Immunogenetic factors that limit the HIV-1 infection in MSM from Medellín, Colombia

Ana Claudia Ossa-Giraldo

Thesis submitted in fulfillment of the requirements for the degree of **Doctor in Basic Biomedical Science**

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A mi esposo y mi familia, el tesoro más grande que tengo y tendré.

Infinitas gracias, amor y admiración a ustedes.

To my husband and my family, the greatest treasure I have and will have.

Infinite thanks, love and admiration to you.

Summary

“A little science takes away from God, but a lot of science returns to Him”.

Louis Pasteur

“Un poco de ciencia aleja de Dios, pero mucha ciencia devuelve a Él”.

Louis Pasteur

Introduction

This section summarizes the methodology and main findings of the study carried out. This thesis aimed to determine the immunogenetic factors associated with HIV-1 resistance in men who have sex with men (MSM) from Medellin, Colombia. This is a cross-sectional study carried out in two phases: (i) the active search of the study population and its epidemiological characterization, and (ii) the determination of immunogenetic profiles of studied subjects. At the beginning of the section, the reader will initially go through the graphic abstract of the study design, where the two phases of the study are made explicit; then, he will find the graphic abstract of the second phase, where the methods used to determine the genetic and immunological profiles of the subjects studied are made explicit. Finally, the reader will find the study's abstract, where the main findings are summarized. Moreover, at the end of the section, the reader will find a systematic summary, which seeks to be a navigation guide, through which the content of each chapter of this thesis is indicated.

ABBREVIATIONS

ABBREVIATION	DEFINITION
6HB	Six helices bundle
95%CI	95% Confidence interval
Abs	Antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
Ang	Angiogenin
APCs	Antigen-presenting cells
APOBE3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
CBA	Cytometric bead array
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CCR5	C-C chemokine receptor type 5
CCR5 Δ32	CCR5 delta 32 mutation
CD4	Cluster of differentiation 4
CI	Confidence interval
CP	Casual partner
CSP	Condomless sexual practice
CTL	Cytotoxic T-cell response
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DC-SIGN	Dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin
DOI	Digital Object Identifier
ECs	Elite controllers
EDN	Eosinophil-derived neurotoxin
Elafin	Specific elastase inhibitor
FcγRIII	CD16 receptor
Fig	Figure
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
gp120	HIV glycoprotein 120
HBD2	Human beta-defensin-2
HBD3	Human beta-defensin-3
hBDs	Human beta-defensins
HESN	HIV-1 exposed but seronegative individuals
HIV	Human immunodeficiency viruses
HLA	Human leukocyte antigen molecules
HNP1	Human alpha defensin-1

hNP-1	Human neutrophil peptide-1
IDUs	Intravascular drug users
IFN-γ	Interferon gamma
IMP7	Importin 7
INS	Instituto Nacional de Salud (Colombia)
ITAM	Activation motifs based on tyrosine
ITIM	Inhibition motifs based on tyrosine
JID	Journal of Infectious Diseases
KIR	Killer-cell immunoglobulin-like receptor
LD	Linkage disequilibrium
LGBTIQ+	Lesbian, gay, bisexual, transgender, queer (or sometimes questioning), and two-spirited
LGMD1F	Limb girdle muscular dystrophy 1F
LGMD1F	Limb-girdle muscular dystrophy 1F
LTNPs	Long-term non-progressors
MDA-5	Melanoma differentiation-associated protein 5
MDC	Myeloid dendritic cells
Me	Median
MHC	Major histocompatibility complex
MHC-I	Major Histocompatibility Complex class I molecules
MHR	Major homology region
MIP1-β	Macrophage inflammatory protein-1β (CCL4)
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
NK	Natural killer
NLS	Nuclear localization signals
OR	Odds Ratio
Pattern-recognition receptors	PRRs
PBMCs	Peripheral blood mononuclear cells
PEP	HIV Post-exposure prophylaxis
PEP	Post-exposure prophylaxis
PIC	Pre-integration complex
pINDEX	INDEX of polyfunctionality
PKC	Protein kinase C
PLWHIV	People living with HIV
PrEP	HIV Pre-exposure prophylaxis
PRRs	Pattern-recognition receptors
qPCR	Real-time polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted (CCL5)
RIG-I	Retinoic acid-inducible gene I
SD	Standard deviation
SDF-1	Stromal cell-derived factor 1

SEB	Staphylococcus enterotoxin B
Serpin A1	Alpha 1-antitrypsin
SIU	Sede de Investigación Universitaria (Universidad de Antioquia)
SIV	Simian immunodeficiency virus
SLPI	Secretory leukocyte protease inhibitor
SLPI	Secretory leukocyte protease inhibitor
SP	Sexual partner (s)
STIs	Sexual transmitted infections
Tfh	Follicular helper T cells
TLR2	Toll-like receptor 2
TNF-α	Tumor necrosis factor alpha
TNPO3	Transportin 3 gen
Treg	Regulatory T cells
TRIM5α	Tripartite motif (TRIM) proteins
UIAI	Insertive unprotected anal intercourse
UNAIDS	Joint United Nations Programme on HIV/AIDS
UPBL1	UL16-binding proteins
URAI	Unprotected receptive anal intercourse
V3	Variable domain 3
VCs	Viremic controllers
VL	Viral load
Vpr	Viral Protein R

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Study and Thesis Structure

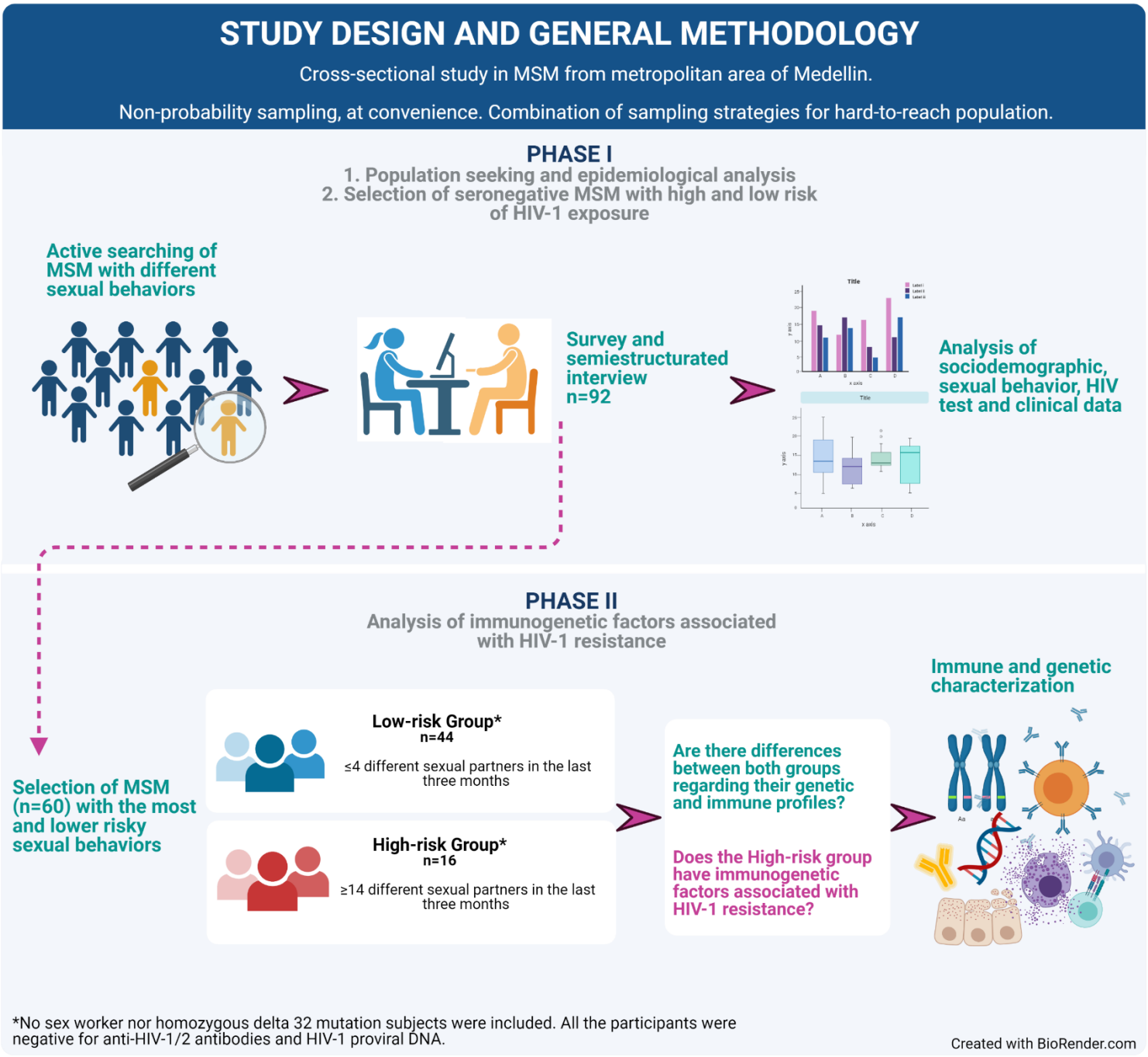
This study has been divided into two phases:

- **Phase I:** This phase encompasses the active search for the target population and the development of the first specific objective.
- **Phase II:** This phase encloses the genetic and immunological analyses, with the development of the third, fourth, fifth, and sixth specific objectives. This phase also includes the general discussion and perspectives.

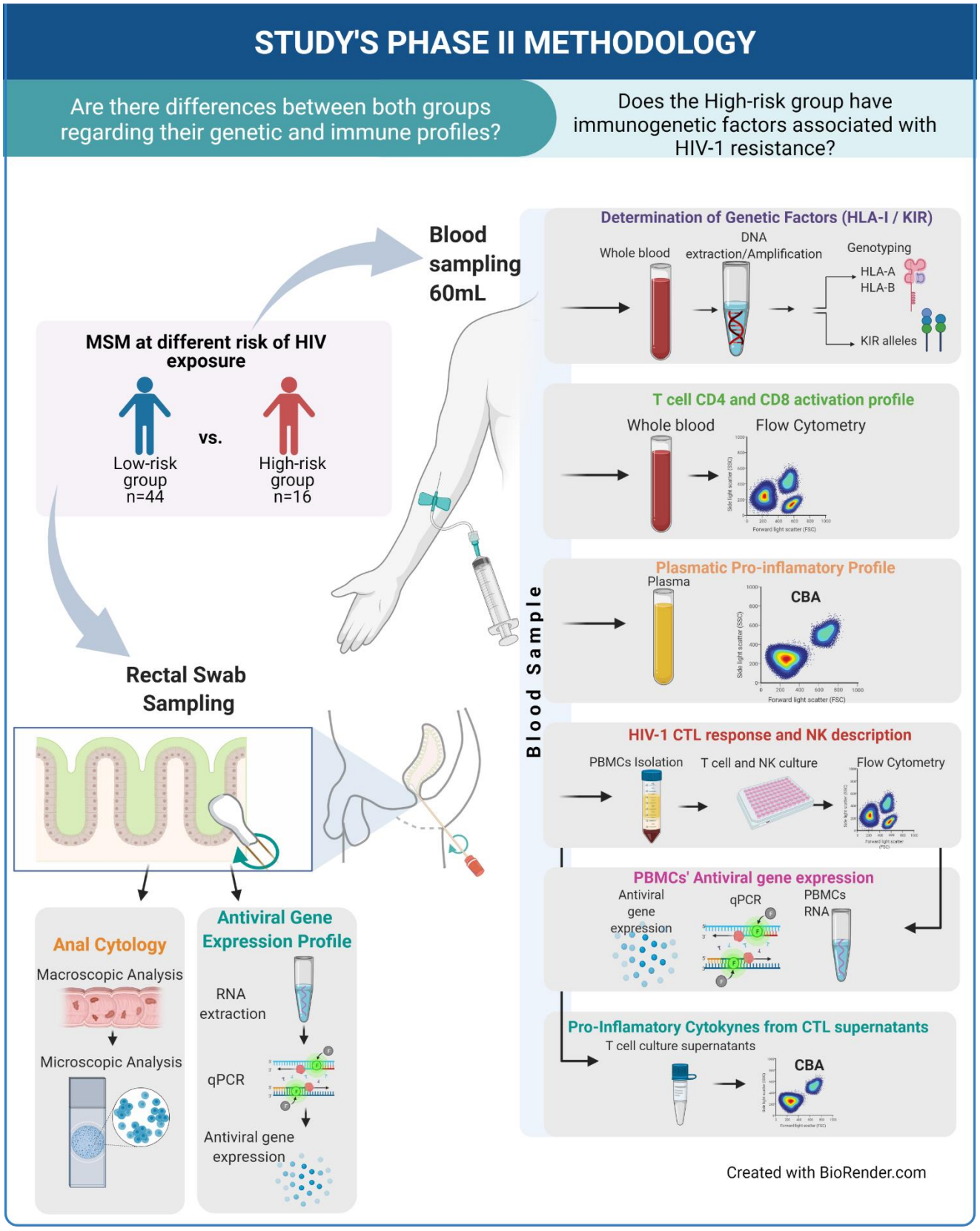
According to the above, the next sections of this thesis correspond to the development of the study´s phases and objectives. Thus, each chapter corresponds to a specific phase/objective as follows:

- **Chapter 2:** Epidemiological analysis. This chapter shows the development of the first specific objective (study's phase I).
- **Chapter 3:** Genetic analysis. This chapter shows the development of the second specific objective (study's phase II).
- **Chapter 4:** Immunological analyses. This chapter shows the development of the third, fourth, fifth, and sixth specific objectives (study's phase II).
- **Chapter 5:** General discussion and perspectives. This chapter shows the general discussion of all results in an integrated manner, but also the perspectives to future studies (study's phase II).

