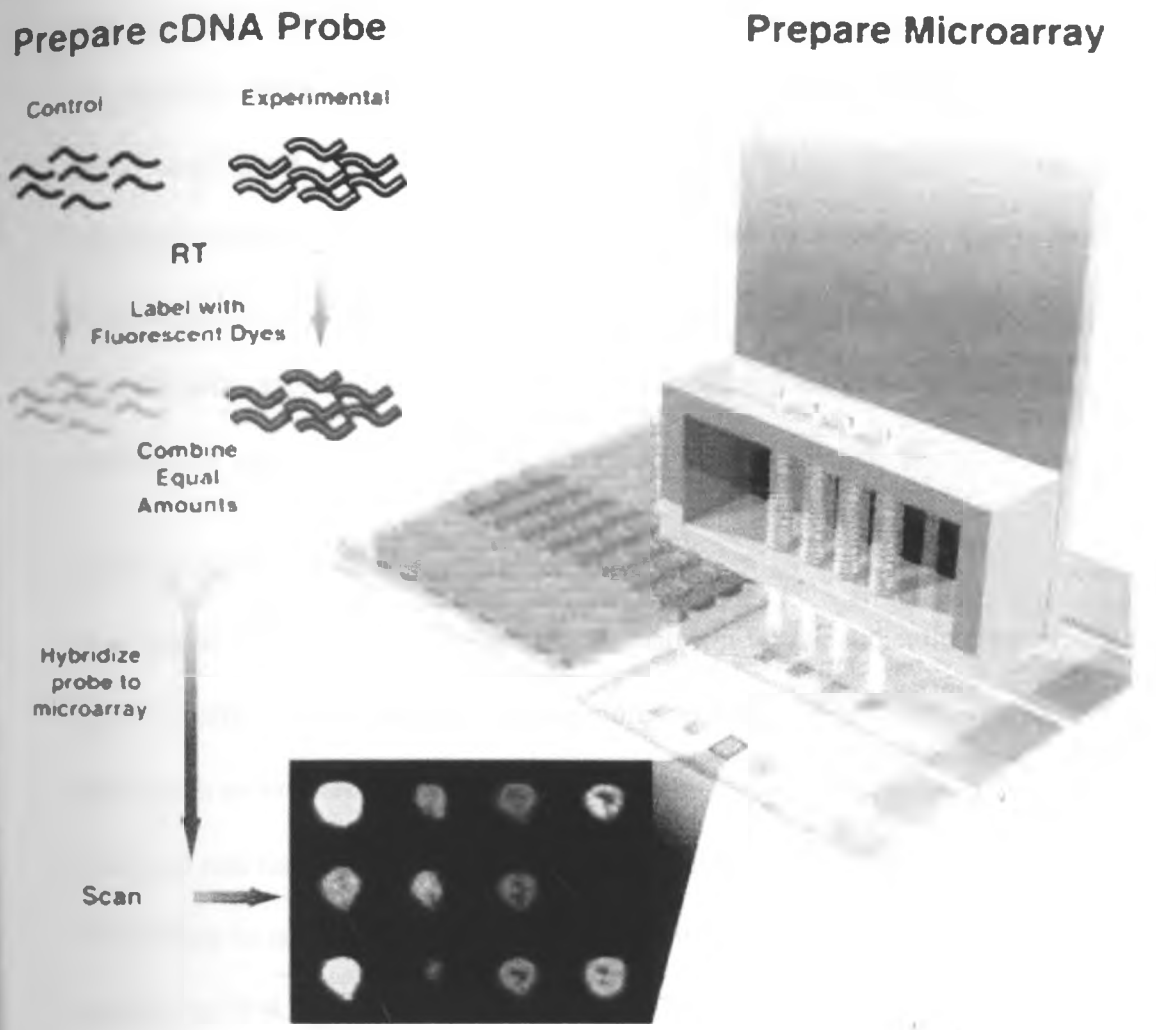
In recent years, the use of DNA microarray technology as a tool for analyzing differentially expressed genes during viral infection ("viral infectomics") has provided valuable insights into the complex and dynamic relationships between the process of infection (Hill, *et al*, 2001; Nam *et al*, 2003, Leong, *et al*, 2005).

The whole process is based on hybridization probing, a technique that uses fluorescently labeled nucleic acid molecules as "mobile probes" to identify complementary molecules/ sequences that are able to base-pair with one another. Each single-stranded DNA fragment is made up of four different nucleotides, adenine (A), thymine (T), guanine (G), and cytosine (C) that are linked end to end. Adenine is the complement of, or will always pair with, thymine, and guanine is the complement of cytosine. When two complementary sequences find each other, such as the immobilized target DNA and the mobile probe DNA, cDNA, or mRNA, they will hybridize.

www.ncbi.nlm.nih.gov/About/primer/microarrays.html

Total RNA or mRNA is extracted from test and reference samples. The samples are labeled with different fluorescent dyes, for example, test sample with Cy3 and reference sample with Cy5. The two samples are combined and hybridized to a microarray slide onto which probes have been immobilized in defined array format. The fluorescence

intensities are then measured providing a pseudo-colored image where red spots indicate upregulation of the corresponding genes in the test sample, while green spots indicate downregulation in the test sample. Typically, the ratio of the average red and the average green intensities within a spot are used as a value for the expression level of the corresponding gene (Hautaniemi, *et al*, 2004).



: Schematic for typical cDNA microarray experiment. Extracted from Hautaniemi, et al, 2004.

The reliability of this technique to detect transcriptional differences representative of the original sample is affected by several factors. Microarray results are influenced by array

production, RNA extraction, probe labeling, hybridization conditions and image analysis. Moreover, a microarray experiment is vulnerable to fluctuations in probe, target and array preparation; in the hybridization process, background overshining effects and effects resulting from image processing. This may include random fluctuation of labeling, varying lengths of DNA after transcription, clone amplification, systematic variations in pin geometry, random fluctuations in target volume and slide inhomogeneities (Der, *et al*, 1998; Schuchhardt, *et al*, 2000). Because of the inherent limitations in reliability, genes identified as differentially expressed must be validated with another method. Real time PCR monitors mRNA levels directly and is the current state-of-the-art for gene quantification, and can be adapted for the validation of differentially expressed genes identified by microarrays (Rajeevan, *et al*, 2001).

1.2.7.2. Real-time Polymerase Chain Reaction

Real time PCR constitutes a highly suitable platform for the authentication of microarray data, as it offers the advantages of being more rapid and sensitive than conventional assays such as Northern blots or RNase protection assays (Max, *et al*, 2001). The optimized real time PCR conditions are reproducible and reliable and thus more relevant and accurate to any investigation at the transcript level (Walker, 2002). The use of quantitative PCR has greatly enhanced the ability of researchers to analyse changes in gene expression in various animal and human experiments (Wecker, *et al*, 2008).
Real time PCR measures the accumulation of the PCR amplicon during the exponential phase of the PCR reaction. It has higher sensitivity and enables the quantification of gene expression, is reliable and accurate. It is also reproducible in the detection of mRNA expression levels for several different genes. Real time PCR allows not only the

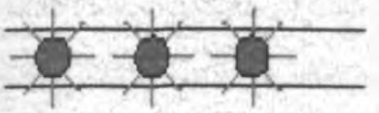
confirmation but also the extension of previous reports of gene expression and correctly quantifies mRNA gene expression during health and disease (Carvalho-Gaspar, *et al*, 2005).

Quantitative real-time PCR (RT qPCR) is a fast, straightforward and reproducible technique which negates the need for post-PCR product handling and is increasingly becoming the method of choice for the accurate profiling of mRNA levels (gene expression) due to its accuracy, wide dynamic range and sensitivity (Bustin, 2002; Gizinger, 2002). RT qPCR enables the investigator to determine the expression levels of a given set of genes in a range of samples and is particularly useful when the sample quantity is limited (Heid, *et al*, 1996). Regardless if the gene expression profiling technology (such as microarrays), once the sequence of the gene of interest is known, the real time PCR approach is well suited for validation of differential expression since it is quantitative and rapid and requires 1000-fold less RNA than conventional arrays (Rajeevan, *et al*, 2001).

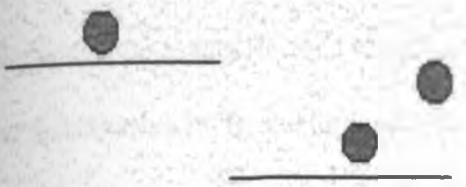
The quantitative aspect at qRT-PCR can be accomplished in several ways, one of which one accurately quantify mRNA species in terms of copy number expressing results as copy numbers per microgram of cDNA, after comparison of samples directly regardless of mRNA synthesis (Whelan, *et al*, 2003). A second method, the SYBR Green method uses the comparative threshold analysis allows to estimate copy numbers in unknown samples, requiring the existence of a standard consisting of template DNA with known number of copies of each of the studied genes, and a housekeeping gene that permits normalization of the quantitative data (Ferriera, *et al*, 2006).

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real time reactions. SYBR Green I dye is a non specific intercalating dye hence the reaction is made specific by using "hot start" PCR and empirically determined annealing and signal acquisition temperatures for each gene-specific primer. Relative expression levels are quantified by constructing standard curves using cDNA dilutions of a sample with highly expressed gene of interest, or housekeeping gene (Rajeevan, *et al*, 2001). The disadvantage is that the SYBR Green will bind to any double stranded DNA in the reaction including primer-dimers and other non-specific reaction products, which may result in an over-estimation of the target concentration. However, the specificity of the reaction can be monitored by determination of the product melting temperature (Tm) defined as the temperature at which half of the DNA helical structure is lost). The "hot start" PCR improves reaction specificity by acquiring a signal at a temperature 1-2°C below the Tm of specific product, avoiding the non-specific signal from primer-dimers that usually melt at lower temperatures. For single PCR product reactions with well designed primers, SYBR Green can work extremely well. SYBR Green binds to the minor groove of double stranded DNA in a sequence independent way. When it binds, its fluorescenct intensity increases 100-fold. As more amplicons are produced, SYBR Green dye signal will increase (Deprez, *et al*, 2002).

SYBR Green 1 assay

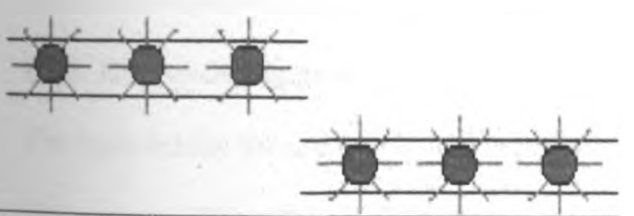


SYBR Green dye attaches when there is double stranded DNA.



When the DNA is denatured the SYBR Green Dye floats free.

Extension phase begins as primers anneal.



Polymerization is complete. SYBR Green Dye binds to the double stranded product and fluoresces.

Real time PCR by SYBR Green Chemistry. Adapted from

www.appliedbiosystems.com/support/tutorials/pdf/rt**pcr**_**vs**_trad**pcr**.pdf

Most of real time PCR data show concordance with the normalized microarray data. Hence, real time PCR validation of high-throughput gene microarray screening is important and necessary before further conclusions on gene expression can be drawn (Liew and Chow, 2006). Comparison of microarray quantitation and quantitative PCR has shown almost identical profiles for a number of genes measured in an experiment i.e. gene expression levels for IL-1 Beta, IL-1 alpha and IL-8 was measured independently using quantitative PCR; the temporal expression profiles obtained were almost identical to those using microarray quantitation (Walker and .Rigley, 2000). Reproducible quantification of gene expression requires an internal reference, for example, a non regulated housekeeper gene, to exclude an essential influence of variations in sample quality or quantity. Real time PCR protocols allow the measurement of the time until fluorescence reaches a pre-defined level during the amplification process, thus providing quantitative data on the amount of nucleic acids in the sample at the beginning of the reaction (Meckelnburg, *et al*, 2007).

1.2.8. Housekeeping genes

The logic behind the use of a housekeeper gene is that its transcriptional levels will not change even if the target gene changes expression patterns dramatically. Housekeeping genes are typically used as endogenous controls because they are constitutively expressed at levels that are assumed to be constant with respect to time and environmental exposures. Hundreds of potential housekeeping genes have been identified by microarray analyses and many of these may have use as controls (Warrington, *et al*, 2000; Overberg, *et al*, 2001). The 'ideal' reference gene for RT qPCR would be one whose mRNA is consistently expressed at the same level in all samples under investigation, regardless of

tissue type, disease state, medication or experimental conditions and would have expression levels comparable to that of the target (Suzuki, *et al*, 2000). Relative gene expression comparison work best when the control gene remains constant and is more abundant in proportion to total RNA, among the samples. Housekeeping genes are defined by specific gene promoter elements which determine that they are expressed constitutively in every cell, but that does not necessarily mean that their expression is not regulated. The use of housekeeping gene expression has allowed relatively inexpensive and simple technologies to be used. Control of RNA quality and cDNA synthesis efficiency without housekeeping gene expression is otherwise difficult (Glare, *et al*, 2002). The choice of housekeeping genes as internal standard in quantitative real-time PCR is critical for the estimation and comparison of mRNA levels in gene expression studies.

Gene expression levels are commonly normalized to distinguish between real (biological) variation and deviations resulting from measurement processes such as run-to-run variation, disparities in mRNA quantity and quality among samples, pipetting errors, and efficiencies of the enzymes used for the reverse transcription and amplification steps (Warrington, *et al*, 2000).

Estimations of gene expression at the mRNA level and comparative analysis of mRNA expression levels of genes of interest in different cells and tissues require methods for accurate normalization. To this end, levels of mRNA for basic structural proteins and genes necessary to maintain basic cellular functions, as well as structural RNA expressed in essentially all cell types have been used as internal standards. Commonly used so-called housekeeping genes are b-actin, glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) and 18S rRNA (Suzuki, *et al*, 2000). Quantitation of the mRNA targets can thus be normalized for differences in the amount of total RNA added to each reaction, for example, by use of Glyceraldehyde-3-phosphatedehydrogenase (GADPH). As a normalizer, it is expressed at a constant level at different time points by the same individual and also by different individuals at the target cell or tissue (Dorak, 2006). The accuracy of 18S rRNA, beta-actin mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as indicators of cell number when used for normalization in gene expression analysis of T lymphocytes at different activation stages has been investigated. Quantitative real-time reverse transcriptase-polymerase chain reaction was used in an experiment to determine the expression level of 18S rRNA, beta-actin mRNA, GAPDH mRNA and mRNA for six cytokines in carefully counted samples of resting human peripheral blood mononuclear cells (PBMCs), intestinal lymphocytes and PBMCs subjected to polyclonal T-cell activation. Cytokine analysis revealed that only normalization to 18S rRNA gave a result that satisfactorily reflected their mRNA expression levels per cell. In conclusion, 18S rRNA was the most stable housekeeping gene and hence superior for normalization in comparative analyses of gene expression levels in human T lymphocytes (Bas, *et al*, 2004). It has been proposed as a reliable standard for the use for relative real time PCR in gene expression. The level of ribosomal RNAs, which constitute up to 80% of the total RNA is thought to be less likely to vary under conditions that affect the expression of mRNAs and moreover, they are transcribed by a distinct RNA polymerase (Goidin, *et al*, 2001). GAPDH and b-actin are both essential for the maintenance of cell function, it is generally assumed that they are constitutively expressed at similar levels in all cell types and tissues. However, several

reports indicate that the expression of these housekeeping genes varies across tissues and cell types, during cell proliferation and development (Mansur, *et al*, 1993).

1.3. STATEMENT OF THE PROBLEM

From previous work it is evident that there is resistance to HIV-1 among commercial sex workers who are continuously exposed to HIV-1 yet they remain uninfected by the virus. This resistance correlates with the presence of HIV-1 specific cellular immune responses and mucosal immune responses, and in some cases is durable for as long as 20 years in a subgroup of women in the cohort.