Graphic abstract of study methodology



Graphic abstract of phase II methodology



ABSTRACT

Background

HIV-1 infection still representing a major public health problem in most of the countries affected by the epidemic, and men who have sex with men (MSM) continue to shoulder a disproportionate HIV disease. In Colombia, the prevalence of HIV in the general population is 0.4% while the prevalence in MSM is 17%. Despite the natural course of HIV-1 infection, small populations of individuals have been identified that, although they are directly exposed to the virus repeatedly, do not present serologic or clinical evidence of the infection; these people are known to be exposed to HIV-1 but seronegative (HESN). The study of seronegative MSM with high-risk behaviors and the possible finding of HESN individuals in this population represents an important opportunity to better understand HIV-1 infection and immune response, to improve the current therapeutic and preventive intervention strategies.

Methods and Results

This study aimed to compare the immunological profile of Colombian seronegative MSM with different risk sexual behaviors. In the first study phase, 92 MSM were recruited through a community-based approach, and the sociodemographic, sexual behavior and clinical data were collected by a structured survey and in-depth interviews. HIV test was made on all participants to know their serostatus. Participants were classified into three groups according to the number of sexual partners in the last three months (0-4, 5-10, and >10 sexual partners), to compare the sociodemographic conditions and sexual behaviors. Multivariate logistic regression was used to compare the groups and to explore the associated factors with condomless sexual practice. The average age was 27 years (Me=25, IQR 22-31). The majority of subjects identified themselves as homosexual (85.9%) and 14.1% as bisexual or pansexual; no participants identified as heterosexual. The overall HIV

estimated prevalence was 4.3%, while the estimated prevalence for MSM with >10 sexual partners in the last three months was 14.8%. This last group showed higher average age (p=0.015), a higher percentage of subjects who have had sex with people living with HIV (p=0.011), and an increased frequency of previous sexually transmitted infections (p=0.014). Having condomless sex with casual partners was associated with the number of sexual partners in the last three months. The number of sexual partners in the last 3 months generates a 5% risk of not using a condom with casual partners (OR, 1.058; 95% CI, 1.00–1.11).

In the second study phase, 60 seronegative MSM with lower and riskier sexual behaviors were selected to study their immunogenetic profiles. They were divided into two groups; the low-risk group (n=44) compound by MSM with ≤4 sexual partners in the last three months, and the high-risk group (n=16), formed by MSM with ≥14 sexual partners in the same period. All included MSM were not sex workers, neither showed the CCR5 Δ32 mutation in their homozygous state. They also remain negative for anti-HIV-1/2 antibodies and HIV-1 proviral DNA. The high-risk group presented a higher frequency of sexual partners throughout life (p<0.001); a major number of sexual partners in the three months before the inclusion of the study (p<0.001), more unprotected intercourses in the last three months (p=0.001) and more frequency of previous sexually transmitted infections (p<0.001).

To explore the basal activation profile of CD4+ and CD8+ T cells, the percentage of cells expressing CD38, HLA-DR, CD69, and Ki-67 molecules was quantified by flow cytometry. The high-risk group showed a low activation profile in T cells with lower percentage of CD4+CD38+ (p=0.002), CD4+HLADR-CD38+ (p=0.027) and CD4+Ki67+ T cells (p=0.048). Likewise, this group had a higher percentage of CD4+HLADR-CD38- cells (p=0.013). Although it was not statistically significant, there was a tendency in the high-risk group to present a lower percentage of CD8+HLA-DR+CD38+ (p=0.058). No differences were found

in the expression of CD69. To identify whether the high-risk group exhibit differences in the basal levels of plasmatic cytokines and soluble factors, the concentrations of IL-6, IL-8 IL-10, IL-12p70, and TNF-α were quantified by CBA, and the mRNA expression of Elafin, Serpin A1, MIP1-β, RANTES, IL-1β, IL-18, IL-22, Caspase 1, and FoxP3, in PBMCs through qPCR. Compared with the low-risk group, the MSM in the high-risk group exhibit higher mRNA levels of Serpin A1 ($p=0.018$) and a tendency to express more MIP1-β ($p=0.06$). No differences were found regards the other soluble factors evaluated.

The intracellular expression of Granzyme B, MIP1-β, TNF-α, and IFN-γ by CD4+ and CD8+ T cells was measured after stimulus with a pool of peptides from HIV-1 subtype B consensus Gag or SEB. Likewise, the levels of IL-10, IL-12, IL-1b, IL-6, IL-8, and TNF-α in the culture's supernatants were quantified by CBA. All the 60 MSM showed a strong specific response to SEB, however, the HIV-1 specific response only was detected in some subjects and there was not a defined pattern of response by risk group. No differences were found in the magnitude nor index of T cell polyfunctionality between the MSM groups in response to HIV-1 Gag peptides. Finally, no statistical differences were found between the groups in the levels of IL-10, IL-12, IL-1β IL-6, IL-8, and TNF-α in the supernatants of HIV-1 stimulated cells.

To explore the different NK cell subpopulations in both MSM groups, the CD56 and CD16 expression was analyzed in the $CD3^-$ cells from fresh peripheral blood. Both groups of MSM showed a similar distribution of NK cell subpopulations. Then, the expression of IFN-γ, Perforin, Granzyme B, and NKG2D was assessed in total NK cells from PBMCs after being stimulated for 48 hours with the combination of IL-12 and IL-15. The high-risk group showed a lower percentage of total NK cells expressing NKG2D ($p=0.021$). No differences were found in the magnitude nor polyfunctional index in those cells.

To explore the macroscopic and microscopic state of anal mucosal tissue and the expression of the antiviral genes HPN1, HBD2, HBD3, SLPI, RNAse7, TRIM5α, and

APOBE3G, an anal sampling was made. In both groups, most MSM presented no symptoms, a healthy anus, and a low frequency of intraepithelial lessons. No statistical differences were found regarding symptoms or the macro and microscopic state of anal mucosa between both groups. Quantification of the mRNA expression of the antiviral genes was no possible due to the low quantity of mRNA obtained, so a qualitative comparison of the detected genes was made between both groups. There were no statistical differences in the detection of the antiviral genes HPN1, HBD2, HBD3, SLPI, RNAse7, TRIM5α, and APOBE3G.

In this study, we managed to reach a population with very difficult access given the conditions of the vulnerability of the LGBTIQ+ population in our country and the inclusion criteria that directed the recruitment of individuals with high-risk sexual behaviors in the absence of sex work. The results of this study show that MSM individuals included in the high-risk group have higher-risk sexual behavior than previously reported associated with seroconversion in international MSM cohorts, which demonstrates their high probability of exposure to HIV. Significant differences were found between the groups of high and low risk of exposure concerning sexual behaviors and the immunological factors that have been previously associated with resistance to HIV-1 and that limit its transmission/acquisition.

Despite we did not find statistically significant differences between the groups of MSM in all the immunological variables evaluated, it is interesting that the group of individuals who presented extremely risky sexual behaviors, presented, too, a low activation profile of T cells. The lower activation profile of T cells has been described previously in some HESN, which have low expression of T cell activation markers and higher percentages of Treg cells. The study of Camara *et al.* shows that HESN from serodiscordant couples of Senegal have lower levels of CD38 expression on CD4+T cells than control subjects. This same low expression of CD38 by CD4Tcells has been described in MSM HESN from the Amsterdam cohort, in

HESN individuals of serodiscordant couples from the Central African Republic, as well as in HESN from the female sex workers of the Pumwani cohort in Kenya. Moreover, it has been described that T cells with low expression of activation molecules have a lower susceptibility of infection in vitro with HIV-1, and the persistent HIV-1 seronegative status is associated with lower T Cell activation.

To our knowledge, this is the first report of a sociodemographic, sexual and immunological characterization of a Colombian cohort of HIV seronegative MSM. Taken together, our results suggest that the protection against the HIV-1 infection in the studied MSM is mediated by a combination of factors/processes: (1) A quiescent immune profile with low basal activation but also a fully functional capacity of CD4+ T cells and NK cells. (2) A protection exerted by HLA-B*18 through the presentation of essential antigens for the HIV-1 viral cycle, leading to a highly efficient specific CTL response by CD8+ cells. (3) The protection led by the HLA-B*18 is, in turn, complemented by the low frequency of HLA-B*35 alleles which are known to recognize and present a reduced repertoire of peptides and are associated with HIV-1 susceptibility. (4) A high responsiveness capacity of NK cells drove by the inhibitory KIR genes/AA haplotype, due to the lower threshold of activation and a stronger response that is exhibited by the NK cells that are licensed by this type of KIRs. Finally (5), A protective anti-viral effect (direct and indirect) by the Serpin A1. It is necessary to continue the study of MSM in high risk of exposure to HIV-1 to better understand their natural response to the virus and improve the prevention and therapy strategies against HIV-1.

Systematic Summary

This thesis consists of five chapters and an annex section. Chapter 1 is a general introduction about the phenomenon of natural resistance to HIV-1 and the characteristics of individuals/cohorts that exhibit said resistance. Chapters two to four present the epidemiological, immunological and genetic profiles of the studied MSM. Chapter five shows a general discussion, and, the annex section shows the goals met during the Ph.D. program, such as awards, oral and poster presentations, as well as other original papers that are the result of side projects of this thesis and I am a co-author.

The first chapter is compounded by three main topics: (i) HIV-1 infection epidemiology, (ii): HIV-1 infection natural history and (iii) Natural Resistance t HIV-1. This final topic is divided by the next subtopics: a. HIV-1 exposed seronegative individuals (HESN); b. MSM as a population to find HESN; c. Genetic factors associated with natural resistance to HIV-1; d. Cellular response and HIV-1 natural resistance; e. Humoral immunity and HIV-1 control, and, f. Immune Quiescence and protection against HIV-1 infection.

The second chapter presents the epidemiological data of the studied population through an original published article and a summary of data. The original article "*Sexual Behaviors and Factors Associated with Condomless Sexual Practice in Colombian Men Who Have Sex with Men at High Risk of HIV Transmission"* published in the Archives of Sexual Behavior journal (Classified as Q1 by Scimago Ranking) shows the analysis of epidemiological data from the 92 MSM recruited in phase I of this study. The summary data shows the epidemiological characteristics of those 60 MSM who were included in the second study phase, with the comparison of that data between the low- and high-risk of MSM.

The third chapter corresponds to the immunological analysis of the studied MSM. This chapter is compounded by the original article *"Seronegative MSM at high-risk of HIV-1 acquisition show Immune quiescent profile with normal immune response against common antigens"*. This paper is pre-published in the bioRxiv Immunology server and submitted to the PlosOne journal (Classified as Q1 by Scimago Ranking).

The fourth chapter shows the MSM's genetic analysis. The data are presented as an original paper (short report) and are going to be submitted in a Q1 journal for its evaluation. The findings of the frequencies of HLA and KIR alleles have been reported at Allele Frequencies Net Database (http://allelefrequencies.net/) and to our knowledge, this is the first report of KIR alleles in the Colombian population.

The fifth chapter presents the general discussion about the found results in this thesis. Finally, the annex chapter shows the other goals met during the Ph.D. process, including awards, published papers, published abstracts and submitted papers.

Published paper: NK cell activity and CD57+/NKG2Chigh phenotype are increased in men who have sex with men at high risk for HIV. DOI: 10.3389/fimmu.2020.537044.

- Accepted paper (written in spanish): "Comparación psiconeuroinmunológica de hombres que tienen sexo con hombres con diferentes comportamientos de riesgo sexual"
- Submitted paper: "A two group's psychoneuroimmunological comparison of men who have sex with men in Medellin, Colombia".

Chapter 1:

Background and General Methodology

“The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom”.

Isaac Asimov

“El aspecto más triste de la vida en este preciso momento es que la ciencia reúne el conocimiento más rápido de lo que la sociedad reúne la sabiduría”

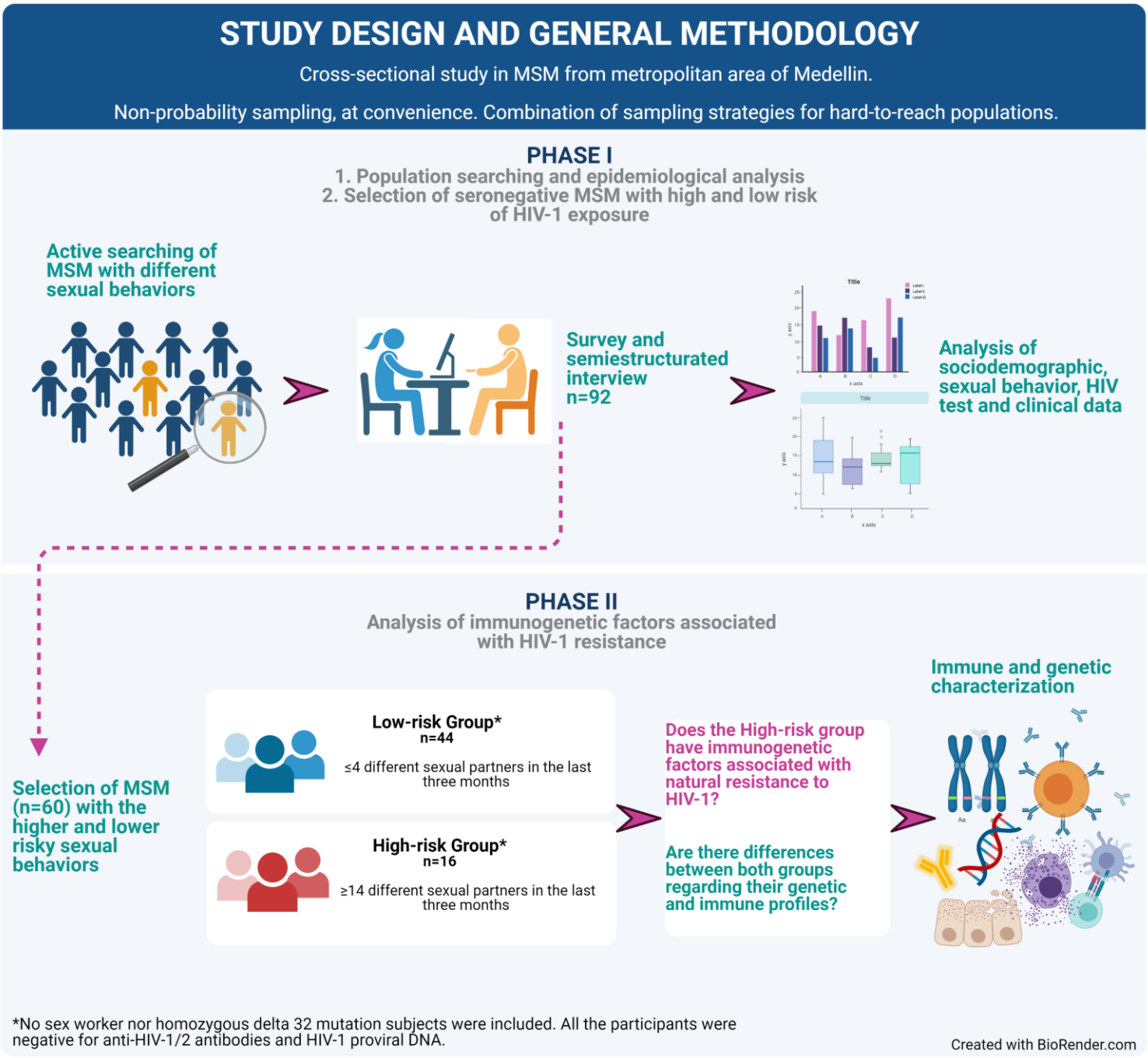
Isaac Asimov

Introduction

Natural resistance to HIV-1 refers to specific characteristics of some individuals that explain how they can prevent infection despite repeated exposure to HIV-1. This phenomenon, far from being unifactorial, involves multiple processes and mechanisms that range from genetic conditions to innate and adaptive immune responses.

This chapter seeks to approach the phenomenon of natural resistance to HIV-1, where its complexity, dynamism, and multidimensionality are evident. The reader will take a journey from the basic conceptualization of the phenomenon of natural resistance, going through the description of the individuals/cohorts that exhibit said resistance, to the identification of the genetic and immunological factors that determine it. Finally, the reader will have an overview of the general study methodology.

Graphic abstract



Background

HIV-1 infection epidemiology

Human immunodeficiency virus (HIV) infection is one of the main global public health problems. According to the *UNAIDS Data 2020* report, approximately 38 million people are currently living with HIV and new infections continue rising by 72% in eastern Europe and central Asia, by 22% in the Middle East and North Africa, and by 21% in Latin America. In 2019, 690,000 lives were lost to AIDS-related illnesses despite the availability of effective treatments[1].

The key populations (sex workers, people who inject drugs, prisoners, transgender people, and gay men and other men who have sex with men) still being the most affected people by HIV infection, who are at an elevated risk to acquire the virus, in part due to discrimination and social exclusion. These populations accounted for 62% of new adult infections globally in 2019, and almost a quarter of them (23%) were in men who have sex with men (MSM)[1]. In many high-income countries, HIV epidemics among MSM continue to climb, even though the overall HIV epidemic is in decline; similarly, in low- and middle-income countries across Africa, Asia, the Caribbean, and Latin America, HIV rates among MSM exceed those of the general population[2,3]. MSM accounted for more than 40% of new infections in Asia and the Pacific and Latin America, and nearly two-thirds (64%) of new infections in western and central Europe and North America. Young MSM (aged 15 to 24 years) are at particular risk in high-income countries of western and central Europe and North America, accounting for 36% of infections in the region in 2019[1].

In Latin America, 2.1 million people are living with HIV (PLWHIV) and 120,000 get the infection in 2019. From the PLWHIV, 44% are MSM, 22% are clients of sex workers and sex partners of all key populations, 6% are transgender people, 3% are sex workers, 2% are people who inject drugs and the 23 remaining percentage are other people[1]. The distribution of HIV infection in Colombia shows a concentrated epidemic, where the highest proportions of reported cases of HIV and AIDS are in the age group of 20 to 39 years old. In 2017, an estimated 150.000 people were living with HIV, with a prevalence in the general population of 0.4% and 17% in MSM, which shows a prevalence of 43 times major in Colombian MSM than the general population[3,4]. The groups with the greatest prevalence are MSM, transgender women, street dwellers, intravenous drug users, women sex workers, and persons deprived of their liberty[5]. The main route of transmission of the virus in Colombia is the sexual one, which corresponded to 83.12% of the transmissions in 2015; in this year, 71.98% of the people who acquired the infection through sexual intercourse were men[5]. In Colombia, there are still prejudices, stigma, and discrimination against the LGBTI population[6]; and there is discrimination against people living with the virus[7].

HIV-1 infection natural history

HIV infection is caused by two types of viruses, HIV-1 and HIV-2, which in turn are classified into groups, subtypes, and recombinant forms. HIV-1 is the most widely distributed, globally causing more than 90% of infections[8]. HIV-1 infection naturally occurs in three stages, starting with the acute phase characterized by high viral

loads, followed by the chronic phase where a drop in viral load is evidenced thanks to the activation of the immune response, and finally, the AIDS phase (acquired immunodeficiency syndrome), in which a marked elimination of CD4+ T cells, high viral loads and appearance of opportunistic diseases and oncogenic processes that eventually lead to death are observed[9,10].

The course of infection in the absence of antiretroviral therapy is heterogeneous[10], with HIV-1 positive individuals who progress to the stage of AIDS in less than 5 years, called rapid progressors, others who do so between 6 and 10 years (mainly between 8 and 10 years), named typical progressors and, those who reach AIDS 10 years after being diagnosed, called long-term non-progressors (LTNPs)[11–13]. The identification of LTNPs is currently hard to address thanks to the broader access to diagnosis and antiretroviral therapy worldwide. However, there is a small subset of PLWHIV that can spontaneously maintain an undetectable viral load (VL) in the absence of previous or ongoing antiretroviral therapy, which would be in the LTNPs classification; these people have been called controllers and are divided into two groups according to the VL they exhibit in absence of treatment: viremic controllers (VCs) who achieve a virologic control characterized by 200 to 2,000 RNA copies/mL while also maintaining elevated CD4+ T cell counts (typically ≤ 500/μL); at their time, elite controllers (ECs) show a major virologic control, showing undetectable levels of viral load (<50 copies/mL), and elevated CD4+ T cell counts (200 to 1000/μL)[14]. Although no strictly defined profile unanimously characterizes each type of progression, multiple studies have shown various immunological, genetic, and

virological differences associated with the different forms of evolution of HIV-1 infection[10,12,15,16].

Natural Resistance to HIV-1

HIV-1 exposed seronegative individuals (HESN)

Despite the natural course of HIV-1 infection, small populations of individuals have been identified who, although repeatedly directly exposed to the virus, do not present serological or clinical evidence of infection; these people are known as HIV-1 exposed but seronegative individuals (HESN)[17]. These individuals have aroused great interest in the scientific community since understanding the factors that generate their resistance to infection not only allows a better understanding of the determining mechanisms in the transmission of the virus, but their study can provide important tools for the development of strategies. Therapeutic and preventive against HIV-1.

HESN comprises different groups, within which people with sexual or other exposure to the virus have been identified. Among HESN with sexual exposure are MSM, sex workers, and individuals who have HIV-1 positive sexual partners (serodiscordant couples). On the other hand, there are HESN in touch with the virus through routes other than sexual, such as intravenous drug users who share contaminated needles, children from HIV-positive mothers, hemophiliacs, and other multi-transfused patients, in addition to health workers who suffer biological risk accidents[17,18]. In

general, the different cohorts of HESN that have been studied are clustered into three main groups: serodiscordant couples, individuals with high-risk sexual behaviors, and individuals not sexually exposed[18].

Thanks to the interest that HESN have aroused and their consequent study, multiple genetic and factors that have been associated with natural protection against HIV-1 or reduced exposure have been described[19–22]; however, only two genetic factors are being related with a direct cause of HIV-1 resistance, (mutation CCR5 Δ32 and the mutation in the Transportin 3 gen (*TNPO3*)). Until now, no HESN having the *TPNO3* mutation are reported, and the CCR5 Δ32 mutation only can explain 3.6% of HESN because of its low frequency[21,23], so, implying the importance of understanding the other mechanisms that must be implied in the natural resistance to HIV-1 infection. When HESN individuals from different cohorts are studied, it has been found that the factors associated with the natural resistance against HIV-1 vary from one individual or cohort to another, suggesting that the mechanisms involved in resistance to HIV-1 infection are complex and act dynamically.

MSM as a population to find HESN

The study of natural resistance to HIV has been directed at HESN that are exposed to the virus in both, sexual and nonsexual routes. However, the finding of HESN is increasingly complex due to their low frequency[18] and the advances in medicine that have positively impacted efforts to mitigate the exposure. The fourth and fifth-generation diagnostic tests[24], the viral load quantification[25], the increasing

opportunity in the application of diagnostic tests[24], the early starting of anti-retroviral therapy[26], the pre and post-exposure prophylaxis (PrEP and PEP, respectively)[27], and the high effectivity of the treatment (that limits to zero the probability of HIV transmission in PLWHIV with undetectable viral loads[28]), have had an irrefutably positive impact in the control of HIV infection[29,30]. Though, those conditions make it less and less likely to identify an individual who will be exposed to the virus in a sustained and long-term way, which reduces the possibility of finding individuals who exhibit a natural resistance to HIV. In this sense, the study of MSM who have risky sexual behaviors acquires great importance as a target population to seek possible HESN. Since the first reports of HIV resistance in 1989[31] to the present, MSM has represented an adequate cohort for the study of HESN individuals[18], being approached both in studies of serodiscordant couples and in cohorts of individuals with risky sexual behaviors[18]. The high prevalence of HIV-1 infection in this population[1] plus the highest probability to acquire the infection through anal sex[32], put MSM who practice risky sexual behaviors (such as anal sex without a condom and having multiple partners), at extreme risk of HIV-1 exposure[33]. Thus, those MSM who in addition to having risky sexual practices do not have access to PrEP, become a population of great interest for the search for HESN individuals and represent an important opportunity to continue the analysis and better understanding of HIV-1 natural resistance. Therefore, in this study, we aimed to find seronegative MSM with high-risk behaviors that do not use PrEP in Medellin-Colombia to study in them possible immunogenetic factors associated with HIV-1 natural resistance.