However, studies have also shown that these associations do not explain the phenomenon of HIV-1 resistance entirely; and indicate that there is at least some genetic basis for the phenomenon. Thus, it is necessary to determine if genetic factors also contribute to the development of protective responses and escape of infection in HIV-1 exposed individuals.

A gene expression profile by cDNA microarray technology was previously performed and genes expressed clustered into pathways. Of these genes, those of the glycolytic pathway showed significant down-regulation.

Hence, the purpose of this study was to monitor gene expression in HIV-1 resistant commercial sex workers to identify which genes are important in susceptibility to HIV-1 through their up-regulation or down-regulation; and more specifically, to validate the preliminary gene expression data obtained after the microarray assay.

1.4. JUSTIFICATION

A detailed knowledge of the differences in gene expression in HIV-1 resistant subjects in comparison to HIV-1 positive and negative susceptible subjects will provide the basis for further understanding the molecular regulation of immunity against HIV-1 infection. The examination and characterization of the expression patterns for these genes will provide valuable information regarding the biological potential in immunologically relevant cell populations.

The search for genetic host factors that might affect the susceptibility to HIV infection was thus the basis of this study.

This study aimed to quantify gene expression in order to investigate the role of these genes involved in the glycolysis, pentose-phosphate and insulin signaling pathways in resistance to HIV-1 infection; as they had showed differential expression in a preliminary microarray study; thereby making it possible to make connections between genes not previously associated and resistance to HIV-1 at the functional level on the basis of temporal expression profiles.

1.5. HYPOTHESES

1.5.1. Null hypothesis

The expression of selected genes in HIV-1 resistant commercial sex workers does not differ from that of HIV-1 susceptible and HIV-1 negative women in the Pumwani Sex Worker cohort.

1.5.2. Alternative hypothesis

The expression of selected genes in HIV-1 resistant commercial sex workers differs from that of HIV-1 susceptible and HIV-1 negative women in the Pumwani Sex Worker cohort.

1.6. OBJECTIVES

1.6.1. General objective:

To determine the differences in expression levels of selected genes suggested to be alternately regulated in HIV-1 resistant commercial sex workers through gene expression profiling.

1.6.2. Specific objectives:

1. To determine the expression levels of 9 selected genes by quantitative real-time PCR.
2. To identify up-regulated and down-regulated genes in HIV-resistant women, uninfected HIV-1 susceptible women and HIV-1 infected women.

CHAPTER 2
METHODOLOGY

2.1. Study population

The subjects are drawn from a commercial sex worker cohort based in the Majengo slum, Nairobi and managed by the University of Nairobi/University of Manitoba collaborative group. The cohort has been studied for well over two decades by the group and is well described (Fowke *et al*, 1996).

The 56 study subjects randomly selected from this population were classified as follows:

1. 14 highly HIV-exposed but uninfected (HIV resistant) commercial sex workers in the cohort >7yrs.
2. 14 newly enrolled HIV uninfected, and therefore susceptible to HIV (New Negative), <3yrs commercial sex workers.
3. 14 HIV infected (Positive) commercial sex workers.
4. 14 non sex worker HIV negative antenatal clinic attendees (MCH) as the control. The MCH cohort members are not commercial sex workers and are considered to be at low risk for contracting HIV (Embree, *et al*, 2000).

During every resurvey, all patients were routinely tested for HIV sero-status using ELISA (Recombigen, Trinity, Carlifonia, USA). Those testing positive are confirmed by a separate immunoassay (Detect HIV 1/2, Montreal, Canada). Only those testing positive for both arrays are considered positive.

2.1.1. Inclusion Criteria

1. The women included in the study were18years and above.

2. The study subject had volunteered out of their own will.
3. The study only included women in the cohort or who had been in the cohort for at least 2 months.
4. Those derived from the commercial sex worker cohort were able to attend the clinic at Majengo every 6 months for sample collection and follow up.

2.1.2. Exclusive criteria

1. Subjects who had not attended clinic at Majengo for the current resurvey (May-August 2007) were not sampled.
2. No sampling was done on women who were not part of the Majengo Sex-worker cohort, Pumwani or the MCH control group.

2.2. Sample Acquistion

Peripheral blood was collected from the Majengo clinic by qualified personnel by collecting peripheral venous blood in PAXgene Blood RNA tubes (Preanalytix, GMBH) containing a proprietary reagent composition based on a patented RNA stabilization technology that protects the RNA from degradation by RNases and minimizes induction of gene expression.

2.3. Ethical Considerations

Informed consent for demographic and behavioural surveys, as well as biological sampling had previously been obtained from all study participants. The study was approved by the Institutional Review Boards of The University of Nairobi through Kenyatta National Hospital and The University of Manitoba Ethical Review boards. All

clinical investigations were conducted according to the Principles of Helsinki Declaration. The consent forms are attached as Appendix A.

2.4. Experimental procedures

2.4.1. RNA extraction

Cellular RNA was isolated from whole blood with PAXgene Blood RNA Kit (Qiagen, USA) from 2.5 ml samples of human whole blood, collected in PAXgene Blood RNA Tubes (Preanalytix, GMBH).

Protocol

Materials and reagents

1. PAXgene blood RNA tubes
2. 10ul-4ml pipettes
3. Buffer BR1
4. Buffer BR2
5. Buffer BR3
6. Buffer BR4
7. Buffer BR5
8. Graduated cylinder
9. Ethanol (96–100%)
10. 1.5 ml microcentrifuge tubes
11. Sterile, aerosol-barrier, RNase-free pipette tips
12. Microcentrifuge (with rotor for 1.5 ml and 2 ml tubes)
12. Shaker-incubator

13. Vortex mixer

14. Disposable gloves

15. RNase-free water

Procedure:

1. Centrifugation of the PAXgene Blood RNA Tubes was done for 10 min at 3750rpm using a swing-out rotor in an IEC CL40R centrifuge..

2. The supernatant was removed; 4 ml RNase-free water was added and the tubes closed using a fresh secondary Hemogard closure.

3. The pellet was thoroughly resuspended by vortexing, and repelleted by centrifugation done for 10 min at 3750rpm. The entire supernatant was removed and discarded.

4. The pellet was thoroughly resuspended in 350 µl Buffer BR1 and mixed by vortexing.

5. The samples were transferred into 1.5ml microcentrifuge tubes and 300 µl Buffer BR2 and 40 µl Proteinase K added. Mixing was done by vortexing for 5 seconds, and incubated for 10 min at 55°C in a shaker-incubator at 400-1400rpm.

6. After mixing, the samples were centrifuged for 3 min at maximum speed in a microcentrifuge. The supernatant was transferred to a fresh tube.

7. 350 µl of 96-100% ethanol was added followed by vortexing, and brief centrifugation (1–2 seconds; ≤ 1000 x g) to remove drops from the inside of the tube lid.

8. 700µl sample was transferred to a PAXgene RNA spin column placed in a 2 ml processing tube, and centrifuged for 1 min at 14,000 rpm. The PAXgene column was placed in a new 2 ml processing tube and the old processing tube containing flow-through discarded.

9. The remaining sample was transferred to a PAXgene column, and centrifuged for 1 min at 14,000rpm. The PAXgene column was placed in a new 2 ml processing tube, the old processing tube containing flow-through discarded.

10. 700 µl Buffer BR3 was transferred into a PAXgene column, and centrifuged for 1 min at 14000rpm. The PAXgene column was placed in a new 2 ml processing tube, the old processing tube containing flow-through discarded.

11. 500 µl Buffer BR4 was added to the PAXgene column, and centrifuged for 1 min at 14,000rpm. The PAXgene column was then placed in a new 2 ml processing tube, the old processing tube containing flow-through discarded.

12. Another 500 µl Buffer BR4 was added to the PAXgene column and centrifuged for 3 min at maximum speed to dry the PAXgene column membrane.

13. To elute, the tube containing the flow-through was discarded; the PAXgene column was transferred to a new 2ml processing tube, and 40 µl Buffer BR5 was added directly to the PAXgene column membrane. This was centrifuged for 1 minute at 14000rpm. The whole membrane was wet with Buffer BR5 in order to achieve maximum elution efficiency.

14. The elution step (step 13) was repeated as described, using 40 µl Buffer BR5.

15. The eluate was incubated for 5 min at 65°C in a shaker incubator. Following incubation, the sample was chilled immediately on ice and the RNA sample stored at –70°C.

2.4.2. RNA quantitation

Quantification and quality analysis of the isolated RNA samples prior to reverse transcription was performed using the Agilent RNA 6000 Nano Kit (Agilent, Ontario, Canada).

1. A gel was prepared for the qauntitation using the Agilent RNA 6000 Nano gel matrix whereby 250µl was placed in a spin filter in a microcentrifuge and spun for 10 min at 4000rpm.
2. The RNA 6000 NAno dye concentrate was vortexed for 10sec and spun down.
3. 1 µl of RNA 6000 Nano dye concentrate was added to a 65µl aliquot of filtered gel.
4. The tube was capped, vortexed thoroughly and visually inspected for proper mixing of gel and dye. The dye concentrate was stored in the dark again.
5. The tube was spun for 10min at room temperature at 14000 rpm. The prepared gel-dye mix was used.
6. To load the gel-dye mix, it was first allowed to equilibrate to room temperature for 30min and protected from light.
7. 9 µl of the gel-dye mix was dispensed into a new RNA Nano chip into each well. Care was taken not to draw up particles that may sit at the bottom of the mix vial and bubble formation that lead to poor results.
8. Ladder aliquots and samples were heat denatured at 70 °C for 2 min to minimize secondary structure. 1 µl of samples and RNA ladder were added into respective wells and the chip was vortexed for 60sec at 2400 rpm.
9. Quantification was then performed in the Agilent 2100 bioanalyzer.

2.4.3. cDNA synthesis (reverse transcription of RNA)

Materials and reagents

QuantiTect Reverse Transcription Kit (Qiagen, Ontario, Canada)

Template RNA

RNase-free water

Reverse Transcription step:

Each RNA sample was standardized to 100ng. Standards for the standard curve were also prepared, standardized and reverse-transcribed in the same manner.

For generation of cDNA from total RNA for real time PCR, the Quantitect Reverse Transcription Kit was used (Qiagen, Ontario, Canada).

1. Genomic DNA elimination was done by mixing the template RNA with 2 µl of gDNA Wipeout Buffer and RNase-free water.

2. This was incubated for 2min at 42°C then placed immediately on ice.

3. The reverse-transcription master mix was prepared by mixing 1 µl Quantiscript Reverse-transcriptase, 4 µl Quantiscript RT Buffer, 1ul RT Primer mix and 1µg template RNA.

4. Template RNA was added to each tube containing the reverse-transcription master mix, mixed and stored on ice.

5. This was then be incubated for 15min at 42°C.

6. Incubation was thereafter done for 3min at 95°C to inactivate the Quantiscript reverse transcriptase.

7. An aliquot for each finished reverse transcription reaction was added to real-time PCR master mix.

2.4.4. Standards:

For the external standards, a control sample was taken and the PBMCs were isolated from the whole blood. The cells were then stimulated with Phytohaemagglutinin (PHA) for the upregulation of genes for 16 hours at 5 ug/ml PHA.

RNA was then isolated using the RNeasy Kit (Qiagen, Ontario, Canada).

Dilutions of the RNA were made 1:10, 1:100, 1:1000 and Undiluted and cDNA prepared for each standard as the rest of the RNA samples. The cDNA obtained was stored at -20°C until use.

2.4.5. Real-time PCR.

Real time PCR was used for the confirmation of gene expression differences between populations. The genes under study are Cyclin-dependent kinase inhibitor 2B; Dipeptidyl peptidase IV; Gastric inhibitory polypeptide receptor; Glucose-6-phosphate dehydrogenase; Insulin receptor substrate 1; Phosphatidyl inositol 3-kinase, catalytic, Gamma; Phosphoglucomutase 1; Pyruvate kinase muscle 2 and 18S r RNA as the endogenoue control. There was generation of standard curves for each gene by amplifying a 10-fold dilution series of total RNA extracted from PHA-stimulated PBMCs pool. All standard curve samples and test samples were run in duplicates. Each real time reaction had a no template control to ensure non-contamination of samples.

Materials and reagents

2x QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Ontario, Canada)

10x QuantiTect Primer Assay (Qiagen, Ontario, Canada)

Template cDNA

Lightcycler™ (Roche Diagnostics, Mannheim, Germany).

Amplification of cDNA and detection of products were accomplished using the Quantitect SYBR Green PCR Kit (Qiagen, Ontario, Canada). Gene specific primers were also used i.e. Quantitect Primer Assay (Qiagen, Ontario, Canada).

Table 1:Primers

GENE DESCRIPTION	GENE SYMBOL	ENTREZ ID
Cyclin-dependent kinase inhibitor 2B	CDKN2B	1030
Dipeptidyl peptidase 4	DPP4	1803
Gastric inhibitory polypeptide receptor	GIPR	2696
Glucose-6-phospahte dehaydrogenase	G6PD	2539
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2597
Insulin receptor substrate 1	IRS1	3667
Phosphatidyl inositol 3-kinase, catalytic, Gamma	PIK3CG	5294
Phosphoglucomutase 1	PGM1	5236
Pyruvate kinase muscle 2	PKM2	
18S rRNA	18S rRNA	

Procedure

1. 2x QuantiTect SYBR Green RT-PCR Master Mix, 10x QuantiTect Primer Assay, (Qiagen, Ontario, Canada) template cDNA, and RNase-free water were thawed. The individual solutions were mixed and placed on ice.

2. A reaction mix was prepared as follows while the samples were still being kept on ice: 25 µl of 2x QuantiTect SYBR Green RT-PCR Master Mix*; 5 µl of 10x QuantiTect Primer Assay; heat-labile Uracil-N-glycosylase (optional) and RNase-free water were mixed thoroughly and dispensed appropriately into the LightCycler capillaries (Roche,GmbH)

4. Template cDNA (¡Ü10 ng/reaction) was added to the individual capillaries containing the reaction mix. For the optional UNG treatment, the samples will be were left for at least 10 min on ice.

5. The LightCycler real time thermocycler (Roche, GmbH) was programmed with PCR initial activation step at 95°C for 15min and 3-step cycling with denaturation for 15 sec at 94°C, annealing for 30 sec at 55°C and extension for 30 sec at 72°C .

Data acquisition was performed during the extension step.

50 cycles were run. Melting curve analysis of the RT-PCR product(s) was performed to verify their specificity and identity.

Table 2: Thermocycling conditions used for qRT-PCR of all genes and samples.

Stage	Temp (°C)	Time	Temp. Transition rate	Detection
Denaturation and "hot start"	95°C	15 min	20°C/sec	None
PCR amplification	94°C	15 sec	20°C/sec	None
Annealing	55°C	20 sec	20°C/sec	None
Product elongation	72°C	20 sec	20°C/sec	Single
x 50 cycles				
Melting	95°C	0-sec hold	20°C/sec	None
	60°C	15 sec	20°C/sec	None
		0-15 sec		
	95°C	hold	0.1°C/sec	Continous

2.4.6. Data management and analysis

Data was recorded in excel spreadsheets and stored in a computer's hard drive, portable disks (flash disk, CD-ROM) plus a hard copy kept as back up.

Relative qauntitation was performed with external standard curves for each primer. Quantification of transcript levels was performed using Light Cycler Data Analysis (LCDA) (Roche, GmbH) using the calibrator normalized relative quantification with efficiency method. The standard curves for each target gene and 18S rRNA were

generated. The standard curves were then exported into RelQuant (Roche, Quebec) from LCDA. The standard curves from each gene were then compared to the standard curve of the control gene, 18S rRNA. This was to correct for any template differences on an individual gene basis, hence a normalized ratio of target gene to reference gene for each sample. These normalized corrected ratios then underwent further analysis.