Genetic factors associated with natural resistance to HIV-1

- **CCR5 delta 32 mutation (CCR5 Δ32)**

The CCR5 delta 32 mutation affects HIV entry into the host cell. The entry of HIV into target cells begins with the approach of the virus to the cell, followed by envelope glycoprotein (gp120) binding to receptors and co-receptors, and finally, the membranes fusion. The adhesion process is relatively nonspecific and increases the efficiency of the infection[34]. The approach and adhesion can occur through the interaction of the virus envelope protein with negatively charged cellular molecules, such as heparan sulfate proteoglycans[34,35], or through the attachment of gp120 or cellular proteins (present in the viral envelope) to target cell components such as DC-SIGN (dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin), or α4β7 integrin[34,36,37].

The binding of gp120 to its primary receptor (CD4) and the co-receptors (CCR5/CXCR4) is essential for the virus entrance into the cell[34]. gp120 binds to CD4 causing a conformational change in the variable domain 3 (V3) and the formation of a bridging sheet, thus producing an "open" position in gp120 that can bind to the co-receptor (CCR5 or CXCR4). This interaction leads to new conformational changes that expose the envelope protein gp41, which inserts into the cell membrane and, through its fusion peptides, forms a six helices bundle (6HB) that brings the viral and cell membranes closer together [Figure 1]. Then, a fusion pore is formed, allowing the capsid release to continue with the viral replicative cycle[34,38].

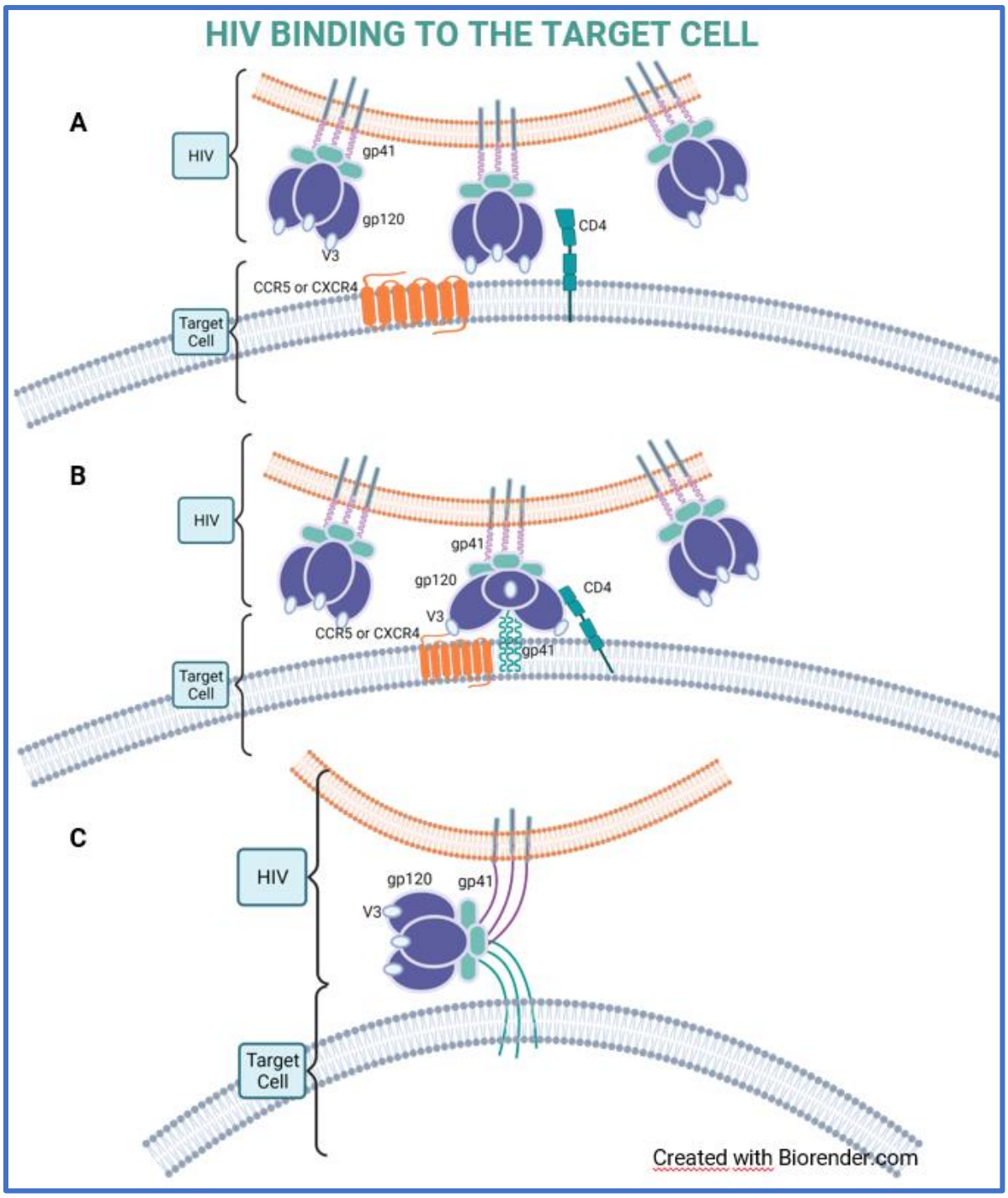
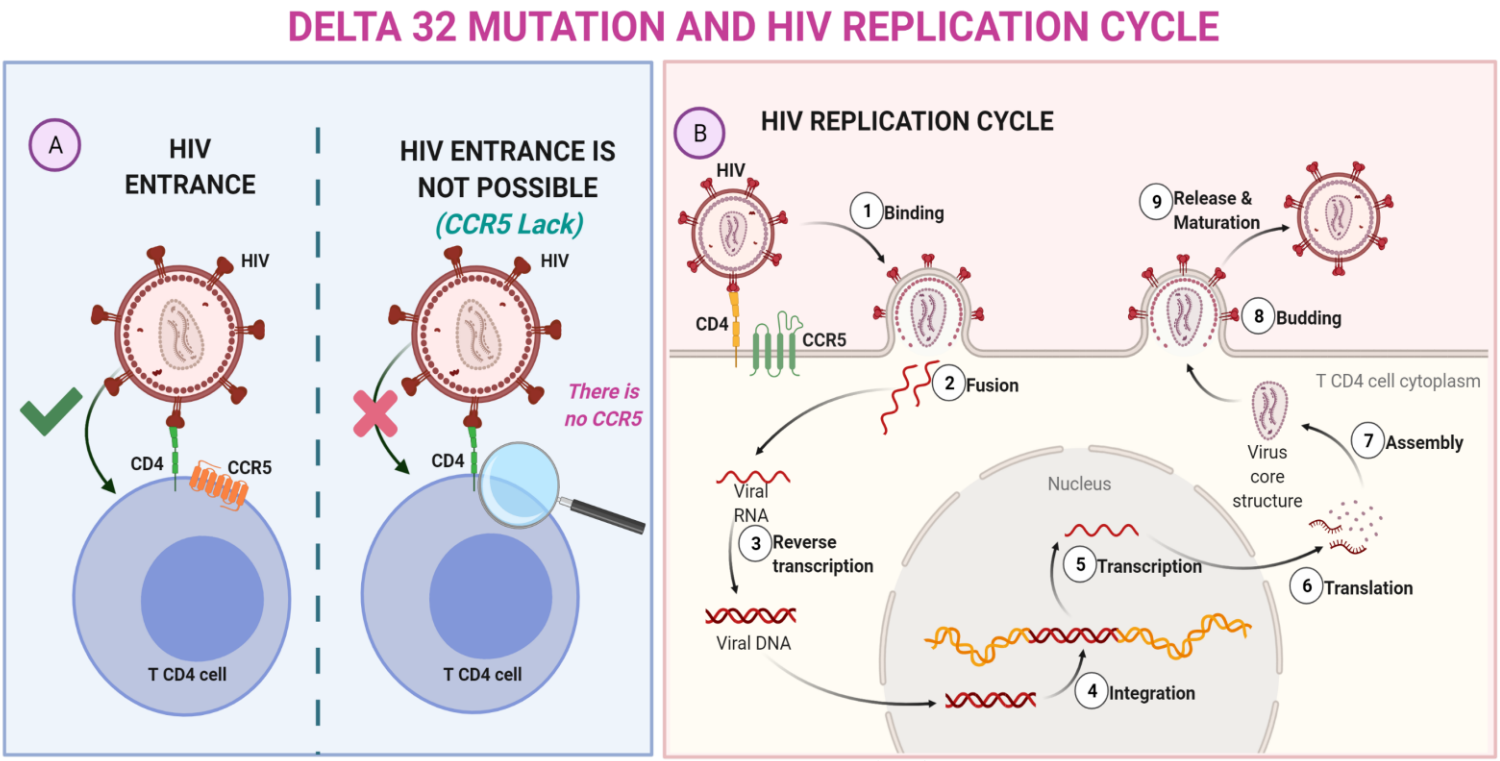


Figure 1. HIV binding to the target cell. A. Illustration of viral and cellular components implied in the HIV binding and entrance into the cell. **B.** The gp120 attaches to CD4 (primary receptor) and suffers a conformational change that permits its interaction with the co-receptor (CCR5 or CXCR4). Other conformational changes expose the gp41, which inserts into the cell membrane. **C.** The six helices bundle formed by gp41 brings the viral and cell membranes closer together. A fusion pore allows the membrane fusion and capsid release to continue the viral replicative cycle.

The CCR5 Δ32 mutation represents a deletion of 32 base pairs in the gene that codes for CCR5, the main co-receptor for HIV-1 entry[39]. This mutation produces an altered protein that is not expressed in the cell membrane of target cells, avoiding their contact with gp120, which prevents the HIV entrance and cellular infection by the HIV strains that use CCR5 as co-receptor (R5 strains) [Figure 2]. Therefore, this mutation confers resistance to infection in homozygous individuals and a slow progression of the disease in heterozygous subjects[40,41]. However, as CCR5 is not the unique co-receptor for HIV entry, people carrying the CCR5 Δ32 mutation are still susceptible to infection by X4 strains (HIV strains that use the CXCR4 as co-receptor), which means they are resistant but not immune to HIV infection[42,43].



Adapted from "HIV Replication Cycle", by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates

Figure 2. Delta 32 Mutation and HIV Replication Cycle. A. To infect cells, HIV must contact the CD4 receptor and a co-receptor. The main co-receptors are CCR5 and CXR4 molecules, expressed in the membrane of several cells, such as the CD4+ T cell. The CCR5 delta 32 mutation in the homozygous state, disables the expression of the CCR5 receptor on the surface of leucocytes, which induces resistance to be infected by HIV. **B**. Once the HIV can be in touch with the CD4 receptor and any co-receptor, the HIV replication cycle starts. 1. Binding: HIV attaches itself to receptors and co-receptors on the cell surface. 2. Fusion: The HIV envelope and the cell membrane join together, allowing HIV to enter the cell. 3. Reverse transcription: HIV converts its RNA genetic material into DNA using the viral enzyme reverse transcriptase. This conversion allows HIV DNA to enter the cell nucleus and fuse with the cell's DNA. 4. Integration: HIV uses the viral integrase to insert its viral DNA into the cell´s DNA. 5. Transcription: Upon HIV DNA's integration, the virus uses the cell's machinery to transcribe DNA to RNA. 6. Translation: Outside the nucleus, the virus uses the cell's ribosomes to make long chains of HIV proteins. 7. Assembly: New HIV proteins and HIV RNA move to the cell's surface and assemble into an immature virus. 8. Budding: Once all the new viral proteins get assembled, immature HIV pushes itself out to the cell. 9. Release & Maturation: The viral protease breaks up the long protein chains in the immature virus, creating the mature (infectious) virus.

- **Mutation in the *TNPO3* gene**

Recently, a new mutation that confers resistance to HIV acquisition was reported, a heterozygous single nucleotide deletion in the stop codon of the nuclear import factor Transportin 3 (*TPNO3* gene), which causes limb-girdle muscular dystrophy 1F (LGMD1F)[44].