Statistical analysis was accomplished by GraphPad Prism 4 Software Version (GraphPad Software, San Diego, USA). For the comparisons, differential expression of genes between groups was evaluated by both fold change and P value. Calculation of P values for each individual gene compared between groups was accomplished by the Students T Tests (non-parametric). P values below 0.05 were considered to show a statistically significant difference.

CHAPTER 3

RESULTS

The gene expression differences between the study groups were expressed by both fold change and P value. The fold changes were determined by dividing the mean expression value for a given gene within the test sample groups (HIV-1 Resistants, New Negatives and Positives) by the mean value of the control condition (MCH).

3.1. Quantification of mRNA levels of HIV-1 resistant women, HIV-negative and HIV positive women by real time PCR.

The differential mRNA expression is shown, as determined by quantitative real time PCR. mRNA expression of some of the genes was clearly different among the three groups. The p values were obtained after comparison with the non sex worker HIV negative antenatal clinic attendees (MCH) as the control. MCH group was used as a baseline to determine levels of expression under 'normal' uninfected state.

Glucose-6-phosphate dehydrogenase (G6PD) showed significantly lower expression in HIV-1 resistant women ($p=0.0298$) as compared to the uninfected HIV-1 susceptible ($p=0.5226$) and HIV-1 positive ($p=0.9533$) women.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also showed significantly lower expression in HIV-1 resistant women ($p=0.0131$) as compared to the uninfected HIV-1 susceptible ($p=0.347$) and HIV-1 positive ($p=0.5824$) women.

The rest of the genes did not show any significant expression changes. However, Gastric Inhibitory Polypeptide Receptor (GIPR), Pyruvate Kinase Muscle 2 (PKM2) and Cycline Dependent Kinase Inhibitor 2B (CDKN2B) showed signs of following the same trend although there was no statistical significance.

Table 3: FOLD CHANGES & FOLD DECREASES

Relative expression differences in terms of fold changes and fold decreases.ss

GENE/GRP	RESISTANTS		NEW NEGATIVES		POSITIVES	
	FOLD CHANGE	FOLD DEC	FOLD CHANGE	FOLD DEC	FOLD CHANGE	FOLD DEC
G6PD	0.2557	3.88	1.4157		0.9783	1.022
GAPDH	0.2993	3.341	1.4357		1.4428	
GIPR	0.4226	2.366	2.2059		0.9527	1.05
CDK2NB	0.4412	2.267	1.2703		0.3061	3.267
IRS1	0.8958	1.116	1.6604		0.5923	1.688
PGM1	0.5256	1.9	0.6162	1.623	0.4026	2.484
PIK3CG	0.6534	1.53	0.5464	1.83	0.4506	2.219
PKM2	0.3942	2.537	0.9244	1.082	0.4738	2.111
DPP4	0.4249	2.353	2.2059		0.1802	5.55

Table 3 shows the relative expression in terms of fold changes/ fold decreases. For fold changes of less than 1, the fold decreases were calculated.

G6PD had a fold change of 0.2557, and a fold decrease of 3.88. That means that G6PD was 3.88 times down-regulated in resistant women. The gene was expressed the same in the HIV-1 susceptible uninfected (1.4157 fold change) and the HIV-1 positive (0.9783 fold change) women.

GAPDH had a fold change of 0.2993 and had 3.341 fold down-regulation in HIV-1 resistant women.

GIPR and CDKN2B also showed double fold down-regulation (2.366 and 2.267 fold down-regulation). IRS1 showed 1.116 fold down regulation.

In the uninfected HIV-1 susceptible women, termed as "New Negatives", the genes GAPDH (p=1.4357), GIPR (p=2.2059), CDKN2B (p=0.1.2703) and IRS1 (p=1.6604) were slightly up-regulated.

Only GAPDH showed up-regulation in HIV-1 positive women (p=1.4428).

The genes PGM1, PIK3CG and PKM2 showed down-regulation in all the study groups.

DPP4 was down-regulated 2.353 times in HIV-resistant women. It was however up-regulated 2.2059 times in New Negatives and down regulated 5.5fold in HIV-positive women.

The following are graphs showing the expression of each of the 9 genes:

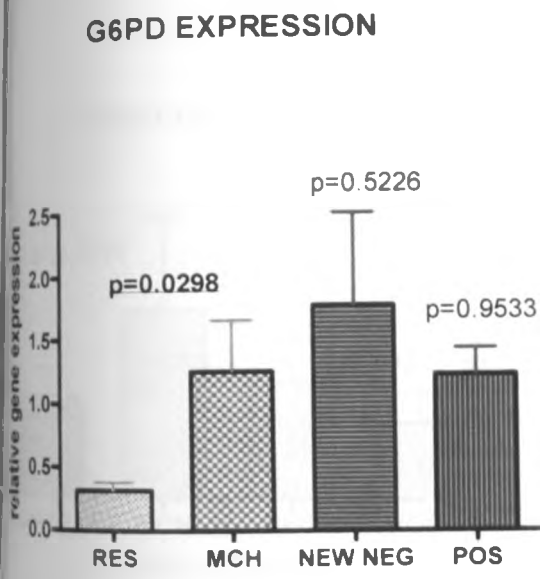


Figure 6:G6PD expression

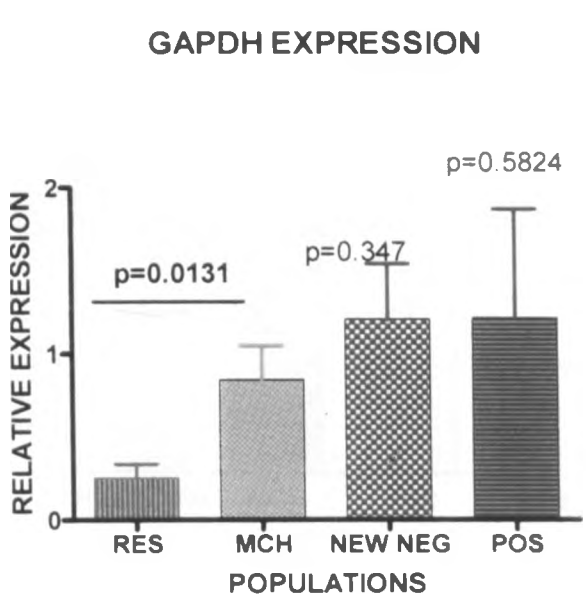


Figure 7:GAPDH expression

Both G6PD and GAPDH show lower significant expression levels as compared to the control, MCH and the other groups New Negatives and Positives.

Figure 8: GIPR expression

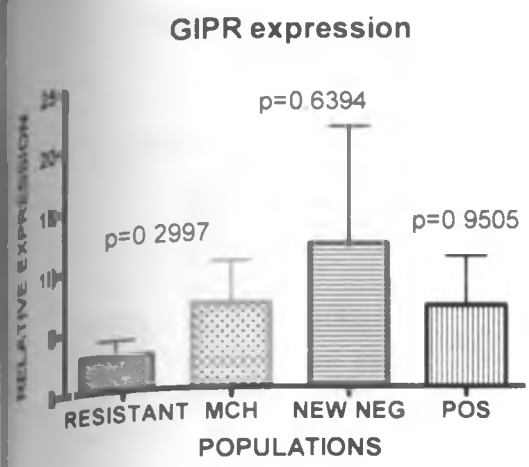
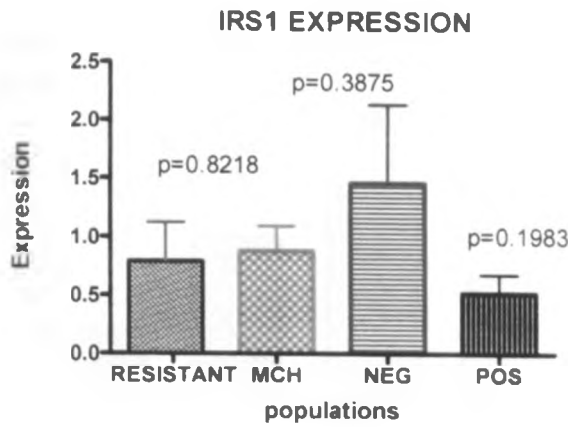


Figure 9: IRS1 expression



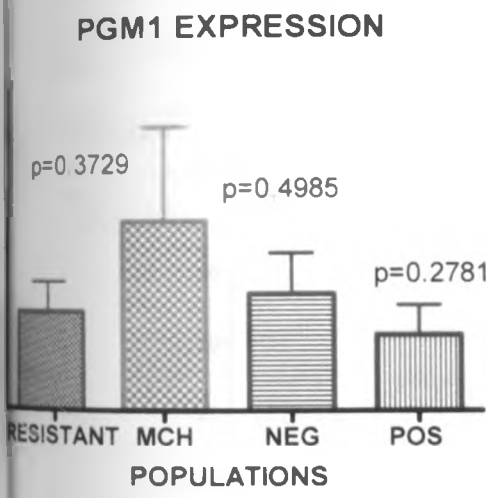


Figure 10: PGM1 expression

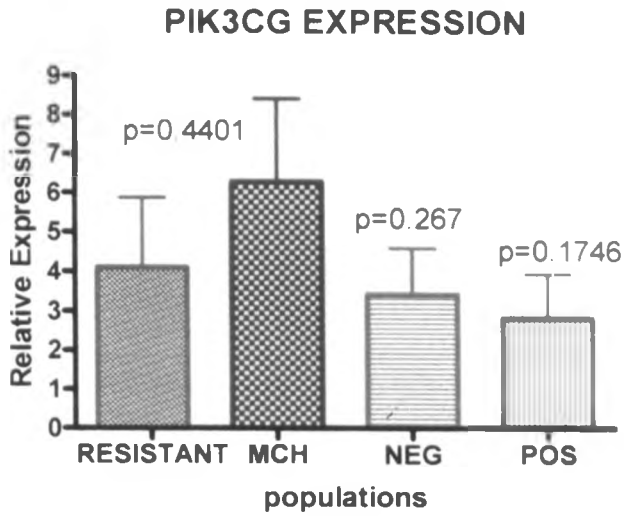
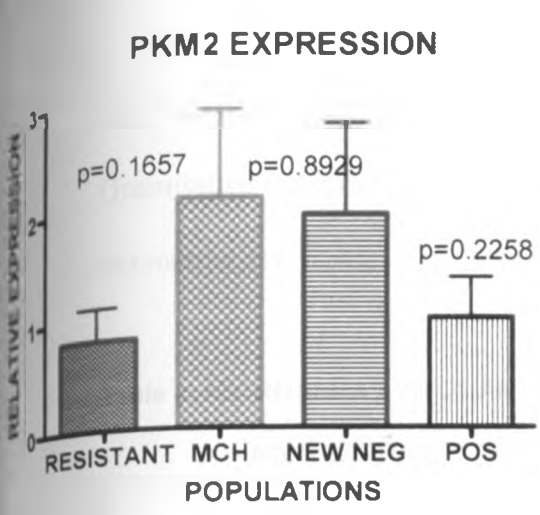


Figure 11: PIK3CG expression

Both PGM1 (p=0.3729) and PIK3CG (p=0.4401) genes were not significantly down regulated in HIV-1 resistant women.



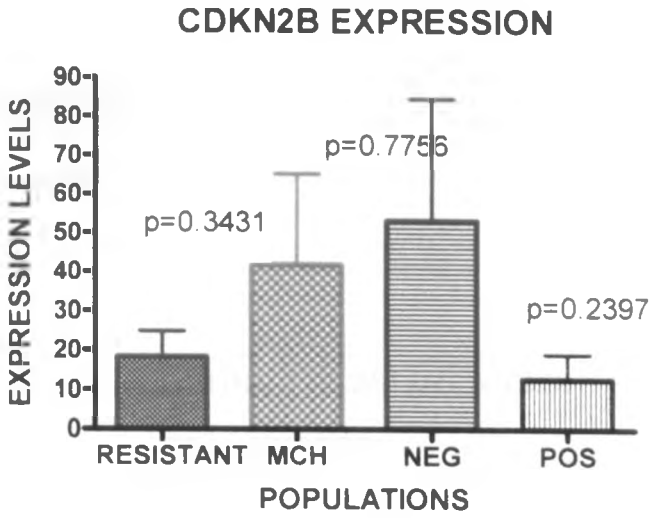


Figure 12: PKM2 expression

Figure 13: CDK2NB expression

The same observation was made for the PKM2 (p=0.1657) and CDK2NB (p=0.3431) genes which were down-regulates in HIV-1 resistant women but this was not statistically significant.

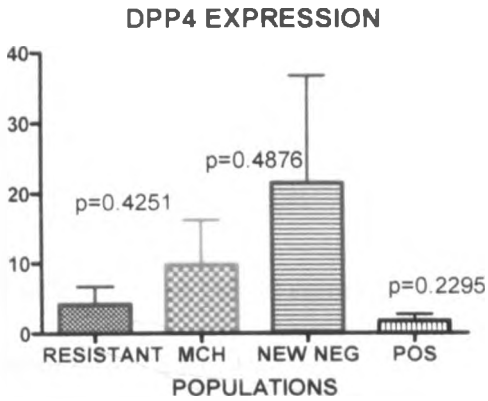


Figure 14: DPP4 expression

DPP4 gene was also down-regulated but not significantly in HIV-1 resistant women. The gene was also down-regulated in HIV-1 infected women.

3.2. Comparison of Microarray data vs Quantitative Real Time PCR

Quantitative Real time PCR was conducted to validate the results found by a preliminary microarray assay.

Table 4: MICROARRAY VS QUANTITATIVE REAL TIME PCR (HIV-1 RESISTANT WOMEN

	MICROARRAY			REAL TIME PCR		
GENE	FOLD CHANGE	FOLD DEC	P-VALUE	FOLD CHANGE	FOLD DEC	P-VALUE
G6PD	0.2557	3.91	0.0294	0.2557	3.88	0.0298
GAPDH	0.3048	3.28	0.0036	0.2993	3.341	0.0131
GIPR	0.1937	5.162	0.001	0.4226	2.366	0.2997
CDK2NB	0.659	1.52	0.2689	0.4412	2.267	0.3431
IRS1	0.608	1.645	0.0424	0.8958	1.116	0.8218
PGM1	0.3892	2.569	0.0006	0.5256	1.9	0.3729
PIK3CG	0.806	1.241	0.5092	0.6534	1.53	0.4401
PKM2	0.876	1.142	0.6338	0.3942	2.537	0.1657
DPP4	2.085		0.0082	0.4249	2.353	0.4251

Table 5 shows the comparison of results from a microarray assay.

The RT-qPCR data of G6PD validates the microarray data showing that G6PD is down-regulated in HIV-1 resistant women. (Microarray p=0.0294, 3.91 fold down-regulation; RT qPCR p=0.0298, 3.88 fold down-regulation).

GAPDH was also validated as down-regulated in HIV-1 resistant commercial sex workers. (Microarray p= 0.0036, 3.28 fold down-regulation; RT-qPCR p=0.0131; 3.341 fold down-regulation).

GIPR. IRS1 and PGM1 genes showed some down-regulation in the microarray. GIPR p=0.001. 5.162 fold down-regulation; IRS1 p=0.0424. 1.645 fold down-regulation and PGM1 p=0.0006. 2.569 fold down-regulation. However, this was not validated by RT qPCR as the differences were not significant. (GIPR p=0.2997. IRS1 p=0.8218 and PGM1 p=0.3729).

CDKN2B. PIK3CG and PKM2 showed no significant differences in both microarray assay and quantitative real time PCR. These genes were all expressed at a normal level.

DPP4 expression in q RT-PCR showed the opposite trend from that seen in the microarray assay. It showed up-regulation in the microarray assay (p=0.0082. 2.085 fold change) whereas in the q RT-PCR assay DPP4 was down-regulated (p=0.4251. 2.353 fold down-regulation).