The mutation (c.2771del) corresponds to a single deletion of adenine nucleotide in the TAG stop codon of *TNPO3* gene, resulting in a TCG codon that encodes a cysteine, and generates an extension of the reading frame by 15 codons of the termination-signal within the transcript[45,46]. As result, a mutated protein is expressed, which has 15 additional amino acids at the C-terminus in comparison with the wild-type protein. The protein encoded by the *TNPO3 gene,* Transportin 3, belongs to the importin β super-family, which transports compounds in and out of the nucleus through the identification of nuclear localization signals (NLS). To have an effective replication, HIV needs the transport of the new viral cDNA into the nucleus to be integrated into the host DNA by the viral integrase [Figure 2][47]. To achieve this, the cDNA is bound to the "pre-integration complex" (PIC) compound by the viral genetic material, viral proteins (including Vpr, matrix and integrase), and host proteins such as host cell LEDGF/p75, that have been identified as possessing potential NLS, through which promote the transport to the cellular nucleus using the Transportin 3 or Importin 7 (IMP7) proteins[47,48]. It has been documented that Transportin 3 binds directly to integrase and, after the transporting into the nucleus, interacts with nucleocapsid proteins and cellular tRNA that are bound to PIC to displace them and facilitate the cDNA integration[49,50]. Rodríguez-Mora *et. al.*

demonstrated that the PBMCs of patients with the mutated TPNO3 protein showed an 18-fold reduced rate of HIV-1 replication compared to healthy controls[44]. Thus, the c.2771del mutation in the *TNPO3* gene is the second resistance factor to HIV-1 infection after the well-known CCR5 Δ32 mutation[44].

- **Polymorphisms in coreceptors and soluble factors**

The protective effect against HIV-1 of soluble factors that are natural ligands of virus entry coreceptors mainly lies in the coreceptor occupancy limiting virus binding[51,52]. Likewise, the occurrence of polymorphisms in the coreceptors or their ligands can confer a significant protective effect[52]. This phenomenon is explained because genetic variants lead to changes in the encoded protein, resulting in an increased binding affinity between the receptor and ligand (generating an increased competition for virus binding) or in the receptor impaired expression/function[51].

The CCR5 and other chemokine receptors play an important but not vital biological role, and the robustness and redundancy of the chemokine system allow the preservation or compensation of their functions[53], as happens in homozygous individuals for the CCR5 Δ32 mutation who do not have evident phenotypic or functional disfuntionalities[43,54]. CCR5 mediates immune response through chemotaxis but also acts as a costimulatory molecule[43,55]. This receptor is expressed by several cell types such as T cells, monocytes, macrophages, dendritic cells, neurons, astrocytes, microglia, epithelial and endothelial cells, fibroblasts and cells from vascular smooth muscle[43,56].

Various polymorphisms in the CCR5 gene have been described in multiple human populations[57,58]; however, most of them do not significantly impact the functionality of the protein[59]. Blanpain *et al.* showed that of the 16 polymorphisms most commonly described in human populations, 10 exhibited cellular expression and function similar to the CCR5 wild-type protein. Nevertheless, the remaining six variants, which were different from the CCR5 Δ32 mutation, showed alterations in the function, linked mainly by intracellular trapping, decreased expression on the cell surface, changes in the cell activation capacity and the binding affinity to the ligands[57].

Several polymorphisms in the *CCR5* and the *CCR2* gene have been associated with lower susceptibility to HIV-1 infection and slow progression of the disease[60,61]. A variant of the promoter region of CCR5, consistently in a C to T substitution 12 kb downstream in the regulatory region of the CCR5 gene has been related to HIV protection[60]. Likewise, the variant CCR5-2459-G (an A to G substitution) is more frequent in HIV-1 low progresors[62,63], and experimental assays have demonstrated that the protein encoded by this variant is expressed in less quantity in both T-helper cells and monocytes, which is related to its association with HIV-1 resistance[64]. In the same line, the variant CCR5-2135T, which is associated with a low CCR5 expression, was related to a protective role in vertical HIV transmission in children from Malawi[65]. Different haplogroups of CCR5 and CCR2 also have been associated with protection against HIV-1. Vega *et. al.* found that the HHC haplotype in CCR5-CCR2 in combination with the haplotypes T-T in CCL3 and G-C in CCL5 was associated with HIV-1 resistance in Colombian serodiscordant couples[66]. Likewise,

Zapata *et. al.* found that the HHF haplotype in CCR5-CCR2 is related to HIV-1 resistance in Colombian HESN[61].

Although rare, R5 strains of HIV can use the CCR2 for cell entry[52]. In several cohorts of HIV-1 infected people worldwide, individuals carrying the CCR2-64I polymorphism in the *CCR2* gene showed slow progression of HIV-1 infection[67]. However, this variant does not appear associated with avoiding HIV-1 acquisition[68]. This polymorphism consists of a substitution of G for A, leading to the replacement of valine by isoleucine at position 64 in the first transmembrane domain of the protein, but is not associated with the protein structure or function alteration[68,69]. The HIV-1 protective mechanism of the CCR2-64I variant remains unclear but appears to be related to the ability of CCR2 to form homo or heterodimers with other chemokines receptors[70]. CCR2-64I can dimerize with CCR5 and CXCR4 sequestering them in the endoplasmic reticulum and thus, reducing their expression[51,70]. The CCR2-64I and CCR5-59653T polymorphisms are in strong linkage disequilibrium, demonstrated in 53 populations of people worldwide, where 96.92% showed the same genotype for both variants[60]. Similar to the CCR2-64I, the CCR2-I allele has been associated with HIV-1 resistance in Colombian serodiscordant couples[61].

Conversely to the frequent polymorphisms in CCR5 and others chemokine receptor regions[57], CXCR4 is a highly conserved protein, even between different species[71], with a low variant frequency observed in studied human populations and not evidenced effect in the function[72,73]. The low frequency of *CXCR4* gene polymorphisms can be explained by its vital function. CXCR4 is expressed by most leukocyte subsets (in circulation, bone marrow and lymphoid organs), stem,

endothelial, stromal and epithelial cells[74]. The binding to their ligands (the canonical ligand SDF-1-CXCL12-, the atypical chemokine receptor 3-ACKR3- and the migration inhibitory factor-MIF-)[71] generates signaling pathways that control cell migration, hematopoiesis, cell homing, cell retention in bone marrow, tissue regeneration, and stem cell activation[75]. Moreover, when CXCR4 or SDF-1 are deleted in mice models, fetal lethality, impaired hematopoiesis, abnormal cerebellum development and cardiac septum are observed[75–77].

Two CXCR4 splice variants have been reported, CXCR4-A and CXCR4-B[74]. While the second variant (-B) is produced by mRNA splicing and is the most commonly expressed form, the first variant (-A) is a product of unspliced mRNA, with a different start codon and four amino acids longer than CXCR4-B[74,78]. Although a low effective function of CXCR4-A has been described[78], there are no functional differences between both proteins, and CXCR4-A is considered a backup receptor for CXCR4-B[74,79]. Nevertheless, in the context of HIV infection, Duquenne *et al.* demonstrated that CXCR4-B and not CXCR4-A produces the effective HIV-1 entrance and cell infection in modified cell lines (CXCR4-A and HOS-CXCR4-B), which expressed the same cell surface density of each variant[79]. Moreover, they observed that infection by R5 strains induces CXCR4-B expression, and the CXCR4-B/CXCR4-A mRNA ratio in PBMCs correlated with HIV RNA plasma levels in infected individuals[79].

Polymorphisms in the chemokines MIP1-α (CCL3), MIP1-β (CCL4) and RANTES (CCL5), the three principal natural ligands of CCR5, have been demonstrated in HESN cohorts and related to HIV-1 infection resistance[66,80]. Vega *et al.* observed an association of the MIP1-α T-T haplotypes and G-C in RANTES with resistance to HIV-

1 infection in Colombian serodiscordant couples[66]. Gonzalez *et al.* showed that AG-containing RANTES haplotype pairs were related to delay progression in a Japanese cohort[81]. Paximadis *et al.* found that the haplotype A1 of MIP1-α (CCL3) was associated with resistance to HIV-1 acquisition in African exposed uninfected infants[82]. Similarly, the SDF1-3'A has been associated with controlling HIV infection in HIV-infected individuals homozygous (SDF1-3'A/3'A) who show a remarkable level of protection against AIDS in pooled or separated cohorts[83]. Some of the chemokine polymorphisms would modify the secretion levels of these proteins influencing the resistance to HIV-1 infection. A high expression of SDF-1 generates a negative signal of CXCR4 expression, limiting its disponibility[84] and the high expression of CCR5 ligands has been associated with a robust CD4+ T cell-specific response against peptides of HIV-1 gp120 in HESN[80].

- **KIR and HLA alleles**

Natural killer (NK) cell activation is mediated by the balance between activating and inhibiting signals from surface receptors. Inhibitory receptors on NK cells interact with human leukocyte antigen molecules -HLA- (also called major histocompatibility complex -MHC-)[85], and the reduced or aberrant expression of these molecules, as occurs during viral infections, leads to NK cells activation. Thus, NK cell activation can occur in the absence of the recognition of their ligands by the inhibitory receptors, as well as after the recognition of cellular stress ligands that interact with the activating receptors[86]. NK cell receptors are grouped into two distinct families, a group of CD94-type C lectins (NKG2) and the immunoglobulin-like receptor

superfamily, which includes KIR receptors (*Killer-cell immunoglobulin-like receptor*), which contain activating and inhibiting receptors[86].

KIR receptors are transmembrane glycoproteins with 2-3 immunoglobulin-like domains with short or long cytoplasmic tails. KIR genes segregate independently of HLA genes and are located in one of the most variable regions of the human genome in terms of gene content and sequence polymorphisms, which contributes to the generation of a wide repertoire in the expression of KIR receptors in NK cells[87]. To date, 15 genes and two pseudogenes have been described at the KIR locus; however, there are polymorphisms of each of these genes. The number of genes contained in the genome varies greatly between individuals, creating haplotypes. Haplotype A contains mainly inhibiting KIRs and an activating KIR, while haplotype B contains mainly activating KIRs[87,88].

The activating receptors, identified with the nomenclature (DS), contain short cytoplasmic tails that are non-covalently associated with adapter molecules such as DAP-12, which in turn has activation motifs based on tyrosine (ITAM), except for KIR2DL4 which, despite having a long tail, is associated with FcεRI containing ITAM motifs[89]. For their part, the inhibitory KIRs contain long cytoplasmic tails (identified with the DL nomenclature), with inhibition motifs based on tyrosine (ITIM) that recruit phosphatases and inhibit the activation of the effector functions of NK cells as well as its adherence to the target cell[90].

KIR receptor ligands correspond to HLA-I molecules[91,92]. HLA class I molecules (HLA-I: HLA-A, -B, and -C; and the non-classic HLA-E, -F, -G, and -H) are expressed by most nucleated cells, and the HLA class II (HLA-II: HLA-DR, -DQ, and -DP) are mainly

expressed by B cells, activated T cells, macrophages, dendritic cells, and also by epithelial cells at the tymus[93]. HLA-I molecules allow the immune system to discriminate between “the self and the non-self”, through the binding of self-peptides in normal conditions[94] and regulate the NK cell function, through the interaction with KIR receptors generating signals to activate or inhibit it[86,94,95]. The HLA class I and class II molecules participate in the production of the adaptative immune response, acting in the immunological synapses since antigen-presenting cells (APCs) use them to show the extracellular non-self-antigens to T CD4+ (HLA-II) and intracellular non-self-antigens T CD8+ (HLA-I) cells, thus, resulting in the cytokine production and the helping to increase the immune response by other cells but also initiating a cytotoxic T-cell (CTL) response[94,96,97]. The HLA system represents one of the most polymorphic regions in the genome. This characteristic gives these molecules a significant capacity to bind and load more diverse types of peptides that will be recognized by CD8+ T cells. Likewise, as these molecules also act as specific ligands for some KIR-type receptors on NK cells, their polymorphisms generate a wide repertoire of NK cell responses, since they are also capable of recognizing polymorphisms in the class I molecules[92,98].

The immune response against HIV-1 can be influenced by the genetic background of each individual, so, the presence of certain HLA and KIR haplotypes confers greater or lesser HIV-1 infection susceptibility. In this sense, it has been identified that the HLA-B57 allele is more frequent in elite controllers[99]. Likewise, the HLA-B27 allele has been associated with a slow progression to the AIDS phase in patients living with the virus[100]. The study of HLA-I in HESN individuals has shown that, as

observed in controller patients, certain HLA alleles confer protection against HIV-1 infection since they are found in a higher proportion in HESN when compares with the general population and PLWHIV. The following HLA alleles have been associated with HIV-1 protection: B*13:02, B*14/14:02, B*27/27:05, B*42:01, B*44:03, B*51, B*52:01, B*57/B*57:01, B*57:02, B*57:03, B*58:01, B*81:01, A*24, A*25/25:01, A*32/32:01, and A*74/74:01[101–103].