The following graphs show a pictorial comparison of the Microarray assay compared to the q RT-PCR:

(i) (ii)

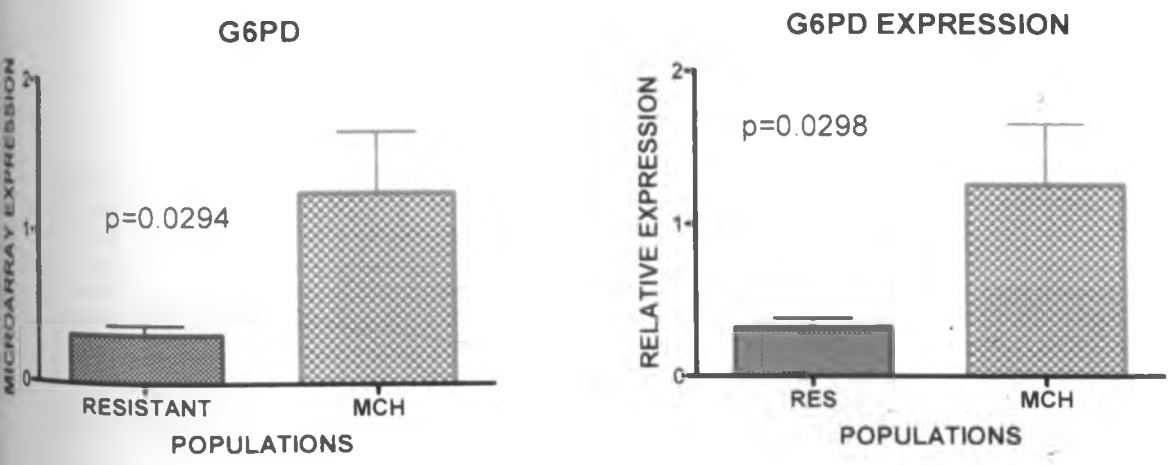
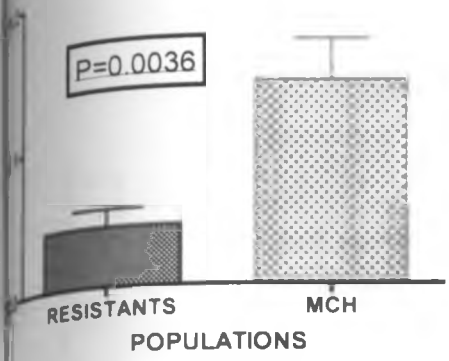


Figure 15 (i) and (ii) shows a comparison of G6PD expression between microarray assay and qRT-PCR

(i)



(ii)

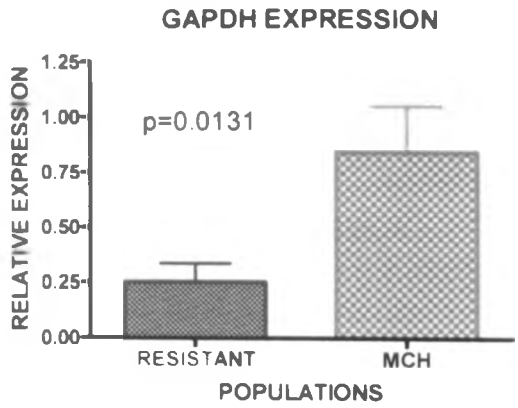
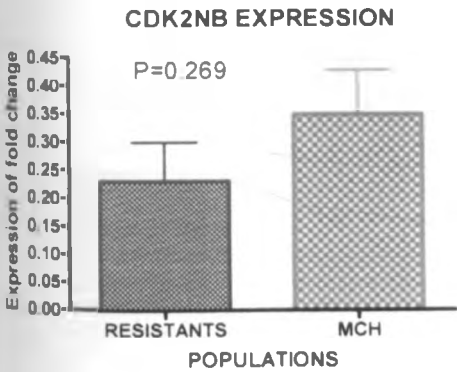


Figure 16 (i) and (ii) shows a microarray and qRT-PCR comparison of GAPDH expression

Both G6PD and GAPDH followed the same trend of significant down-regulation (lower levels of both genes) in HIV-1 resistant women in both the microarray assay and q RT-PCR.

(i)



(ii)

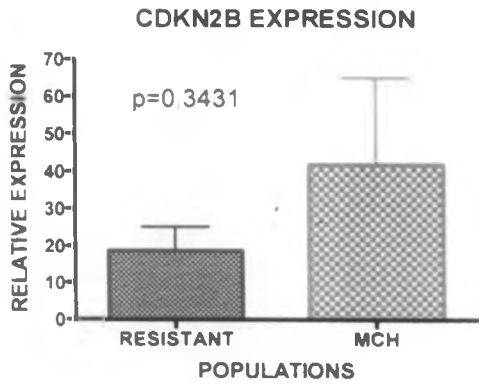


Figure 17 compares CDNK2B microarray and qRT-PCR expression

CDKN2B showed no significant difference in the expression levels above.

(i) (ii)

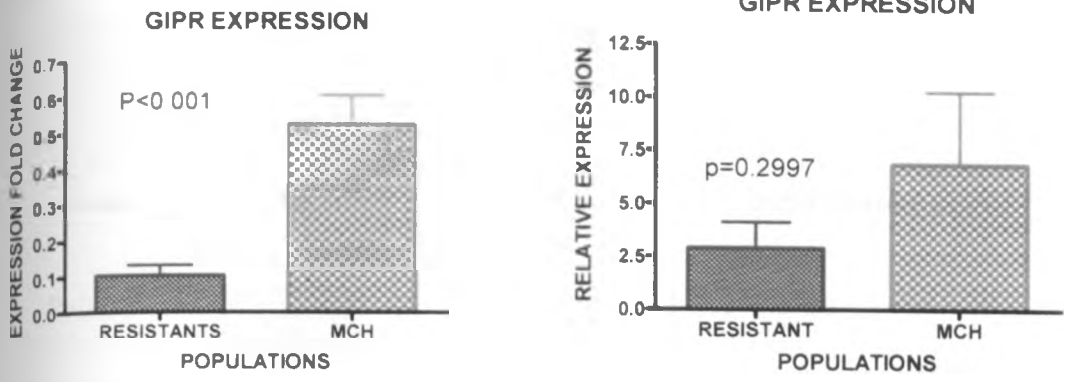


Figure 18 (i) and (ii) shows a comparison between GIPR microarray and qRT-PCR expression.

GIPR showed significant down-regulation in HIV-1 resistant women after the microarray assay ($p<0.001$). However, only a slight down-regulation was noted in the HIV-1 resistant women although it was not significant statistically.

(i) (ii)

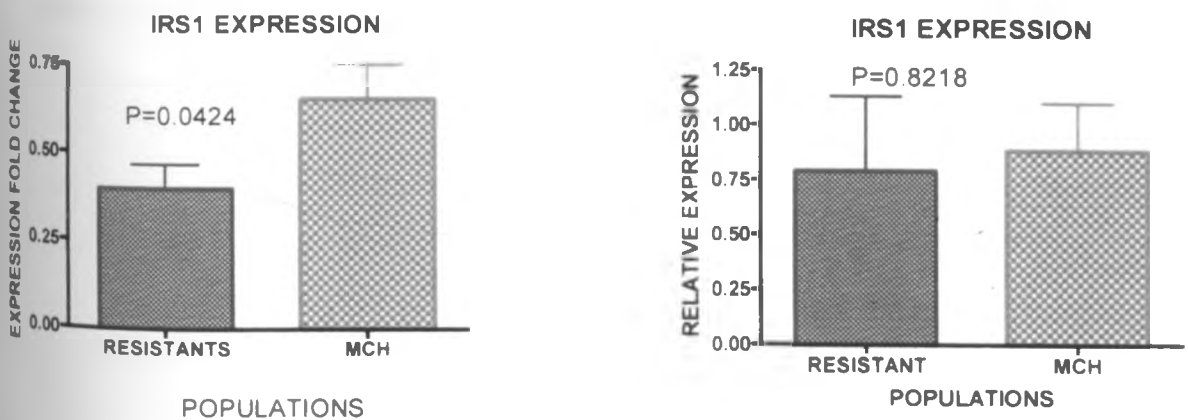


Figure 19 (i) and (ii) shows a comparison between IRS1 microarray and qRT_PCR expression

IRS1 gene showed significant under-expression in HIV-1 resistant women in the microarray assay. However this under-expression was not confirmed by the qRT-PCR as it showed no significant difference in the expression.

(i) (ii)

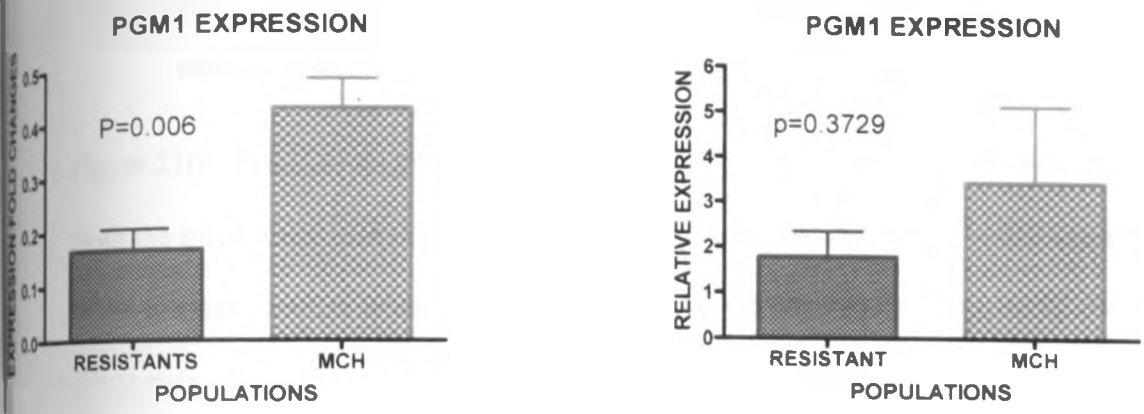
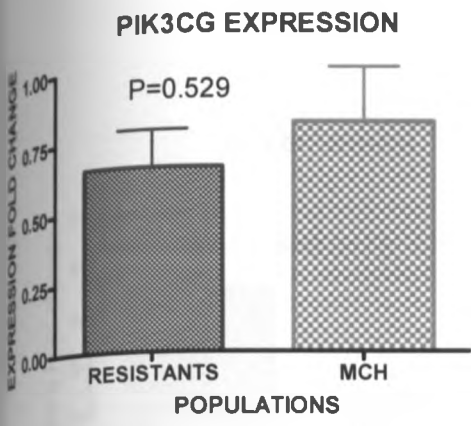


Figure 20 (i): PGM1 microarray expression (ii): PGM1 qRT-PCR expression

The same trend was seen in PGM1 whereby the microarray data showed significant overexpression while qRT-PCR did not.

(i)



(ii)

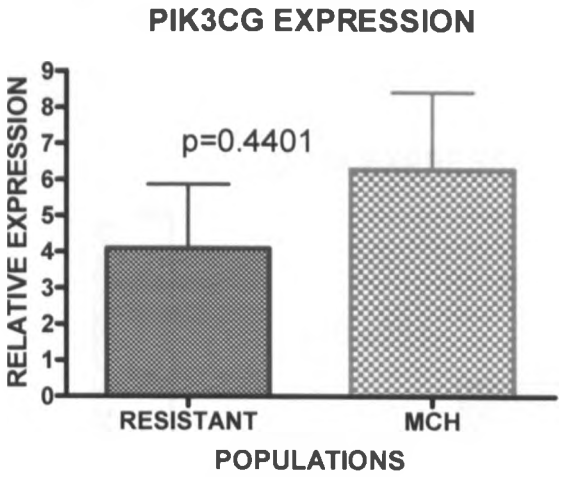
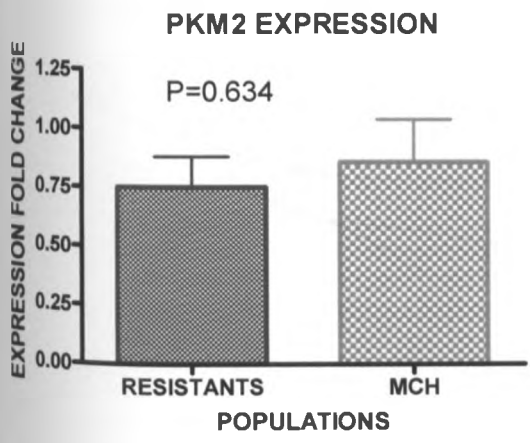


Figure 21(i): PIK3CG microarray expression (ii): PIK3CG qRT-PCR expression

Both the microarray assay and qRT-PCR did not show any significant under-expression in the resistant women when compared with the low exposure non-commercial sex workers (control) in both PIK3CG and PKM2 genes (shown below).

(i)



(ii)

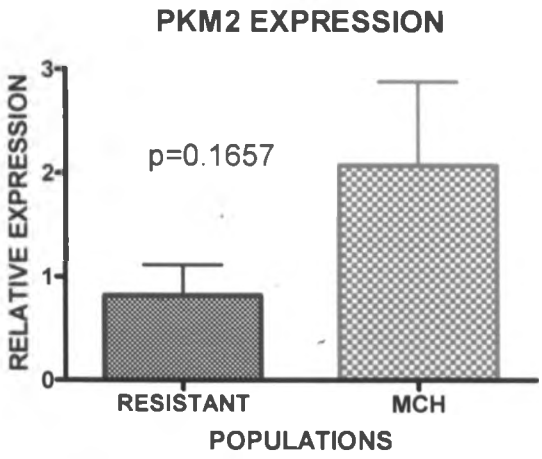


Figure 22 (i) and (ii) show microarray and qRT-PCR expression of PKM2.

(i) (ii)

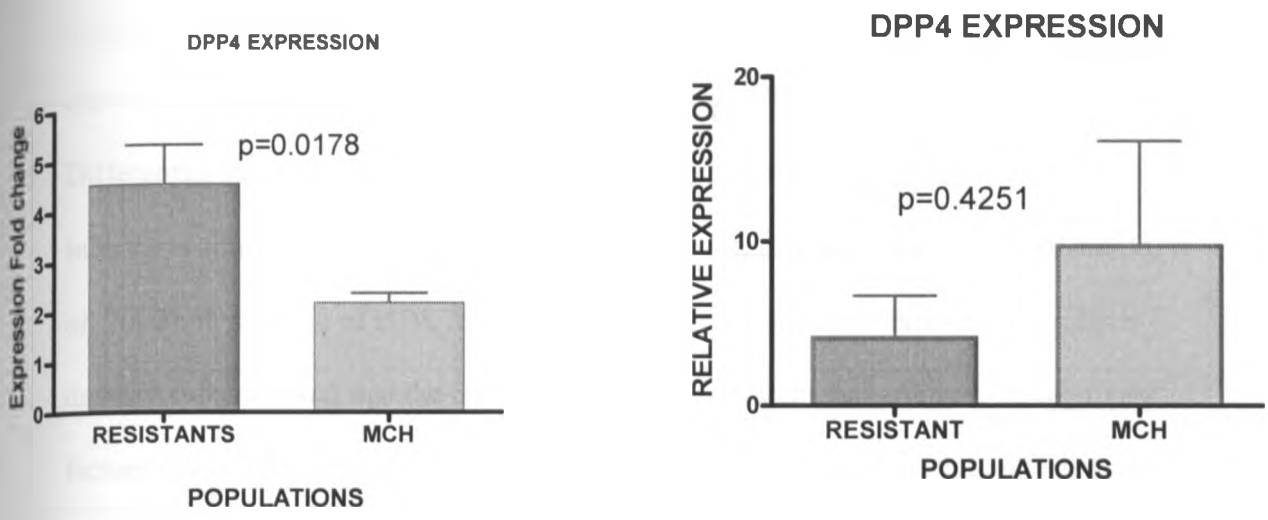


Figure 23 shows microarray (i) and qRT-PCR (ii) expression of DPP4.

DPP4 showed considerable up-regulation in the microarray assay (p=0.0082) whereas it showed a low level of down regulation (p=0.4251) in the q RT-PCR.

CHAPTER 4

DISCUSSION

Host genetic factors have a major impact on the pathology of infectious diseases in humans. These genetic differences between populations studied could be a determinant of the variability in the results obtained (McIlroy, *et al*, 2006).

Differential susceptibility to infection and disease progression is observed with almost all infections although often the reasons for the heterogeneity are unknown (MacDonald, *et al*, 2000). In the case of HIV, it has been recognized that not all exposed individuals develop infection and that the course of infection is extremely variable. Among many factors responsible are viral characteristics, infectious co-factors, individual behaviors, and in the case of this study, host genetics (Royce, *et al*, 1997). Evidence is increasing for multiple mechanisms of resistance and susceptibility.

Prior to this study, microarray technology was used as a screening tool. These microarray results needed to be validated by real time PCR. A real time LightCycler assay was used as the validation procedure as it can quantify relative change in expression of a large number of genes with limited RNA rapidly and precisely (Mangalathu, *et al*, 2001). Genes with less than four-fold difference in expression can be validated by the LightCycler. In fact, the largest groups of differentially expressed genes in many studies using DNA arrays are those with two-to four fold differences in expression (Mangalathu, *et al*, 2001).

In this study, the gene expression differences between the study groups: HIV-1 Resistant CSW, Uninfected HIV-1 susceptible CSW (New Negatives) and HIV-1 infected CSW (HIV-1 positives) respectively and the control group of low susceptible-non-CSW HIV-1

negative antenatal clinic attendees (MCH) were studied. The genes studied were key genes of the glycolytic/pentose phosphate/insulin signaling pathways. The novel selection of these genes was due to their differential expression in the preliminary microarray experiment. In addition, the novelty of these findings, as discussed, these genes have not been previously associated with HIV.

The results show that there was differential expression of genes after quantification by real time PCR. mRNA expression of Glucose-6-Phosphate Dehydrogenase (G6PD) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) had significantly lower expressions in HIV-1 resistant commercial sex workers as compared with the expression levels HIV-1 susceptible and HIV-1 positive commercial sex workers. This can be seen clearly in figures 7 and 8.

G6PD enzyme catalyses the first and critical step in the pentose phosphate pathway during glucose metabolism. It is also the rate-limiting enzyme of this pathway (Beutler, 1994). Its over-expression provokes dysregulation of lipid metabolism and adipocytokine expression, causing insulin resistance and oxidative stress (Park, *et al*, 2005; Park, *et al*, 2006). However, as shown by this study, G6PD was significantly under-expressed or down-regulated in HIV-1 resistant women. (Tables 2 and 3, Figure 7) This down-regulation suggests decreased concentration of G6PD enzyme, which has been shown to have a protective role against infection with *P. falciparum* malaria. In fact, G6PD is one of the best examples of genes that have a role in resistance to infectious diseases whose lack/deficiency causes resistance to malaria (Tishkoff, *et al*, 2001; Saunders, *et al*, 2002). Both malaria and HIV are concentrated to an extent in the same geographical regions, Sub-Saharan Africa (Storey, 1998; W.H.O., 2000). Although the significance of this

observation is not yet known, it could partially account for resistance to HIV-1 infection and thus the down-regulation of this gene in the context of resistance/susceptibility to HIV-1 infection is worth further investigation. However, studies have shown that oxidative stress increases susceptibility of G6PD-deficient cells to viral infection as demonstrated also suggests that antioxidant treatment may protect G6PD deficient subjects from viral infection (Wu, *et al*, 2008).

GAPDH gene also showed similar significant down-regulation in HIV-1 resistant CSWs, as compared to its levels in HIV-1 infected and uninfected HIV-1 susceptible CSWs, which were up-regulated although not significantly. (Tables 2 and 3; Figure 8) GAPDH is a multifunctional protein and plays many and diverse roles (Sirover, 2005; Zhou, *et al*, 2008). GAPDH has recently been shown to have the capacity to regulate gene expression at the level of mRNA stability. (Zhou, *et al*, 2008) This might suggest that the low expression of GAPDH in HIV-1 resistant CSWs may subsequently affect the expression of other genes involved in the cellular pathways under study. The level of activity of the enzyme in the resistant women should be examined.

The preliminary analysis by cDNA microarrays identified the 9 genes as differentially expressed functioning in the glycolytic pathway (Songok E.M, in press). These genes have been reported to function as a variety of cellular and non cellular processes. These genes were presumed to play a role in host cell defense mechanisms as they were down-regulated significantly in HIV-1 resistant CSWs as compared to non-CSW antenatal clinic attendees (MCH). In general, the direction of the differential gene expression obtained by cDNA analysis and the qRT-PCR was similar for 5 of the genes studied. The decrease in G6PD and GAPDH expression demonstrated by the RT-PCR assay was

consistent with that observed in the microarray experiment for both genes as shown in Table 5 and Figures 16 and 17.

An interesting observation of this study was that although the other genes studied showed no significant down-regulation, the genes were still expressed at lower levels in the HIV-1 resistant CSWs than in the Uninfected HIV-1 susceptible and HIV-1 infected subjects. The genes CDKN2B, PIK3CG and PKM2 were also down-regulated but not to a statistically significant extent in both arrays. They showed the same trend although not as significant as GAPDH and G6PD. Probably, a larger sample size would show significance.

For genes shown as differentially expressed by microarray assay, the levels of gene expression could be quite different in Rt-PCR. Although the genes IRS1, GIPR and PGM1, were shown to be significantly down-regulated in microarray data, qRT-PCR showed that this down-regulation was not significant. These transcripts thus showed smaller changes by RT-PCR. This difference may be due to limitations inherent in the study of a large number of genes by microarray technology hence the need for validation by RT-PCR (Der, *et al*, 1998). Interestingly, IRS1 was not significantly down-regulated as opposed to PIK3CG which IRS1 plays a role in its activation (Taniguchi, *et al*, 2005). It may be of interest to examine the expression of other genes which take part in PIK3CG activation and maintenance of its full signal. Moreover, a reduction of IRS1 expression results in a significant increase in the mRNA abundance of essential gluconeogenic enzymes and may also be worth further investigation.

DPP IV expression showed significant over-expression in the microarray assay (Table 5, Figure 24) as opposed to the insignificant down-regulation shown by qRT-PCR. The reasons behind this difference are still unclear and this warrants further investigation. Hence, these genes, especially GAPDH and G6PD, have been revealed as candidates that might help explain the resistance to HIV-1 infection phenomenon and are key areas of future research.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. CONCLUSION

In conclusion, the results of this study show that the gene expression patterns of HIV-1 resistant commercial sex workers were most clearly distinct from those obtained from uninfected HIV-1 susceptible and HIV-1 infected commercial sex workers in terms of 2 genes, Glucose-6-phosphate dehydrogenase and Glyceraldehyde-3-phosphate deydrogenase (G6PD and GAPDH). These two genes were found to be significantly down-regulated in the HIV-1 resistant women in comparison to the uninfected HIV-1 susceptible and HIV-1 infected women, all relatively compared with non commercial sex workers MCH clinic attendees considered to be at low risk of contracting HIV-1. This validation was made possible by qRT-PCR.

Moreover, these showed that the under-expression of these glycolytic enzymes in the resistant women suggest that these enzymes play a previously unknown, essential role in the resistance to HIV-1 infection. Further studies are warranted.

The study also showed that the rest of the genes GIPR, IRS1, CDK2NB, PGM1, PIK3CG, PKM2 and DPP4 did not show any significant expression changes, although they were also under-expressed lowly, though not enough to show significant down-regulation. Perhaps the quantification of RNA from more study subjects would give more information.

In addition, the study continued to emphasize on the need of qRT-PCR to validate differential expression and biological confirmation of genes seen in cDNA microarray assays, especially of genes with less than four-fold difference in expression.

5.2. RECOMMENDATIONS

In the Pumwani commercial sex worker cohort, new ground was covered by this study when expression of a few genes of the glycolysis/insulin signaling/pentose phosphate pathways was validated by quantitative real time PCR. However, due to time this study was not able to cover the validation of all the genes of these pathways identified as differentially expressed in HIV-1 resistant women. Further studies of genetic factors involved in resistance to HIV-1 infection may help to elucidate other protective mechanisms to this phenomenon and decipher why it occurs in only a few exposed individuals.

Therefore I would recommend further investigation into the following areas:

A. Further investigation on the enzymatic activity in the down-regulated G6PD gene in order to assess if there is G6PD deficiency, and in turn, the effects of this deficiency in HIV-1 resistant women. This should be done hand in hand with the degree of normal enzyme activity checked.
B. Further investigation of the function of the multifunctional GAPDH gene to assess the role that this gene plays in HIV-1 resistance, and also to investigate the effects that the down-regulation may cause in HIV-1 resistant women.
C. Although the other genes studied showed no significant down-regulation, the genes were still expressed at lower levels in the HIV-1 resistant CSWs than in the Uninfected HIV-1 susceptible and HIV-1 infected subjects. Therefore, it is recommended that validation of these genes using sample numbers greater than 14 used in this study that may further consolidate this trend.

D. Further studies need to be done for G6PD and GAPDH to find their association with the HIV-1 susceptibility/resistance phenomenon, and the consistency and strength of this association identified.

REFERENCES

1. Naghavi M. J., Hatziioannou T., Gao G., Goff S. P. **Overexpression of fasciculation and elongation protein-1 (FEZI) induces a post entry block to retroviruses in cultured cells.** *Genes and Development* 2005; **19**:1105-1115

2. Harris R. S., Bishop K. N., Sheehy A. M., Craig H. M., Petersen-Mahrt S. K., Watt I. N., Neuberger M. S., Malim M.H. **DNA deamination mediates innate immunity to retroviral infection.** *Cell* 2003; **113**:803-809

3. Carvalho-Gasper M., Billing J. S., Spriewald B. M, Wood K. J. **Chemokine gene reception during allograft rejection: comparison of two quantitative PCR techniques**. *Journal of Immunological Methods* 2005; **301**:41-52

4. Guerra S., Lopez-Fernandez L.A., Conde R., Pascual-Montano A., Harshman K., Esteban M. **Microarray analysis reveals characteristic changes of host cell gene expression in response to attenuated modified vaccinia virus Ankara infection of human HeLa cells.** *Journal of Virology* 2004; **78**:5820-5834

5. DeRisi J., Penland L., Brown P.O., Bittner M. L., Meltzer P. S., Ray M., Chen Y., Su Y. A., Trent J. M. **Use of a CDNA microarray to analyze gene expression patterns in human cancer.** *Nature Genetics* 1996; **14**,457

6. Schena M., Shalon D., Heller R., Chai A., Brown P. O., Davis R. W. **Parallel human genome analysis: microarray-based expression monitoring of 1000 genes.** *Proc. Natl. Acad.* 1996; **93**: 10614

7. Walker J. and Rigley K. **Gene expression profiling in human peripheral blood mononuclear cells using high density filter-based cDNA microarrays**. *Journal of Immunological Methods* 2000; **239**:167-179

8. DeRisi J. L., Iyer V. R., Brown P.O. **Exploring the metabolic and genetic control of gene expression on a genomic scale.** *Science* 1997; **278**, 680

9. Meckelnburg I., Weckermann D., Zippelius A., Schoberth A., Petersen S., Prang N., Riethmuller G., Kufer P. **A multimarker real time RT-PCR for MAGE-A gene expression allows sensitive detection and quantitation of the minimal systemic tumor load in patients with localized cancer**. *Journal of Immunological Methods* 2007; 10525

10. Overberg L., Valcky D., Waer M., Mathieu C. **Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR.** *Cytokine* 2001; 305-312

11.Whelan J.A., Russel N. B., Whelan M. A**. A method for the absolute quantification of cDNA using real time PCR**. *Journal of Immunological Methods* 2003; 261-269

12. Ferreira I. D., Rosari V. E., Cravo P. V. **Real time quantitative PCR with SYBR Green 1 detection for estimating copy numbers of 9 drug resistance candidate genes in Plasmodium falciparum.** *Malaria Journal* 2006; **5**:1

13. Fowke K. R, Kaul R., Rosenthal K. L. **HIV-1 specific cellular immune responses among HIV-1 resistant sex workers.** *Immunology Cell Biology* 2000; **78**:586-595

14. Fowke K. R., Nagelkerke N. J., Kimani J., Simonsen J. N., Anzala A. O., Bwayo J. J. **Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya.** *Lancet* 1996; **348**:1347–1351

15. Plummer F. A., Ball T.B., Kimani J., Fowke K.R. **Resistance to HIV-1 infection among highly exposed sex workers in Nairobi: What mediates protection and why does it develop?** *Immunology Letters* 1999; **66**:27–34

16. Alimonti J.B., Koesters S.A., Kimani J., Matu L., Wachihi C., Plummer F.A., Fowke K.R. **CD4R T cell responses in HIV-exposed seronegative women are qualitatively distinct from those in HIV-infected women.** *Journal of Infectious Diseases* 2005; **91**:20–24.

17. Trivedi H.N., Plummer F. A., Anzala A. O., Njagi E., Bwayo J. J., Ngugi E. N. **Resistance to HIV-1 infection among African sex workers is associated with global hyporesponsiveness in interleukin 4 production.** *Journal of the Federation of American Societies for Experimental Biology* 2001; **15**:1795–1797.

18. Fowke K.R., Dong T., Rowland-Jones S.L., Oyugi J., Rutherford W.J., Kimani J, **HIV type 1 resistance in Kenyan sex workers is not associated with altered cellular susceptibility to HIV type 1 infection or enhanced beta-chemokine production.** *AIDS Research and Human Retroviruses* 1998; **14**:1521–1530.

19. Dunand V.A., Ng C.M., Wade J.A., Bwayo J., Plummer F.A., MacDonald K.S. **HLA-DR 52- and 51-associated DRB1 alleles in Kenya, East Africa.** *Tissue Antigens* 1997; **49**:397–402.

20. MacDonald K.S., Fowke K.R., Kimani J., Dunand V.A., Nagelkerke N.J., Ball T.B. **Influence of HLA supertypes on susceptibility and resistance to human immunodeficiency virus type 1 infection.** *Journal of Infectious Diseases* 2000; **181**:1581–1589.

21. Ball T. B., Ji H., Kimani J., McLaren P., Marlin C., Hill A. V. S., Plummer F. A. **Polymorphisms in IRF-1 associated with resistance to Hiv-1 infection in highly exposed uninfected Kenyan sex workers.** *AIDS* 2007; **21**:1-11

22. Kroger A., Koster M., Schroeder K., Hauser H., Mueller P. P. **Activities of IRF-1**. *Journal of Interferon Cytokine Research* 2002; **22**:5–14.

23. Mamane Y., Heylbroeck C., Genin P., Algarte M., Servant M. J., LePage C. **Interferon regulatory factors: the next generation**. *Gene* 1999; **237**:1–14.