Similar to certain HLA, the KIR genes have been associated with natural resistance to HIV-1, but with divergent results about the specific genes that could contribute to the HIV-1 resistance. Although some KIR, such as 3DS1, are more frequent in HESN than HIV-1 infected individuals or the general population, studies report no association with natural resistance to HIV. The sample sizes and methodologies could affect the different observations; however, from a biological point of view, this divergence could be explained by how polymorphic the KIR are[104]; the haplotype patron of inheritance, which leads to variability in the proportion of activating/inhibitory genes; the SPNs generating different levels of expression and affecting the ability to bind the ligands, and the interaction with polymorphic HLA molecules[104].

In 2011, Guerini *et. al.* reported a reduced frequency of the inhibitory KIR3DL1 and the KIR3DL1/Bw4 inhibitory complex and an increased frequency of KIR3DS1 and the activator Bw4/3DL1-/3DS1 complex in Italian HESN individuals compared with HIV patients[105]. Conversely, higher frequencies of both activating KIR2DS5 and inhibitory KIR2DL3 were found in Indian HESN infants as compared to HIV-1 positive infants[106]. A study developed in serodiscordant couples from Argentina reported

that KIR3DS1(3DS1/3DL1)-Bw4 combination was significantly higher in HESN patients versus the discordant and PLWHIV, while the KIR3DL1/KIR3DL1 homozygosity was significantly decreased[107]. On the other hand, Paximadis *et. al.* found that KIR2DL2/KIR2DL3 was underrepresented in intrapartum HIV-transmitting mothers from South Africa, compared to non-transmitting mothers. Moreover, they found that KIR2DL3 in combination with its HLA-C1 ligand (C1) as well as homozygosity for KIR2DL3 with C1C2, were underrepresented in the infected infants compared to HESN infants[108]. Despite the differences in the frequency of KIR and KIR/HLA found in the HESN populations previously described, Vince *et. al.* did not find a protective role of KIR against HIV infection in hemophilic HESN from the USA, UK, Switzerland, Italy, Netherlands, Spain, Greece, Germany, and Japan[109]. To our knowledge, there is no information about KIR genes in Colombian HESN nor the general population.

Although the findings are different between HESN populations regarding the specific KIR and do not allow to affirm with certainty which are the KIR genotypes or haplotypes that most contribute to the phenomenon of natural resistance against HIV-1 infection, there seems to be a pattern in which there is a higher presence of activating KIRs and a lower frequency of inhibitory KIRs or their complexes in HESN individuals when compared to PLWHIV and healthy controls.

To date, the pathways by which the HLA and KIR molecules protect against the progression of the disease and HIV-1 infection are not clear; however, some observations give ideas of how they would be intervening. It has been evidenced that specific HLA alleles provide significant protection due to the ability to induce a

cytotoxic T lymphocyte (CTL) response against HIV-1 Gag peptides (see the CTL response below), at the same time, other HLAs present in progressor HIV+ individuals tend to generate responses against viral accessory and envelope proteins[110]. Thus, the heterozygosity HLA is an advantage as well in the response to HIV-1 infection, since individuals who are heterozygous at the HLA locus will be able to present a broader repertoire of antigenic peptides to T cells as compared to homozygotes, thereby exerting greater pressure on the pathogen to escape the CTL responses that may, in turn, affect pathogen fitness[94,111]. Recently, Arora *et al.* (2020) assessed the contribution of HLA heterozygosity in the control of HIV-1 infection in people from Australia, Europe, and the USA[112]. They found that the heterozygous individuals present a broader diversity of HIV-1 peptides than HLA homozygous individuals and this diversity was negatively correlated with the viral load. Moreover, the HIV-1 peptide diversity was more evidenced in heterozygous individuals for the HLA-B, evidencing the strong effect of HLA heterozygosity in the HIV-1 evolution and behavior[112]. Similar findings have been shown in Caucasian HESN, where HLA heterozygosity is more frequent than HIV-1 infected or healthy people[113]. However, in the Japanese population, the control of HIV-1 was evidenced by specific HLA alleles (*B52:01 and C*12:02) than the HLA heterozygosity[114].

On the other hand, the HIV-1 protection generated by the KIR receptors seems to reside in a significant capacity to induce NK cell activation. This effect depends on the type of KIR, the quantity of expression and the interaction with HLA-I molecules (their ligands)[94,115]. In homeostatic conditions, the interaction between KIR receptors and HLA molecules generates inhibitory signaling into NK cells to prevent auto-

aggression. Otherwise, the downregulation of HLA molecules in NK-target cells, by viral infections, together with the interaction between stress molecules and their receptors, initiates the NK cell activation[116,117]. But the interaction of KIR with HLA molecules can also generate inhibitory or activating signals to NK cells[116,117]; therefore, the balance of inhibitory/activating NK signals depends not only on the presence of KIR/HLA molecules but also on their type and expression.

The activating receptor KIR3DS1 in combination with HLA Bw4 alleles (its ligand) and the absence of the inhibitor KIR allele 3DL1, resulted in a powerful effector response of the NK cell[94,118]. In 2007, Alter *et al.* demonstrated *in vitro* that KIR3DS1+ NK cells exhibited a more robust cell contact depend-inhibition of HIV-1 replication in infected cells that expressed HLA-B Bw4-80I in comparison with KIR3DS1- NK cells[119]. The HLA-B molecules are differentiated in Bw4 or Bw6 regarding their polymorphisms at positions 77-83. While Bw6 alleles do not interact with KIR receptors, the Bw4 is the ligand for KIR3DL1/S1[120,121]. HLA-B Bw4-80I corresponds to alleles that encode a specific amino acid motif with an Isoleucine at position 80[120]; the presence of those alleles together with KIR3DL1/S1, are associated with HIV-1 control, generating slow progression and preventing the infection.

The KIR and HLA receptors also can modulate the NK cell responsiveness through licensing. To be functional, an NK cell must recognize a self-HLA-I through a self-specific KIR receptor (or Ly49 receptor in mice). NK cells from mice knockout for MHC-I have a low capacity to kill MHC-I-deficient cells and NK-susceptible tumor targets and impaired cytokine production; moreover, those mutant mice are unable to reject allogenic bone marrow[117,122]. Likewise, the human NK cell responsiveness

depends on this licensing. NK cells that express self-MHC-I-specific KIRs show a wide response and cytokine production compared to self-KIR-negative NK cells[117]. The unlicensed NK cells have a weakened recognition of HLA-I-deficient target cells, nevertheless, they appear to be important to respond in particular situations such as when the target cells increase the expression of MHC-I, to avoid NK cell recognition and response interacting with the inhibitory receptors[117]. Therefore, the combination of specific KIR/HLA alleles in HESN would drive the effector function of NK cells, which could explain a proportion of the natural resistance to HIV-1 infection.

Cellular response and HIV-1 natural resistance

- **CTL Response**

As an intracellular infection, the cellular response against HIV is vital for the protection and control of the infection. Almost all of PLWHIV exhibit a broad cellular response against virus antigens (HIV-specific CTL response), characterized by CD4+ and CD8+ T cells that generate virus control and lysis of infected cells through Fas/FasL interaction, production of perforins and granzymes, and release of cytokines such as IFNγ, TNFα, MIP-1β, and RANTES, in response to the stimulus (*ex-vivo*) or by contact with cells infected with the virus (*in vivo*)[123,124]. Although this response is expected in infected people and IFN-γ measurement is frequently used as an indicator of specific CTL response, there is no direct correlation between this response and infection control[125]. However, it has been observed that T cells function (characterized by cytokine release, either individually or in combination -

polyfunctionality- and proliferation capacity[126]) is more significant in elite controller individuals than progressors[127]. This suggests an important role of CTL response in the spontaneous control of infection. Additionally, this response seems to be involved in controlling the establishment of the infection, since strikingly, HIV-specific CTL response has been observed in HESN [111,128–130]. This unexpected specific CTL response would be explained by a possible previous ineffective or "controlled" infection (especially considering that HESN are people with high exposure to the virus) as some HESN populations exhibited a low quantity of naïve T cells, an increased number of activated T CD8+ effector cells[111,131,132]. However, this differential distribution of T cell subsets could be, by itself, a particular characteristic of HESNs which is associated with their ability to prevent or control infection. Higher proportions of terminally differentiated CD8+ T cells have also been seen in HESN, and, the terminally differentiated CD8+ T cells are characterized by their high production of perforin, which is one of the most important mechanisms in the response to HIV-1[132,133]. Another possibility that could explain the unexpected specific CTL response is its broad cross-reactivity, which opens the probability that this response to HIV-1 in HESN could correspond to a heterologous response by T cells to other viruses[134–136].

Alimonti *et. al.* found that almost 40% of women in the Pumwani commercial sex worker cohort in Nairobi, Kenya, exhibited an HIV-specific CTL response, and this response was five times lower in magnitude compared to PLWHIV[137]. Nevertheless, some HESN women from this cohort had a late seroconversion, associated with the waning of HIV-specific CD8+ T cells responses due to reduced antigenic exposure

because of a reduction in sex work over the year[138,139]. Although HIV-specific CTL responses have been observed in cohorts of serodiscordant couples from Uganda[140,141], Spain[111,130], Italy[142,143] and USA[144], in none of these cohorts the response was found in all HESN. Moreover, this HIV-specific CTL response was not found in serodiscordant couples from Zambia[145] nor MSM from Seattle, USA[146].

- **NK cells**

Natural killer (NK) cells are classified by their CD56 and CD16 expression. $CD56^{bright}$ $CD16^{neg}$ cells represent approximately 10% of total NK cells in adults and have a limited cytotoxic capacity but a significant cytokine production. Meanwhile, $CD56^{dim}$ cells represent the majority of NK cells, have a low proliferation capacity and a strong cytotoxic profile. Finally, there is a group compound by the $CD56^{neg}$ $CD16^{pos}$ NK cells, which have a limited expression of lineage markers[147,148]. The cytotoxic activity and cytokine production by NK cells are driven by the balance between activating and inhibitory signals[149]. The suppression of HIV-1 infected cells by NK cells could be developed by cytotoxic activity and the secretion of IFNγ and β-chemokines. The cytotoxic activity from NK cells evokes their degranulation and release of granzyme and perforin, which can be generated by (i) infected cells showing stress signals such as UPBL1, 2, and 3, activating NK cells through NKG2D receptors; (ii) through the binding of anti-HIV antibodies (Abs) to CD16 receptor (FcγRIII) (antibody-dependent cell-mediated cytotoxicity -ADCC), and (iii) by activation signals through KIR receptors in response to downregulation of HLA class I molecules on infected cells[150]. Activated NK cells produce β-chemokines, which exert anti–HIV-1 activity by competing with glycoprotein gp120 from binding to

CCR5 and by promoting CCR5 endocytosis[151]. Moreover, NK cells can eliminate CD4+ T cells and follicular helper T cells (Tfh), reducing the HIV reservoirs[152].

Ghadially *et. al.* showed that in comparison with uninfected women, NK cells from HESN women more efficiently killed autologous immature dendritic cells, secreted interferon IFNγ, and induced the secretion of IL-12 in immature dendritic cells[153]. Similarly, a study in Vietnamese intravascular drug users (IDUs) showed that cytolytic activities of NK cells against both the NK-susceptible K562 cell line and the NK-resistant Daudi cell line were increased in HESN compared with either controls or seroconverters before or after seroconversion[154]. On the other hand, Lima *et. al.* found that $CD56^{bright}$ and $CD56^{dim}$ NK cells from HESN individuals showed higher expression of NKG2D compared with HIV-infected patients and healthy control subjects[155]. Similar to those studies, we found that NK cells from seronegative MSM at high-risk of HIV sexual exposure showed higher cytotoxic capacity and IFN-γ production in response to K562 stimuli in comparison with MSM at low-risk of exposure[156]. However, our group found a lower frequency of NK cells expressing the activation marker CD69 in Colombian elite controllers and serodiscordant couples[157].

Humoral immunity and HIV-1 control

Three or four weeks upon HIV-1 infection establishment, non-neutralizing anti-HIV-1 antibodies are developed and detected in the sera of patients[158]. It seems that B cells are primed during the acute viremic peak of the initial outburst of HIV

replication in the host; the rise of humoral response is correlated with the control of acute infection[159]. Neutralizing antibodies arise approximately three months after the primary infection and select for viral escape mutants[160]. To ensure a broad antibody response, it is necessary long-lasting antigen stimulation, as the titer and neutralizing capacity of antibodies increase over time in the infected individuals[159]. Although HIV-specific serum IgG and IgA are detected in PLWHIV, some HESN showed HIV-specific serum IgA in the absence of IgG[161]. The IgA has an essential role in avoiding HIV infection in sexual transmission, the leading entry site. HIV-1-specific IgA has been detected in urethral swabs and vaginal wash samples from Italian HESN[162,163], and, in the foreskin tissue from HESN men from Uganda[164], but not in the vaginal swabs of uninfected sex workers from the Gambia[165].

Soluble factors

Several soluble factors inhibit the HIV-1 replication without the elimination of the infected cells and are reported to be more increased in HESN people compared with PLWHIV and healthy controls[151].

- **β-chemokines:** The β-chemokines RANTES, MIP1α, MIP1β (CCL chemokines), and SDF-1 (CXCL chemokine) avoid HIV entry into the target cell. These molecules are natural ligands of the HIV co-receptors (CCR5 and CXCR4), used for the viral entry to the cells; therefore, these chemokines inhibit the entry by competing with the virus and subsequently, through downregulation of these receptors.

Zagury *et. al.* showed that activated PBMCs from Italian hemophilic HESN produced more RANTES, MIP1α, and MIP1β compared to healthy controls[166]. Similarly, the production of MIP1α was increased in Italian HESN IDUs[167]. In the Pumwani cohort of sex workers, the findings have been contradictory; Iqbal *et. al.* found higher quantities of RANTES in the cervical mucosa HESN sex workers[168], while Yao *et. al.* found lower levels of this chemokine in the cervicovaginal lavage of women from the same cohort[169]. Our group found higher mRNA levels of MIP-1β and RANTES in the genital mucosa of Colombian HESN compared to healthy controls[170].

- **α- and β-Defensins:** the anti-HIV-1 effect of cationic peptides can be explained by direct mechanism when they bind to viral gp120 and the CD4 receptor[171] or the inhibition of the protein kinase C (PKC), which is involved in the viral transcription[172]. These peptides can induce the expression of MIP1α, and MIP1β by infected macrophages[173]. Our group found higher levels of human neutrophil peptide-1 (hNP-1) and human beta-defensins (hBDs) mRNA in the oral mucosa of Colombian HESN compared to healthy controls[170].
- **SLPI** (Secretory leukocyte protease inhibitor): this protease inhibits the viral replication through a high-affinity interaction with annexin II (a cell surface molecule); thus, SLPI does not allow the interaction of phosphatidylserine residues present in the viral envelope with annexin II on the cell membrane of susceptible macrophages[174]. Pillay et. al. evidenced that the SLPI concentrations were lower in vaginal fluid samples from women whose babies became infected with HIV-1 than in samples from non-transmitting

women[175]. Likewise, higher levels of SLPI were found in the saliva of Kenyan babies who received maternal feeding from their HIV+ mothers and were not infected than in babies who acquired the infection[176]. Similarly, our group found an increased expression of SLPI in the oral mucosa of Colombian HESN when compared with chronically HIV-1-infected individuals and in healthy controls[177].

- **RNAses:** these peptides have antimicrobial activity through ribonuclease function. Higher levels of recombinant eosinophil-derived neurotoxin (EDN), RnaseA (Rnase1), angiogenin (Ang), and Rnase7 were detected in the genital mucosa of Colombian HESN compared to healthy controls[170,178].
- **TRIM5α** (Tripartite motif (TRIM) proteins): TRIM5α consists of the RING, B-box 2, coiled-coil and SPRY (B30.2) domains; the RING domain is frequently found in E3 ubiquitin ligase and TRIM5α is degraded via the ubiquitin-proteasome pathway during HIV-1 restriction, thus, this molecule binds to the viral capsid inhibiting the viral cycle at the uncoating step. Increased expression of TRIM5α in genital mucosa from Colombian HESN was reported[178].
- **APOBEC3G** (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G): this molecule exerts anti-HIV-1 activity by deamination of cytidine to uridine in minus-strand reverse transcripts, resulting in guanosine to adenosine hypermutation of plus-strand DNAs and the impairment of proviral integration into the host genome[179]. Vázquez-Pérez et. al. found that Mexican HESN expressed higher APOBEC3G than healthy controls, and the expression

significantly decreased after a year from the HIV diagnosis and subsequent treatment of their sexual partners[180]. Likewise, Colombian HESN showed a higher mRNA expression of APOBEC3G in the oral mucosa[178] and Italian HESN in PBMCs and cervical tissues[181] than healthy control or PLWHIV.

- **Elafin** (Specific elastase inhibitor): this antimicrobial and anti-inflammatory molecule inhibits neutrophil elastase and proteinase 3. Elafin is released from the N-terminus of Trappin-2 by proteolysis, its precursor, mainly by mast cell tryptase[182]. The recombinant Trappin-2/ELAFIN inhibits both X4/T-tropic IIIB and R5/M-tropic BaL HIV in a dose-dependent manner, suggesting an anti-HIV function, possibly through direct interaction with the virus [183]. The study of Jasinghe et. al. suggests Elafin interferes with HIV replication at the early steps of its cycle[184]. This protein was found to be overexpressed in both HESN women from the Pumwani Cohort compared to HIV-uninfected and infected women[185], and in oral mucosa from Colombian HESN[178].
- **Serpin A1** (Alpha 1-antitrypsin): this serine protease inhibitor exhibits anti-HIV-1 activity preventing the damage of the mucosal tissue integrity, avoiding the inflammatory response[186] and transmigration of the virus to other tissues, as well as inhibiting proteases and reactive oxygen species[187]. Our group found that Colombian HIV-controllers exhibited higher levels of SerpinA1 in gut-associated lymphoid tissue (GALT)[178].

Immune Quiescence and protection against HIV-1 infection

HIV acquisition, transmission, and pathogenesis have been associated with inflammation and immune activation. In the context of HIV-1 exposure, the presence of activated cells and inflammation at the site of HIV entry increases the risk of infection, through the recruitment of target cells and damage of epithelial barrer[188]. The increased levels of proinflammatory cytokines in blood and the entry tissue have been associated with a significant risk of HIV transmission and acquisition, as reported in people with clinical or subclinical sexual transmitted infections (STIs) and mucosal inflammatory process[189–192]. In the simian immunodeficiency virus (SIV)-macaque model, CD4+ T cells are recruited in the cervix and vagina through MIP-3α and plasmacytoid dendritic cells[193], and elevated genital levels of MIP-1α, MIP1β, IL-8, and IP-10 increase more than three-fold the risk of HIV acquisition[194]. This evidence suggests that a reduced immune activation could be favorable to HIV control and acquisition by less availability of target cells. Precisely, this phenomenon has been observed in sooty mangabeys, which despite the infection with SIV do not progress to AIDS[195]. These animals exhibit a lower frequency of CD4+ CCR5+ T cells (systemic and at mucosal tissues)[196], reduced type 1 IFN reponses[197] and regulated immune activation through IL-10 and regulatory T cells (Treg)[195]. A similar phenomenon, called immune quiescence has been observed in HESN populations and proposed as a model of HIV protection[198,199]. This phenomenon is characterized by a low activation profile of target cells, low levels of generalized gene transcription as well as low levels of proinflammatory cytokine and chemokine production in the periphery and genital mucosa, and it has been found in several HESN cohorts, including men who have sex with men (MSM)[169,198,200].

In previous studies in the Pumwani cohort, reduced susceptibility to HIV-1 infection was related to a lower frequency of activated CD4+ CD69+ T cells and a high number of Treg cells[201]. Likewise, a lower expression of CD38 in both CD4+ and CD8+ T-cell subsets was observed in exposed female sex workers from Puerto Rico[202]. This low activation profile of T cells was found in MSM from the Amsterdam Cohort, which showed lower expression of HLA-DR, CD38, and CD70 in CD4+ T cells and, lower proliferating CD4+ and CD8+ T cells (Ki67+) in comparison with MSM that became HIV positive[203].

McLaren *et al*. showed that HESN from Pumwani and Mother-Child Health cohorts in Kenya exhibited a lower basal expression of IL-6, TNFα, and IL-10 by PBMCs in comparison with controls; this difference was not evidenced upon PBMCs stimulation with phytohemagglutinin, *Candida albicans,* or flu peptides, showing an important difference between quiescence and immunosuppression[204]. In the same Pumwani cohort, Yao *et. al.* found lower levels of IL-1β, IL-8, and RANTES in cervicovaginal lavage of HESN sex workers and lower expression levels of pattern-recognition receptors (PRRs) TLR2 (Toll-like receptor 2), RIG-I (retinoic acid-inducible gene I), and MDA-5 (melanoma differentiation-associated protein 5) as well as TLR7 and TLR8 in their cervical mononuclear cells[169].

Contrariwise, other studies have shown that the immune activation state is related to protection. Biasin *et. al.* reported an increased expression of IL-6, IL-10, IL-12, IFNγ and, TNFα, and TNFβ mRNA as well as CCR5 and CXCR4 mRNA in PBMCs and cervical biopsies of HESN women[205]. Increased percentages of CD4+/CD25^{low}, CD8+/CD38+ T lymphocytes, and memory T cells were found by Saulle *et. al.* in

HESN serodiscordant couples from Italy, independently of microbial translocation[206]. Not only the adaptative immune activation has been described in HESN. IDU HESN from Philadelphia -USA exhibited innate immune cell activation by CD69 and CD107a upregulation on NK cells and CD40 and CD83 on myeloid dendritic cells (MDC)[20]. These results suggested the role of immune quiescence in HIV-1 protection is not clear.

Research Problem

HIV-1 is the cause of one of the most problematic infections in recent times. Its wide worldwide distribution, the large number of people affected since the beginning of the epidemic, the absence of a vaccine, and the stigma and discrimination that this infection has been associated with, evidence that HIV-1 infection is still a big challenge worldwide. Even though there are very effective anti-retroviral treatments, none provide a definitive cure for the infection or disease. In addition, the emergence of mutant genotypes of the virus with resistance to commonly used anti-retroviral drugs means that treatment options are increasingly smaller in many cases.

The particular panorama of HIV-1 infection makes it increasingly necessary to continue developing studies in multiple directions that allow a deeper understanding of the pathophysiology of the infection to elucidate possible critical points on which prophylactic or therapeutic intervention options can be formulated in the future. In this sense, the study of mechanisms involved in natural resistance against HIV-1 represents an interesting way to open unknown doors in the comprehension of HIV-1 infection and how the immune system naturally avoids or controls it. Although multiple studies have analyzed cohorts of people who exhibit a natural resistance to and control HIV-1, the evidence shows that this phenomenon is very complex, and the results are not the same for different populations. Thus, there is a significant necessity to continue the study of natural resistance of HIV-1, mainly in populations where there is a significant gap in the knowledge, such as Colombian MSM. The deep comprehension of the complex synergy or molecules, cells, and processes

involved in this phenomenon allows the possibility of applying them in the design of prophylactic treatment and cure strategies.

Research questions

Does seronegative Colombian MSM at high risk of infection have immuno-genetic factors associated with natural resistance to HIV-1?

Are those immuno-genetic factors different between MSM with high- and low-risk of infection?

Hypothesis

Seronegative Colombian MSM at high risk of infection present genetic (HLA and KIR protective alleles) and immune (quiescent profile*) factors associated with natural resistance to HIV-1, in comparison with seronegative MSM at low risk of infection.

*Regarding the immune factors, we hypothesized that seronegative MSM at high risk of infection would present:

- Lower levels of pro-inflammatory cytokines in PMBCs and plasma; higher levels of antiviral molecules in anal mucosa; lower percentage of activated CD4+ and CD8+ T cells and NK cells, and specific CTL response against HIV-1 Gag peptides.

General objective

To determine the immuno-genetic factors associated with natural resistance to HIV-1 in MSM at high risk of infection from Medellín, Colombia.

Specific objectives:

1. To describe the sociodemographic, clinical, and sexual behavior data from MSM from Medellin-Colombia.
2. To determine the frequency of HLA-Bw4 and KIR3DL1/DS1 alleles in MSM from Medellin-Colombia.
3. To quantify in the anal mucosa the mRNA levels of the antiviral genes HBD-2, HBD-3, HNP-1, cathelicidin, RNases, SLPI, Trim5α, APOBEC3G, MIP-1β, and RANTES.
4. To establish the basal immune profile of MSM's by measuring the expression of T cell activation biomarkers (HLA-DR, CD38, CD69, and Ki67) in unstimulated PBMCs; the plasmatic concentrations of IL-6, IL-8 IL-10, IL-12p70, and TNF-α; and, the mRNA expression of Elafin, Serpin A1, MIP1-β, RANTES, IL-1β, IL-18, IL-22, Caspase 1, and FoxP3, in unstimulated PBMCs.
5. To determine specific *ex vivo* response of MSM's CD4+ and CD8+ T cells to HIV-1 by measuring the expression of TNFα, IFNγ, MIP1β, and Granzyme B in PBMCs stimulated with Gag peptides.
6. To detect the effector functions and activation of NK cells from PBMCs stimulated with IL-12/IL-15 by measurement of IFN-γ, Perforin, Granzyme B, and NKG2D expression.