24. Mcllroy D., Meyer L., Dudoit Y., Samri A., Delfraissy J. F., Autran B., Debre P., Theodorout I**., and the SEROCO, IMMUNOCO and ALT study groups**. *International Journal of Immunogenetics* 2006; **33**:73-79

25. Hogan C. and Hammer S. **Host determinants in HIV infection and disease Part 1: Cellular and humoral responses.** *Annals of International Medicine* 2001a; **134**, 761-766

26. Dorak M. T. (Ed.) **Real-time PCR** (Advanced Methods series). *Oxford: Taylor and Francis*, 2006

27. Hogan C. and Hammer S. **Host determinants in HIV infection Part 2: Genetic factors and implications for antiretroviral therapeutics.** *Annals of International Medicine* 2001b; **134**, 978

28. Zhang H., Yang B., Pomerantz R. J., Zhang C., Arunchalam S. C., Gao L. **The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA.** *Nature* 2003; **424**:94-98

29. Xia J., Wu H., Smith H. **APOBEC3G levels predict rates of progression to AIDS**. *Retrovirology* 2007; **4**:20

30. Sheehy A. M., Gaddis N. C., Choi J. D., Malim M. **H. isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein.** *Nature* 2002; **418**: 646-650

31. Harris R. S. and Liddament M. T. **Retroviral restriction by APOBEC3 proteins**. *Nature Review of Immunology* 2004; **4**:868-877

32. Cullen B. R. **Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors.** *Journal of Virology* 2006; **80**: 1067-1076

33. Jin X., Brooks A., Chen H.,Bennet R., Reichman R., Smith H. **APOBEC3G/CEM15 (Ha3G) mRNA levels associate inversely with Human Immunodeficiency Virus viremia**. *Journal of Virology* 2005; **79**:11513-11516

34. Cron R. Q. **HIV-1, NFAT and cyclosporine: Immunosuppression for the immunosuppressed?** *DNA cell Biology* 2001; **20**: 761-767

35. Pessler F. and Cron R.Q. **Reciprocal regulation of the nuclear factor of activated T cells and HIV-1.** *Genes and Immunity* 2004; **5:**158–167

36. Martin M. P., Qi Y., Gao X., Yamada E., Martin J. N., Pereyra F., Colombo S., Brown E. E., Shupert W. L., Phair J., Goedert J. J., Buchbinder S., Kirk G. D., Telenti A., Connors M., O'Brien S. J., Walker B.D., Parham P., Deeks S. G., McVicar D. W., Carrington M. **Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1.** *Nature Genetics* 2007; **39**:733-740

37. Bas A., Forsberg G., Hammarstrom S., Hammarstrom M.L. **Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes.** *Scandinavian Journal of Immunology* 2004; 59(6):566-573.

38. Mansur, N. R., Meyer-Siegler, K., Wurzer, J. C., and Sirover, M. A. **Cell cycle regulation of the glyceraldehyde-3-phosphate dehydrogenase/uracil DNA glycosylase gene in normal human cells.** *Nucleic Acids Research* 1993; **21,** 993–998

39. Suzuki T, Higgins PJ, Crawford DR. **Control selection for RNA quantitation.** *Biotechniques* 2000; **29**:332–7

40. Heeney, J.L., Beverly P., McMichael A. **Immune correlates of protection from HIV and AIDS- more answers but yet more questions.** *Immunology Today* 1999; **20**:247-251

41. Goidin D., mamessier A., Staquet M.J., Schmitt D., Berthier-Vergnes O. **Ribosomal 18S rRNA prevails over Glyceraldehyde-3-phosphate dehydrogenase and Beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive**

and non-invasive human melanoma cell subpopulations. *Analytical Biochemistry* 2001; **295**:17-21

42. Pederson R. A. **Gastric Inhibitory Polypeptide.** *In: gut peptides: Biochemistry and Physiology.* Walsh J. H., Dockray G.H. Raven Press, New York, 1994. pp 217-259

43. Gromada J., Bokvist K., Ding W.G., Holst J.J, Nielsen J.H., Rorsman P. **Glucagon-like peptide 1(7-36) amide stimulatesexocytosis in human pancreatic Beta cells by both proximal and distal regulatory steps in stimulus secretion coupling.** *Diabetes* 1998; **47**:57-65

44. Ding W.G, Gromada J. **Protein kinase A-dependent stimulation of exocytosis in mouse pancreatic beta cells by glucose dependent insulinotropic polypeptide.** *Diabetes* 1997; **46**:615-621

45. McIntosh C.H.S., Wheeler M.B., Gelling R.W., Brown J.C., Pederson R.A. **GIP receptors and signal-transduction mechanisms.** *Acta Physiologica Scandinavica* 1996; **157**:361-365

46. Ehses J.A., Lee S.S., Pederson R.A., McIntosh C.H.S. **A new pathway for glucose-dependent insulinotropic polypeptide (GIP) receptor signaling: evidence for the involvement of phospholipase A2 in GIP-stimulated insulin secretion.** *Journal of Biological Chemistry* 2001; **276**: 23667-23673

47. Fehman H. and Burkhard G. **Characterization of GIP (1-30) and GIP (1-42) as stimulators of proinsulin gene transcription**. *Peptides* 1995; **16**:1149-1152

48. Wang Y., Montrose-Rafizadeh M., Adams L., Raygada M., Nadiv O., **Agan J.M. GIP regulates glucose transporters, hexokinases and glucose induced insulin secretion in RIN 1046-38 cells.** *Molecular and Cellular Endocrinology* 1996; **116**:81-87

49. Demulth H. and Heins J. **Catalytic mechanism of dipeptidyl peptidase IV. In dipeptidyl peptidase IV (CD26) in metabolism and the immune response. Fleisher B, Ed RG. Landes, Georgetown TX, 1995 pp 1-35**

50. Mentlein R. **Dipeptidyl peptidase IV (CD26): Role in the activation of regulatory peptides.** *Regulatory Peptides* 1999; **85**:9-25

51. Deacon C.F., Danielsen P., Klarskov L., Olesen M. and Holst J.J. **Dipeptidyl peptidase IV inhibition reduces the degradation and clearance of GIP and**

potentiates its insulinotropic and hyperglycemic effects in anaesthized pigs. *Diabetes* 1999; **50**:1588-1597

52. Krarup T., Holst J.J., Larsen K.L. **Responses and molecular heterogeneity of IR-GIP after intraduodenal glucose and fat**. *American Journal of Physisology* 1985; **249**:E195-E200

53. Nauck M.A., Bartels E., Orskov C., Ebert R., Creutzefeldt W. **Additive insulinotropic effects of exogeneous synthetic human gastric inhibitory polypeptide and glucagon-like peptide 1-(7-36) amide infused at near physiological insulinotropic hormone and glucose concentrations.** *Journal of Clinical Endocrinology and Metabolism* 1993; **76:**912-917

54. Mari A., Sallas W.M., He Y.L., Foley J.E. **Vyldagliptin, a dipeptidyl peptidase IV inhibitor, improves model-assessed beta-cell function in patients with type 2 Diabetes**. *Journal of Clinical Endocrinology and Metabolism* 2005; **90**:4884-4894

55. Sedo A. and Malik R. **Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities?** *Biochimica et Biophysica Acta* 2001; **1550**:107-116

56. De Meester I., Korom S., Van Damme J., Scharpe S. **CD26, let it cut or cut it down.** *Immunology Today* 1999; **20**:367-375

57. Marguet D., Baggio l., Kobayashi T., Bernard A.M., Pierres M., Nielsen P.F., Ribel U., Watanabe T., Drucker D.J., Wagtmann N. **Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26**. *Proceedings of the National Academy of Sciences USA* 2000; **97**:6874-6879

58. Ahren B., Simonsson E., Larrson H., Landin-Olson M., Bavenholm P., Efendic S., Eriksson J.W., Dickinson S., Holmes D. **Inhibition of dipeptidyl peptidase IV improves metabolic control over a 4-week study period in type 2 Diabetes**. *Diabetic Care* 2002; **25**:860-875

59. Henriksen E.J., Jacob S., Fogt D.L., Dietze G.J. **Effect of chronic bradykinin administration on insulin action in an animal model of insulin resistance.** *American Journal of Physiology* 1998; **275**: R40-R45

60. Zhu L., Iamvakopoulos C., Xie D., Dragovic J., Shen X., Fenyk-Melody J.E., Schmidt K., Bagchi A., Griffin P.R., Thornberry N.A., Sinha R.R. **The role of dipeptidyl peptidase IV in the cleavage of glucagons family peptides: in vivo metabolism of**

pituitary adenylate cyclase activating polypeptide –(1-38) *Journal of Biological Chemistry* 2003; **278**:22418-22423

61. Cappadoro M., Giribaldi G., O'Brien E., Turrini F., Mannu F., Ulliers D., Simula G., Luzzatto L., Arese P. **Early phagocytosis of Glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes parasitized by Plasmodium falciparum may explain malaria protection in G6PD deficiency.** *Blood* 1998; **92**:2527-2534

62. Rosen C.A. **Regulation of HIV gene expression by RNA-protein interactions**. *Trends in Genetics* 1991; **7** (1): 9-14

63. Rieber P. and Bauerle P.A. *Embolism Journal* 1998; 10 (8):2247-2528

64. Ursini M.V., Lettieri T., Braddock M., Martini G. **Enhanced activity of human G6PD promoter transfected in HeLa cells producing high levels of HIV-1 Tat.** *Virology* 1993; **196**:338-343

65. Boonacker E., Van Noorden C.J.F., **The multifunctional or moonlighting protein CD26/DPPIV**. *European Journal of Cell Biology* 2002; **82**:53-73

66. Ejiri S. **Moonlighting functions of polypeptide elongation factor 1: from actin bundling to zinc finger protein R1-associated nuclear localization**. *Biosciences, Biotechnology and Biochemistry* 2002; **66**:1-21

67. Jeffrey C.J. **Moonlighting proteins**. *Trends in Biochemical Sciences* 1999; **24**:8-11

68. Valenzuela A., Blanco J., Callebaut C., Jacotot E., Lluis C., Hovanessian A.G., Franco R. **Adenosine deaminase binding to human CD26 is inhibited by HIV-1 envelope glycoprotein gp120 and viral particles.** *Journal of Immunology* 1997; **158**:3721-3729

69. Herrera C., Morimoto C., Blanco J., Mallol J., Arenzana F., Lluis C., Franco R. **Comodulation of CXCR4 and CD26 in human lymphocytes**. *Journal of Biological Chemistry* 2001; **276**:19532-19539

70. Blazquez M.V., Madueno J.A., Gonzalez R., Jurado R., Bachovchin W.W., Pena J., Munoz E. **Selective decrease of CD26 expression in T-cells from HIV-1 infected individuals.** Journal of Immunology 1992; **149**:3073-3077

71. Schmitz T., Underwood R., Khiroya R., Bachovchin W.W., Huber B.T. **Potentiation of the immune response in HIV+ individuals**. *Journal of Clinical Investigations* 1996; **97**:1545-1549

72. Pauly R.P., Rosche F., Wermann M., McIntosh C.H., Pederson R.A., Demuth H.U. **Investigation of glucose glucose-dependent insulinotropic polypeptide (1-42) and glucagon-like peptide 1-(7-36) degradation in vitro by dipeptidyl peptidase IV using matrix-assisted laser desorption ionization time of flight mass spectrometry.** *Journal of Biological Chemistry* 1996; **271** (38):23222-23229

73. Popssilik J.A., Stafford S.G., Demuth H.U., McIntosh C.H., Pederson R.A. **Long-term treatment with dipeptidyl peptidase IV inhibitor improves hepatic and peripheral insulinsensitivity in the VDF Zucker rat. A Euglycemic-hyperinsulinemic clamp study.** *Diabetes* 2002; **51**:2677-2683

74. Ahren B., Holst J.J., Martensson H. and Balkan B. **Improved glucose tolerance and insulin secretion by inhibition of dipeptidyl peptidase IV in mice**. *European Journal of Pharmacology* 2000; **404**:239-245

75. Deacon C.F., Danielsen P., Klarkov L., Oleen M., Holst J.J. **Dipeptidyl peptidase IV inhibition reduce the degradation and clearance of GIP and potentiates its insulinotropic and hyperglycic effects in anaesthized pigs.** *Diabetes* 2001; **50**:1588-1597

76. Sudre B., Broqua P., White R.B., Ashworth D., Evans D.M., Haigh R, Junien J.L., Aubert M.L. **Chronic inhibition of circulating dipeptidyl peptidase IV by FE 999011 delays the occurrence of diabetes in male zucker diabetic fatty rats**. *Diabetes* 2002; **51**:1461-1469

77. Bohm S.K., Gum J.R., Erickson R.H., Hicks J.W., Kim Y.S. **Human dipeptidyl peptidase IV gene promoter: tissue-specific regulation from a TATA-less GC-rich sequence characteristic of a housekeeping gene promoter.** *Biochemistry Journal* 1995; **311**:835-843

78. Working Group, World Health Organization. **Glucose-6-phosphate dehydrogenase deficiency.** *Bull WHO* 1989; **67**:601

79. Denney P.A., Seiden D.S., Stevenson D.K. **Neonatal hyperbilirubinemia: pathophysiology, preventon and treatment**. *New England Journal of Medicine* 2001; **344**:581-590

80. Elliot R.M., Morgan L.M., Tredger J.A., Deacon S., Wright J., Marks V. **Glucagon-like peptide-1 (7-36) amide and glucose-dependent insulinotropic polypeptide**

secretion in response to nutrient ingestion in man: acute post parandial and 24-hour secretion patterns. *Journal of Endocrinology* 1993; **138**:159-166

81. Baggio L.L, Drucker D.J. **Biology of incretins: GLP-1 and GIP**. *Gastroenterology* 2007; **132**:2131-2157

82. Isken F., Pfeiffer A.F., Nogueiras R., Osterhoff M.A., Riston M., Thorens B., Tschop M.H., Weikert M.O. **Deficiency of glucose-dependent insulinotropic polypeptide receptor prevents ovariectomy induced obesity in mice.** *American Journal of Physiology, Endocrinology and Metabolism.* 2008; **295**:E350-E355

83. Yip R.G. and Wolfe M., **GIP biology and fat metabolism**. *Life Sciences* 2000; **66**:91-103

84. Xu G., Kaneto H., Laybutt D.R., Duvivier-Kali V.F., Trivedi N., Suzuma K., King J.L., Weir G.C., Bonner-Wier S. **Down-regulation of GLP-1 and GIP Receptor expression by hyperglycaemia, possible contribution to impaired incretin effects in Diabetes.** *Diabetes* 2007; **56**:1551-1558

85. Yan Q., Briehl M., Crowley C.L. **The NAD^+ precursors, nicotinic acid and nicotinamide upregulate glyceraldehydes-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase mRNA in Jurkat cells.** *Biochemistry and Biophysisology Research Communication* 1999; **255**:133-136

86. Ravichandran V., Seres T., Moriguchi T. **S-thiolation of GADH induced by phagocytosis-associated respiratory burst in blood, monocytes**. *Journal of Biological Chemistry* 1994; **269**:25010-25015

87. Park J., Choe S.S., Choi S.H., Kim K.H., Yoon M.J., Suganami T., Ogawa Y., Kim J.B**. Increase in glucose-6-phosphate dehydrogenase in adipocytes stimulates oxidative stress and inflammatory signals.** *Diabetes* 2006; **55**:2939-2949

88. Park J., Rho H.K., Kim H.K., Choe S.s., Lee Y.S., Kim J. **Overexpression of G6PD is associated with lipid dysregulation and insulin resistance**. *Molecular and Cellular Biology* 2005; **25**:5146-5157

89. White M.F. **The IRS-signaling system, a network of docking proteins that mediates insulin action**. *Molecular and Cellular Biochemistry* 1998; **182**:13-22

90. White M.F. **The insulin signaling system and the IRS-proteins**. *Diabetologia* 1997; **40** (Suppl 2): S2-S17

91. Kulkarni P.S., Butera S.T., Duerr A.C. **Resistance to HIV-1 infection: Lessons learned from studies of highly exposed persistently seronegative (HEPS) individuals.** *AIDS Review* 2003; **5**:87-103

92. Brown P.O and Botstein D. **Exploring the new world of the genome with DNA microarrays**. *Nature Genetics* 1999; **21**:33-37

93. Lipshutz R.J., Fodor S.P., Gingeras T.R. and Lockhard D.J. **High density synthetic oligonucleotide arrays**. *Nature Genetics* 1999; **21**:20-24

94. Hawkins P.T. and Stephens L.R. **PI3K γ is a key regulator of inflammatory responses and cardiovascular homoestasis**. *Science* 2007; **318**:64-66

95. Ruckle T., Shwarz M.K., Rommel C. **PI3KG inhibition. Towards an aspirin of the 21st century.** *Nature Reviews Drug Discovery* 2006; **5**:903-918

96. Hirsch E., Giuseppe L., Giuseppe M., Rommel C., Costa C., Barberis L. **Signaling through PI3K γ: a common platform for leukocyte, platelet and cardiovascular stress sensing.** *Thrombosis and Homoestasis* 2006; **95**:29-35

97. Suire S., Condliffe A.M., Ferguson G.J., Ellson C.D., Guillou H., Davidson K., Welch H., Coadwell J., Turner M., Chilvers E.R., Hawkins P.T., Stephens L. **G, β, γ, S and then Ras binding domain of P110 γ are both important regulators of PI(3)K γ signaling in neutrophils**. *Nature Cell Biology* 2006; **8**:1303-1309

98. Wacker M.J., Tehel M.M., Gallagher P.M. **Technique for quantitative RT-PCR analysis directly from single muscle fibers.** *Journal of Applied Physiology* 2001; **105**:308-315

99. Walker E.H., Perisic O., Reid C., Stephens L., Williams R.L. **Structural insights into Phosphoinositide-3-kinase catalysis and signaling**. *Nature* 1999; **402**:313-320

100. Martignetti J.A., Desnick R.J., Aliprandis E., Norton K.I., Hardcastle P., Nade S., Gelb B.D. **Diaphyseal medullary stenosis with malignant fibrous hisiocytoma: a hereditary bone dydplasia/cancer syndrome maps to 9p21-22.** *American Journal of Human Genetics* 1999; **64**:801-807

101. Kovacs P., Hanson R.L., Lee Y.H., Yang X., Kobes S., Permana P.A., Bogardus C., Baier L.J. **The role of insulin receptor substrate I gene (IRS-1) in type 2 Diabetes in Pima Indians.** *Diabetes* 2003; **52**:3005-3009

102. Max N., Willhauck M., Wolfe K., Thilo F., Reinhold U., Powlita M., Thiel E., Keilholz U. **Reliability of PCR-based detection of occult tumor cells: lesson from real-time RT-PCR**. *Melanoma Research* 2001; **11**:31-38

103. Nam J.H., Hwang K., Yu C.H., Kang T.H., Shin J.Y., Choi W.h., Kim I.B., Joo Y.R., Cho H.W., Park K.Y. **Expression of interferon inducible genes following Hantaan virus infection as a mechanism of resistance in A549 cells.** *Virus Genes* 2003; **26**:31-38

104. Leong W.F., Tan H.C., Ooi E.E., Koh D.R., Chow V.T. **Microarray and real time PCR analyses of differential human expression patterns induced by severe acute respiratory syndrome (SARS) coronavirus infection of vero cells**. *Microbes and Infection* 2005; 7:248-159

105. Walker N.J. Technique sight- **A technique who's time has come.** *Science* 2002; **296**:557-559

106. Giri M.S., Bumgarner R.E., An M.C., Agy M.B., Wout A.B., Hammersmark E., Carter V.S., Upchurch D., Mullins J.I., Katze M.G. **Large scale monitoring of host cell gene expression during HIV infection using cDNA microarrays.** *Virology* 2000; **266**:8-16

107. Glare E.M., Dijvak M., Bailey M.J., Walters E.H. **β actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalizing mRNA levels**. *Thorax* 2002; **57**:765-770

108. Warrington J.A., Nair A., Mahadeveppa M., Tsyganskaya M. **Comparison of human adult and fetal expression and identification of 535 housekeeping maintenance genes**. *Physiological Genomics* 2000; **2**:143-147

109. Suzuki T., Higgins P.J., Crawford D.R. **Control selection for RNA quantification**. *Biotechniques* 2000; **29**:332-337

110. Bustin S.A. **Quantification of mRNA using real-time Reverse Transcription PCR (RT-PCR): Trends and problems**. *Journal of Molecular Endocrinology* 2002; **29**:23-39

111. Gizinger D.G. **Gene quantification using real time quantitative PCR**: **An emerging technology hits the mainstream**. *Experimental Hematology* 2002; **30**:503-512

112. Heid C.A., Stevens J., Livaka K.J., Williams P.M. **Real time quantitative PCR**. *Genome Research* 1996; **6**:986-994

113. Romano A.H. and Conway T. **Evolution of carbohydrate metabolic pathways**. *Research in Microbiology* 1996; **147** (6-7): 448-455

114. Rajeevan M.S., Ranamukhaarachichi D.G., Vernon S.D. and Unger E.R. **Use of real time quantitative PCR to validate the results of cDNA array and diferential display PCR technologies.** *Methods* 2001; **25**:443-451

115. Der S.D., Zhou A., Williams B.R.G., Silverman R.H. **Identification of genes differentially regulated by interferon α, β or γ using oligonucleotide arrays.** *Proceedings of the National Academy of Sciences of USA* 1998; **95** (26): 15623-15628

116. Schuchhardt J., Beule D., Malik A., Wolski E., Lehrach H., Henzel H. **Normalization studies of cDNA microarrays**. *Nucleic Acids Research* 2000; **28**(10): E47

117. Collins F.S., Green E.D., Guttmacher A.E., Guyer M.S. **A vision for the future of genomics research**. *Nature* 2003; **422**:835-847

118. Tishkoff S.A., Varkonyi R., Cahinhinan N., Abbes S., Argyropoulos G. Destro-Bisol G., Drousiotou A., Dangerfield B., Lefranc G., Loiselet J., Piro A., Stoneking M, Tagarelli A., Tagarelli G., Touma E.H., Williams S.M., Clark A.G. **Haplotype diversity and linkage disequilibrium at human G6PD: recent origin of alleles that confer malaria resistance.** *Science* 2001; **293**:455-462

119. Saunders M.A., Hammer M.F., Nachman M.W. **Nucleotide variability at G6PD and the signature of malarial selection in humans.** Genetics 2002; **162**:1849-1861

120. Rajeevan M., Vernon S.D., Taysavang N., Unger E.R. ***V*alidation of array-based gene expression profiles by real time (kinetic) RT-PCR.** *Journal of Molecular Diagnostics* 2001; 3 (1): 26-31

121. Hauteniemi S., Ringner M., Kauraniemi P., Autio R., Edregen H., Yli-Harja O., Astola J., Kallioniemi A., Kallioniemi O. **A strategy for identifying putative causes of gene expression variation in human cancers.** *Journal of the Franklin Institute* 2004; **341**:77-88

122. Deprez R.H.L., Fijnnvandrat A.C., Ruijter J.M., Moorman A.F.M. **Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions.** *Analytical Biochemistry* 2002; **307**:63-69

123. Gloria-Bottini F., Lucarini N., Palmarino R., La Torre M., Nicotra M., Borgiani P., Cosmi E. and Bottini E. **Phosphoglucomutase genetic polymorphism of newborns.** *American Journal of Human Biology* 2001; **13**:9-14

124. Beutler E. G6PD deficiency. ***Blood*** 1994; **84**:3613-3636

126. Embree J.E., Njenga S., Datta P., Nagelkerke N.J.D., Ndinya-Achola J.O., Mohammed Z., Ramdahin S., Bwayo J.J., Plummer F.A. **Risk factors for post-natal mother-child transmission of HIV-1.** ***AIDS*** 2000; **14**:2535-2541

127. White M.F. **The insulin signaling system and the IRS proteins**. ***Diabetologia*** 1997; **40**:S2-S17

128. Araki E., Sun X., Haag B.L. **Human skeletal muscle insulin receptor substrate-1 characterization of the cDNA, gene and chromosomal localization**. *Diabetes* 1993; **42**:1041-1054

129. White M.F. **Insulin signaling in health and disease**. ***Science*** 2003; **302**:170-181

130. MacDonald K.S., Fowke K.R., Kimani J., Dunand V.A., Nagelkerke N.J.D., Ball T.B., Oyugi J., Ephantus N., Gaur L.K., Brunham R.C., Wade J., Luscher M.A., Krausa P., Rowland-Jones S., Ngugi E., Bwayo J.J., Plummer F.A. **Influence of HLA supertypes on susceptibility and resistance to Human Immunodeficiency Virus type 1 infection.** *The Journal of Infectious Diseases* 2000; **181**:1581-1589

131. Royce R.A., Sena A., Cates W. Jr., Cohen M.S. **Sexual transmission of HIV**. *New England Journal of Medicine* 1997; **336**:1072-1078

132. Mentlein R. **Dipeptidyl-peptidase IV (CD26) - role in the inactivation of regulatory peptides**. *Regulatory Peptides* 1999; **85**:9-24

133. Ahren B., Holst J.J., Martensson H., Balkan B. **Improved glucose tolerance and insulin secretion by inhibition of dipeptidyl peptidase IV in mice.** *European Journal of Pharmacology* 2000; **404**:239-245

134. Gizinger D.G. **Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream**. *Experimetnal Hematology* 2002; **30**:503-512

135. Bird R.C. **Role of cyclins and cyclin-dependent kinases in G1 phase progression** in Boonstra J. (Ed), **G1 phase progression**. *Kluwer Academic Plenum*, New York, pp 40-57 ISBN : 0-306-47831-5

136. Taniguchi C.M., Ueki K., Kahn C.R. **Complementary role of IRS-1 and IRS-2 in the hepatic regulation of metabolism**. *Journal of Clinical Investigation* 2005; **115:**718-727

137. Weihofen W.A., Liu J., Reutter W., Saenger W., Fan H. **Crystal structures of HIV-1 Tat derived nonapeptides Tat (1-9) and Trp-Tat-(1-9) bound to the active site of Dipeptidyl-peptidase IV (CD26)**. *Journal of Biological Chemistry* 2005; **280** (15): 14911-14917

138. Luzzatto L., Mehta A. in "**The metabolic basis of inherited diseases**" (Scriver C.R., Beaudet A.L., Sly W.S., Valle D. Eds) pp 2237-2265, *McGraw-Hill*, London, 1988

139. Wu Y., Tseng C., Cheng M., ho H., Shih S., Chiu D.T. **Glucose-6-phosphate dehydrogenase deficiency enhances human coronavirus 229E infection.** *Journal of Infectious Diseases* 2008; **197**:812-816

140. pao M., Kulkarni A., Gupta V., Kaul S., Balan S. **Neonatal screening for glucose-6-phosphate dehydrogenase deficiency.** *Indian Journal of Pediatrics* 2005; **72** (7): 835-837

141. Zhou Y., Yi X., Stoffer J.B., Bonafe N., Gilmore-Hebert M., McAlpine J., Chambers S.K. **The multifunctional protein glyceraldehydes-3-phosphate dehydrogenase is both regulated anc controls colony-stimulating factor-1 messenger RNA stability in ovarian cancer.** *Molecular Cancer* 2008; **6** (8):1375-1384

142. Sirover M.A. **New nuclear functions of the glycolytic protein, glycrealdehyde-3-phosphate dehydrogenase, in mammalian cells**. *Journal of Cellular Biochemistry* 2005; **95**:45-52

143. Germain R.N. **MHC-dependent antigenic processing and peptide presentation providing ligands for T-lymphocyte activation.** *Cell* 1994; **76**:287-299

144. Bird T.G., Kaul R., Rostron T., Kimani J., Embree J., Dunn P. **HLA typing in a Kenyan cohort identifies novel class 1 alleles that restrict cytotoxic T-cell responses to local HIV-1 clades.** *AIDS* 2002; **16**:1899-1904

145. MacDonald K.S., Fowke K.R., Kimani J., Dunand V.A., Nagelkerke N.J., Ball T.B. **Influence of HLA supertypes on susceptibility and resistance to HIV-1 infection.** *Journal of Infectious Diseases* 2000; **181**:1581-1589

146. Hardie R, Luo M., Bruneau B., Knight E., Nagelkerke N.D., Kimani J., Wachihi C., Ngugi E.N., Plummer F.A. **Human leukocyte antigen-DQ alleles and haplotypes and their associations with resistance and susceptibility to HIV-1 infection.** *AIDS* 2008; **22**:807-816

147. www.who.int/malariaandhivaids.html

148. www.appliedbiosystems.com/support/tutorials/pdf/rt**pcr**_**vs**_trad**pcr**.pdf

149. www.genome.ad.jp/dbget-bin/show_**pathway**?hsa00030+5238

146. www.genome.ad.jp/**kegg**/**pathway**/ot/ot00010.html

150. www.genome.ad.jp/**kegg**/**pathway**/hsa/hsa04910.html

APPENDIX A: CONSENT FORM

Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya

Majengo Research Clinic

Resurvey: Patient Information and Consent Form

This information will be communicated orally in English, Swahili or other Kenyan dialect of potential participant's preference.

Investigators:
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Background information

The University of Nairobi and its collaborators from Canada have been working for many years to fight the epidemics of AIDS and other sexually transmitted infections that we are facing in Kenya. This basic science research program is conducting studies to determine the relationship between immunity and susceptibility to sexually transmitted infections (STI) with the goal of developing vaccines or treatments for STIs. You are being asked to participate in this study because you are:

a) at a very high risk of acquiring an STI or are already infected with an STI or

b) at a low risk of acquiring an STI; or

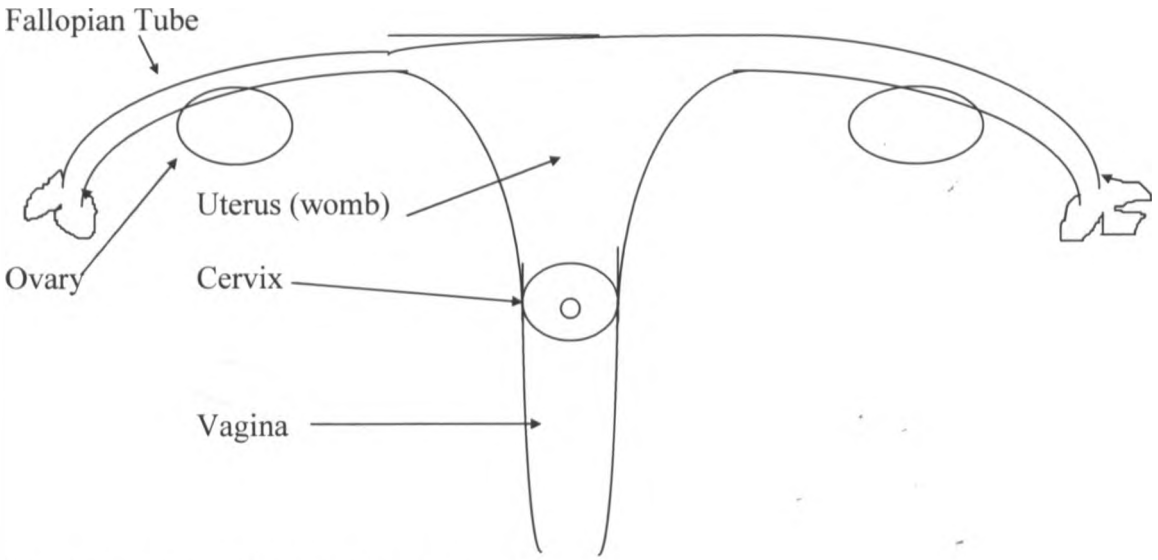
c) the relative of a person in group a) or b).

The purpose of this research program is to determine if there are factors that could protect individuals from acquiring sexually transmitted infections (STI) especially HIV. It is important to keep free of other sexually transmitted diseases, as the presence of these infections may increase your risk of becoming infected with HIV. If you have an STI, you should seek treatment for it as quickly as possible. However, sometimes you may have an STD and not know it, because you may not have any symptoms and thus advised to visit the clinic monthly for free check ups.

Why Is This Study Being Done?

This study is being done to find out why some people are more or less likely to get the Human Immunodeficiency Virus (HIV), the virus that causes AIDS. There is more and more evidence that the immune system in some people is able to protect them against infection with HIV. Since most people get HIV through sexual exposure to an HIV infected partner, the first contact with the virus occur in the genital tract, the vagina and cervix in women. We know from some of our previous work that some women, who seem to be protected against HIV, have a special type of immune response that it not present in women who get HIV. The purpose of this study is to try to find out the targets of this immune response in the vagina, uterus and cervix and to try to find out what is special about the immune system of these few individuals. This work may be helpful in eventually making a vaccine for HIV.

To help you understand what is involved in the study a drawing of the vagina, cervix and uterus of the female genital tract is shown below.



How Many People Will Take Part in the Study

About 3000 participants mainly women will take part in this study.

What Is Involved in the Study?

You have been invited to voluntarily participate in this study because all are at risk of becoming infected with STDs and HIV. Some sexual behavior especially among sex workers or those who use sex as an income generating activity exposes those involved or their partners to a higher risk of contracting HIV. If you agree to participate in the study, you will be given additional counseling, advised on appropriate STIs prevention strategies and requested to practice safer sex. You can also choose to leave sex work at any time but you will be asked to return to the clinic every month for free check ups. Again, the results of these tests will be ready after one week or less, and you will be informed of the results and given the correct treatment if you have an infection. You will also be encouraged to come to the clinic for examination and treatment at any other time that you feel ill. If you forget to return to the clinic for one of your scheduled visits, a clinic staff member will contact you by phone, SMS or send one of your friends to remind you of the missed appointment. **All study participants will also be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on HIV infection status every three months.** In addition, we will store specimens from your blood for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

Clinic visits

First visit and semi-annual visits (All study participants)

We will ask you general questions about your life, about problems you are having, and about your sexual history.

The doctor will examine your body, including your female parts.

Swab and washing from your vagina to look for germs and to collect samples for studying your immune response.

Swab from your cervix to look from germs and to collect samples for studying your immune response.

A thin plastic tube will be placed in your cervix (opening to your womb) to get some of the mucous your cervix makes.

Urine to look for germs.

Three tablespoons of blood will be taken for testing syphilis, and HIV and for studying your immune response. We will inform you of your results at your one month visit. We also will test your spouse for 0the HIV virus free of charge if he/she wishes.

Monthly visits

Questions will be asked about you, and what problems you are having.

If you have any complaints the doctor will examine your body, including your female parts.

Every third month all study participants will be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on individuals HIV infection status.

Follow-up visits (All study participants)

You will be asked to return 3 to 7 days after every visit to be given you laboratory results.

You will be treated for new infections, free of charge.

How Long Will I Be in the Study?

The study will last 5 years. Although we would appreciate if you stayed in the study for the entire period you may choose to leave the study at any time without any penalty to you.

What Are the Risks of the Study?

Risk of blood and cervical collection

This study requires the use of your blood. In order to get the blood we will need to insert a needle into a vein in your arm so that the blood can be removed. There will be some pain associated with the needle stick but this will be only for a short period of time. There may be some bruising around the needle site and, although we will sterilize the site to minimize infection, there is a very minimal risk of infection at the site. There is also some discomfort associated with taking specimens from your cervix.

HIV test

Non-physical risks:

If you are HIV positive, learning so may cause you to become depressed. We will counsel you about your HIV test results if you are negative or positive. If you are HIV positive, we have antiretrovirals in the clinic to manage your condition. We will also test one boyfriend for HIV virus if he wants.

Risks of taking antibiotics / Antiretrovirals

If we find that you have an STD or AIDS we will provide you with the appropriate treatment. With any drug there is some potential for side effects. For the antibiotics/ antiretrovirals you might receive, the following side effects are possible.

Very likely:

Sick to your stomach

Headache

Metallic taste in mouth

Diarrhea

If a woman - infection of your vagina by yeast (a white discharge with itching). If this happens, we will give you medicine to put inside your vagina to treat the yeast infection.

Less likely but serious:

Less than 1 person in 100 will have a severe allergic reaction to one of the antibiotics/ antiretrovirals.

Are There Benefits to Taking Part in the Study?

The benefits that you will get from this study are that you will be examined regularly, and if you are found to have an STD or AIDS, you will receive appropriate and effective medication. Medical care will also be provided for other illnesses that you might have. You will also be informed about what you are suffering from, and you will be informed about the future implications of these STDs and of HIV.

What about Confidentiality?

Efforts will be made to keep your personal information confidential. We will record your information only by a special number assigned to you. The number will only be known to the clinic staff and yourself.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: the researchers, members of the local and international ethics teams and the National Institutes of Health in the United States of America. The research results will be published, but your identity will remain secret.

What Are My Rights as a Participant?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. If the participation in the study results in you becoming ill, the study team will provide you with medical care for the problem for free.

Although you will not be paid to participate in the study, you will be offered a small payment of two hundred shillings (KSh 200) for the resurvey visits only to compensate you for your transportation to the clinic.

We will also provide you with any new information and findings from the study that may affect your health, welfare, or willingness to stay in this study.

All information that is obtained will be kept strictly confidential, and your identity will not be known, except to those providing your medical care.

At the end of every year, we will be holding baraza's at the different clinics to give progress reports and share any new findings from the study with all members of the different clinics.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, call or contact Dr. Wachihi any one of the researchers named above at the Medical Microbiology Annex at the University of Nairobi

For questions about your rights as a research participant, contact Professor Bhatt, who is the chairperson of the Ethical Review Committee at the University of Nairobi, by calling 725452, or make an appointment to see her in the Department of Medicine, at the University of Nairobi.

Statement of Consent:

I have read the attached written information and / or received verbal information on the above study. I have been given the opportunity and time to have any questions about the research answered to my satisfaction. I consent to take part in the study and I am aware that my participation is entirely voluntary. I understand that I may withdraw at any time without giving a reason and without this affecting my future care.

By signing this information and consent form I agree that my personal data, may be used as described in this consent form and may be consulted by qualified representatives from sponsor the Ethics Committee or the health authorities.

I understand that the following (check the box only if you fully understand and agree with each statement):

☐ goals of this research program are to study resistance and susceptibility to sexually transmitted infections

☐ lment is completely voluntary and I can withdraw from the study at any time

☐ od, cervical and vaginal specimens will be required for this study and may be used for genetic studies

☐ blood specimens previously collected may be used for this study

☐ a portion of my blood, cervical and vaginal specimens will be stored for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

I am willing to participate in the study.

Name of Study Participant_____

Signature/Thumb print: _____ Date: _____

For clinic staff:

I, _____, have explained the nature and purpose of the

above study to _____

Name of Clinic Staff: _____

Signature: _____ Date: _____

Assigned Study Number / Clinic Number ____ ____ ____

Standards of Medical Care for Participants in the Research Clinics

This document outlines the standard of medical care for all participants in the Majengo, MCH Pumwani, Kindred, Kibera and Korogocho cohorts, regardless of HIV-1 serostatus. It should be emphasized that any member of the said cohorts may freely decline to take part in any cohort substudy, and that this decision will in no way affect their access to this standard of care. All care outlined is provided free of charge, thereby significantly improving health care access and outcomes for all members of the cohorts... The nature of the medical care will vary depending on HIV-1 serostatus of the participants, as outlined below.

1. **General medical care for all participants, regardless of HIV-1 status**.

HIV and STD prevention services: provision of the male condom, and peer-based and clinic-based counseling regarding safer sexual practices.

Family planning services as directed in the *Kenyan National Family Planning Guidelines*

Rapid and effective treatment of sexually transmitted diseases in accordance with the *Kenya National Guidelines for the Syndromic Management of Sexually Transmitted Diseases*

Medical care for acute and chronic illnesses, both infectious and non-infectious

Access to diagnostic testing in haematology, biochemistry, infectious diseases, immunology, radiology

Prompt referral for specialist consultation and hospitalization when indicated

2. **Management of Opportunistic Infections in HIV-1 Infected Participants.**

Primary Prophylaxis: Trimethoprim-Sulphamethoxazole (Septrin): all participants with a CD4+ T cell count <200/mm^3 or on Anti-TB must use Septrin for prevention of PCP, toxoplasmosis and bacterial infections (bacterial pneumonia, bacteremias, some bacterial diarrhoea), according to *National AIDS/STD Control Program (NASCOP) Guidelines.*

However, all HIV infected individuals with CD4+ <350 should be encouraged to use Septrin according to the latest WHO guidelines (2006)

Secondary Prophylaxis: Septrin: offered to all participants regardless of CD4+ T cell count after an episode of PCP, toxoplasmosis, or severe bacterial infection. Fluconazole: provided for secondary prevention of Cryptococcus

Treatment

Herpes. simplex/Herpes zoster infection: acyclovir

Candidiasis (oral, esophageal, vaginal): nystatin, clotrimazole, Fluconazole

Tuberculosis (pulmonary or extra pulmonary): referral to National TB Programme

Toxoplasmosis: referral for inpatient therapy

Cryptococcus: referral for inpatient therapy

PCP: Septrin (with prednisolone, if severe)

Kaposi's Sarcoma: ARV and referral to Clinical Oncologist

3. **Antiretroviral therapy.**

Antiretroviral therapy rollout in Kenya is supported and directed by NASCOP and The Ministry of Health. Kenya is a recipient of ARVs and infrastructure support through the *Presidents Emergency Plan for AIDS Relief (PEPFAR)* a US government international development initiative.

ARV drugs and infrastructure support has been secured by the University of Manitoba from NASCOP/PEPFAR and CDC PEPFAR to provide HIV basic and ARV care for **all** cohorts members who are eligible as per the *"Guidelines for Antiretroviral Drug Therapy in Kenya"* (NASCOP-2005 edition). Such medical treatment and its requisite follow-up, integrated with the above standard of care, will also be provided at no cost.

APPENDIX B: DATA SUMMARY

Relative expressions of genes (normalized values) for each subject.

SAMPLE NO.	G6PDH	GAPDH	IRS-1	PGM-1	PGM-1*	PIK3CG	CDNK2B	PKM2	DPP4	GIPR
ML 1394	0.4	0.04	0.142	0.156	0.21	0.541	1.728	0.047	0.136	0.496
ML 1437	0.35	0.085	0.84	0.708	5.528	21.21	76.23	0.991	4.194	72.64
ML 1482	0.18	1.143	1.72	93.61	5.572	6.947	13.61	0.71	1.851	14.59
ML 1515	0.45	0.31	0.46	13.93	2.241	1.754	6.75	1.274	0.448	1.165
ML 1573	0.2	0.303	1.58	53.448	2.509	16.121	32.64	13.68	4.159	8.508
ML 1589	0.03	0.013	0.043	0.414	0.712	2.015	4.67	1.496	0.72	0.915
ML 1635	0.75	0.188	0.013	0.679	0.534	0.331	5.52	0.077	0.575	1.37
ML 1668	0.032	0.0004	0.041	0.244	0.798	0.422	4.283	0.188	0.39	2.284
ML 1700	0.37	0.07	0.03	0.049	0.057	0.123	1.097	0.049	0.176	0.019
ML 1747	0.07	0.08	0.024	0.023	0.034	0.045	0.68	0.028	0.048	0.018
ML 1814	0.31	0.56	0.145	0.256	0.244	0.573	11.91	0.703	2.927	0.124
ML 1817	0.75	0.146	4.65	0.485	2.131	6.318	62.965	3.867	36.91	6.823
ML 1833	0.41	0.53	1.265	0.172	4.53	0.891	34.38	1.193	5.148	1.164
ML 1851	0.16	0.06	0.075	0.003	0.011	0.039	0.482	0.016	0.054	0.014
ML 2302	0.17	0.05	0.133	0.141	0.031	0.414	0.421	0.047	0.096	0.53
ML 2314	0.56	0.377	0.51	1.037	2.004	6.03	6.195	0.235	0.448	7.514
ML 2346	2.89	2.089	0.375	48.93	9.963	15.61	25.55	0.828	2.459	4.513
ML 2361	0.26	0.262	1.21	6.057	1.807	1.096	2.442		0.366	0.915
ML 2363	0.35	0.312	3.5	10.377	1.357	3.056	2.51	1.273	0.468	1.037
ML 2394	0.71	0.249	0.054	0.539	1.778	2.965	3.817	2.097	0.393	1.244
ML 2438	78.6	33.5	7.95	63.89	124.82	41.574	437.78	36.05	212.9	133
ML 2448	0.84	0.326	0.09	0.629	0.979	0.404	3.07	0.231	0.987	2.238
ML 2453	2.26	2.15	0.357	0.65	0.642	1.793	12.982	0.779	3.093	1.576
ML 2528	1.86	1.34	0.494	0.631	0.805	0.719	11.413	0.693	2.649	1.127

ML 2632	0.8	1.97	0.487	0.567	0.597	1.21	55.981	1.568	3.308	1.312
ML 2650	9.31	3.9	8.037	0.461	1.969	4.486	15.671	4.279	8.65	1.65
ML 2660	52.8	28.77	52.81	1.553	5.132	6.281	148.61	11.19	62.15	4.486
ML 2673	1.16	1.48	2.27	0.109	0.281	0.542	13.114	0.905	1.834	0.474
ML 2303	0.97	0.38	0.272	0.846	0.526	0.023	4.762	0.294	0.096	3.397
ML 2306	2.08	0.568	0.947		0.705	5.318	9.82	0.178	0.422	52.5
ML 2312	2	0.48	0.856	13.31	1.082	3.716	14.07	0.192	1.155	1.431
ML 2377	2.57	0.9	0.545	35.15	7.397	4.668	11.97	2.619	0.757	6.543
ML 2403	0.91	0.48	0.402	1.84	1.349	1.325	3.808	1.04	0.264	1.033
ML 2465	1.43	0.475	0.139	3.67	0.701	4.443	8.617	2.67	0.503	1.927
ML 2516	0.44	0.423	0.116	0.75	0.775	0.623	1.398	0.498	0.627	1.622
ML 2566	1.13	0.464	0.14	4.67	1.758	0.82	6.791	0.236	0.647	2.722
ML 2582	1.55	9.12	2.074	3.09	2.313	14.65	92.581	4.63	14.21	10.68
ML 2596	0.39	0.619	0.191	0.369	0.363	0.342	4.929	0.257	0.478	0.423
ML 2606	0.251	0.396	0.072	0.2	0.499	0.285	2.995	0.2	0.885	0.305
ML 2619	1.71	0.66	0.919	0.079	0.229	0.401	8.304	0.292	3.133	1.788
ML 2631										
ML 2667	0.42	0.834	0.102	0.028	0.164	0.103	3.902	0.221	0.434	0.149
MCH 100129	0.02	0.63	1.12	1.709	0.443	5.749	7.064	0.508	1.228	6.08
MCH 100367	2.83	0.1	0.748	1.948	0.337	13.9	45.9	0.755	2.913	48.72
MCH 114277	0.17	0.614	3.36	53.19	15.819	28.02	33.94	1.459	7.37	9.466
MCH 103625	1.58	1.346	1.01	18.42	4.429	3.777	25.35	4.811	2.758	1.963
MCH 106527	0.65	0.264	0.445	1.192	0.756	2.929	4.373	0.906	0.415	0.718
MCH 108172	0.84	0.36	0.106	0.647	0.438	6.315	9	1.95	1.219	1.094
MCH 111280	1.53	1.063	0.308	2.42	2.81	2.266	28.25	1.56	5.463	8.175
MCH 113039	0.53	0.377	0.212	2.139	0.794	1.25	11.55	0.837	1.721	2.197
MCH 113261	1.67	3.07	1.392	1.731	0.606	5.566	42.217	3.17	12.89	2.637
MCH 115344	0.5	1.42	0.34	0.704	0.763	1.081	17.21	0.959	4.457	0.617

MCH 115641	5.7	1.21	1.07	5.1	20.219	16.184	338.6	11.51	91.97	12.91
MCH 116459	0.33	0.355	0.695	0.064	0.153	0.458	6.316	0.269	2.202	0.261
MCH 116588	0.53	0.48	0.937	0.035	0.087	0.135	8.255	0.243	0.684	0.3
MCH 116715	0.57	0.5	0.567	0.05	0.131	0.113	4.215	0.124	0.604	0.416