General Methodology

Study Design

A Cross-sectional study in MSM from the metropolitan area of Medellin-Colombia. This study was divided into two phases: [i] the seeking of the target population and the analysis of sexual behaviors, HIV status, and epidemiologic data of all MSM who accepted to participate in the project, and, [ii] the analysis of immuno-genetic factors associated with natural resistance to HIV in the MSM who met the inclusion criteria to be enrolled in this second phase.

Population

MSM from the metropolitan area of Medellin-Colombia.

Sampling

Non-probability sampling, by the combination of sampling strategies for hard-to-reach populations.

Population inclusion and exclusion criteria

Phase I:

Inclusion criteria

- To identify himself as MSM.
- Being ≥18 years old.
- To be a resident of Medellin and its metropolitan area.

- Accept voluntarily be part of the study and sign the informed consent.

Exclusion criteria

- Being a sex worker.

Phase II:

Inclusion criteria

- High-risk group: MSM with ≥14 different sexual partners in the last three months before the study enrollment.
- Low-risk group: MSM with ≤4 different sexual partners in the last three months before the study enrollment.

Exclusion criteria

- Homozygous delta 32 mutation.
- Positive for anti-HIV-1/2 antibodies and HIV-1 proviral DNA.

Specific Methodologies

To achieve each objective, specific methodologies were developed. These methodologies are described in each chapter according to the relationship with each specific objective. Those methodologies can be reviewed in the “Materials and Methods” section.

Ethics

This study was carried out respecting the research ethics guidelines determined by the Declaration of Helsinki and Resolution 8430 of 1993 of Colombia. According to this, it is considered a study with minimal risk for the participating subjects. Respect

for the privacy and reserve of the subjects' data, which will only be known by the researcher responsible for the study, was guaranteed. The results of this research have been and will be published in national and international scientific journals, preserving the confidentiality of the information of the participants. In addition, this study was submitted for approval by the Ethics Committee of the School of Medicine from Universidad de Antioquia (Act No.007, May 22th, 2014).

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Chapter 2

Epidemiological Analysis

Study Phase I

“Not all of us can do great things. But we can do small things with great love”.

Mother Teresa

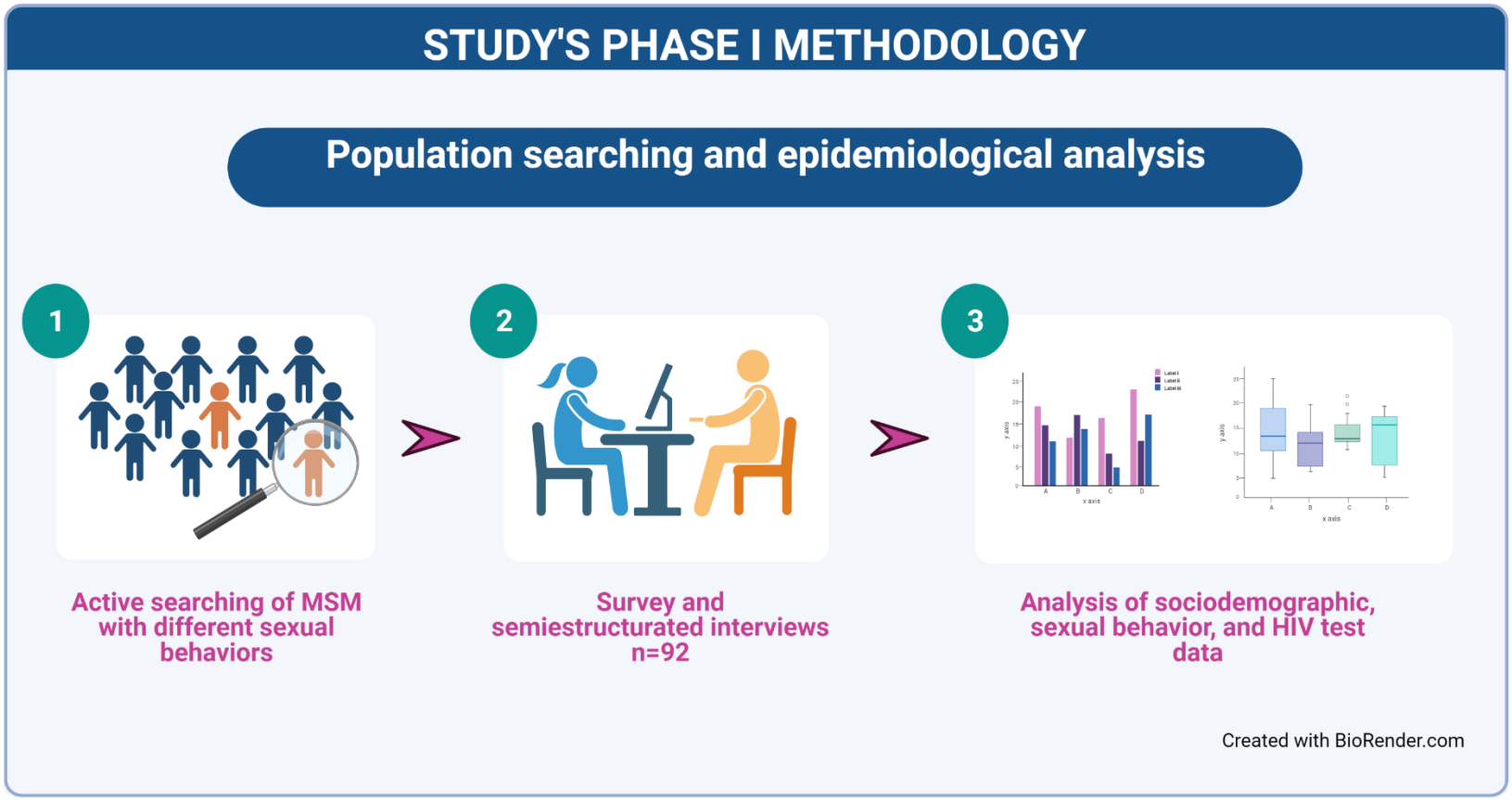
“No podemos hacer grandes cosas, pero sí cosas pequeñas con un gran amor”.

Madre Teresa de Calcuta

Introduction

To find possible HESN in MSM from Medellin, we searched for HIV-1-seronegative MSM individuals exhibiting high-risk sexual behaviors to assess their immune and genetic profiles, compared to seronegative MSM with low-risk sexual behaviors. The research was divided into two phases: [i] the analysis of sexual behaviors, HIV status, and epidemiologic data of 92 MSM who accepted to participate in the project, and [ii] the analysis of immuno-genetic factors associated with natural resistance to HIV in 60 MSM who met the inclusion criteria of this phase. In this chapter, the reader will find the results of the study's Phase I, published on the original paper *"Sexual Behaviors and Factors Associated with Condomless Sexual Practice in Colombian Men Who Have Sex with Men at High Risk of HIV Transmission"* (DOI: 10.1007/s10508-020-01856-y).

Graphic Abstract



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ORIGINAL PAPER

Sexual Behaviors and Factors Associated with Condomless Sexual Practice in Colombian Men Who Have Sex with Men at High Risk of HIV Transmission

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Abstract

Men who have sex with men (MSM) have a disproportionate burden of HIV infection worldwide. In Colombia, the prevalence of HIV in MSM is ~ 43 times higher than in the general population (17% vs. 0.4%). This study determined the sexual behaviors, HIV serostatus, and associated factors with condomless sexual practice with both regular and casual partners in 92 MSM from Medellín, Colombia. The subjects were recruited through a community-based approach, and the data were collected by a structured survey and in-depth interviews. Participants were classified into three groups according to the number of sexual partners in the last three months, to compare the sociodemographic conditions and sexual behaviors. Univariate analysis was described by absolute and relative frequencies; bivariate analysis and multivariate logistic regression were used to compare the groups and to explore the associated factors with condomless sexual practice. The overall HIV estimated prevalence was 4.3%, while the estimated prevalence for MSM with > 10 sexual partners in the last three months was 14.8%. This last group showed higher average age, higher percentage of subjects who have had sex with people living with HIV, and increased frequency of previous sexually transmitted infections. Having condomless sex with casual partners was associated with the number of sexual partners in the last three months. This study demonstrates that Colombian MSM continue to have a high risk of HIV infection/transmission and reinforce the need to implement adequate prevention programs, PrEP and guarantee access to treatment for people living with HIV.

Keywords Sexual behavior · HIV · Men who have sex with men · High-risk sex · Sexual orientation · PrEP

Introduction

In the last decade, steady progress has been made to reduce the global burden of AIDS-related deaths; however, preventing new HIV infections remains a challenge (UNAIDS, 2019). Globally, 37.9 million of people live with HIV, and 1.7 million correspond to new infections. Men who have sex with men (MSM) are the most affected population by HIV infection (Beyrer et al., 2016). In high-income countries, the HIV infection among MSM continues to rise, while in low- and middle-income countries, MSM rates far exceed those of the general population (Beyrer et al., 2012; UNAIDS, 2018, 2019). In 2018, MSM accounted for 17% of all of the new HIV infections worldwide, and 40% of the new infections in Latin America (UNAIDS, 2019). In Colombia, the overall prevalence of HIV is 0.4%, and MSM and transgender women are the most affected population, accounting for 17% and 21.4% of HIV prevalence, respectively (UNAIDS,

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2017b, 2019).

The risk of HIV acquisition is the result of a combination of sociostructural, behavioral, and biological factors (Harrison, Colvin, Kuo, Swartz, & Lurie, 2015; McKinnon et al., 2014; Sapsirisavat et al., 2016). Although MSM and people that practice anal sex have a greater biologic risk to acquire HIV (Centers of Disease Control and Prevention, 2019; Patel et al., 2014), and some of them practice other risky behaviors (having multiple sex partners, substance abuse, etc.), there are several social and structural conditions that drive the HIV infection in MSM, such as low income, immigration status, religion, and lower access to education and health services (Kelley et al., 2012; Shao & Williamson, 2012; Xia et al., 2016). Each of the biological, sociostructural and behavioral conditions is independently associated with HIV transmission and acquisition; however, from a syndemic perspective, there are interactions between most of them, where exist co-occurrence and the same time influence among them, generating a synergistic phenomenon that contributes to increasing the vulnerability to HIV and the excessive burden of this infection in MSM (Mustanski, Garofalo, Herrick, & Donenberg, 2007; Wilson et al., 2014).

Colombian MSM have a disproportionate burden of HIV, with a prevalence ~ 43 times higher than in the general population (17% vs. 0.4%), which is a reflection of the accumulative effect of several sociostructural conditions besides behavioral and biological factors. A recent study of Colombian MSM (Berbersi Fernández et al., 2019) shows that MSM with a low-educational level have 6.53 times more probabilityof have HIV than those with a higher level of education; likewise, the unemployed MSM have three times more probability to have HIV than those who have a job. Despite that is was not statistically significant, this study evidenced a tendency of MSM with low income to be more affected by HIV infection than other MSM. Other structural conditions such as lackof health services, machismo, and discrimination because of sexual orientation are associated with the high burden of HIV in Colombian MSM as well (Alvarado, Mueses, Galindo, & Martínez-Cajas, 2020; Quevedo-Gómez, Krumeich, Abadía-Barrero, Pastrana-Salcedo, & van den Borne, 2011).

Considering the syndemic model of HIV, UNAIDS promotes the implementation of combination prevention programs to reduce HIV incidence. These programs encompass biomedical, behavioral, and structural interventions designed to meet the HIV prevention needs of specific people and communities (UNAIDS, 2015). In the case of MSM, a combined prevention program includes access to treatment for people living with HIV, pre-exposure prophylaxis (PrEP) and other health services; condoms and lubricants distribution programs, community/peer-led outreach services, new media approaches, and empowerment of MSM addressing their rights and the laws which involved them (UNAIDS, 2010, 2015).

To establish effective programs targeting MSM, it is nec-

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essary to better know and understand their sexual behaviors without stigma (Stahlman, Hargreaves, Sprague, Stang, & Baral, 2017; UNAIDS, 2015). However, this is a challenge to face in Latin America including Colombia, where HIV prevalence in MSM is high and there is limited informa- tion about MSM's sexual behaviors and associated factors (García, 2014; Rubio Mendoza, Jacobson, Morales-Miranda, Sierra Alarcón, & Luque Núñez, 2015; Xia et al., 2016). In fact, to obtain this information is not easy because most MSM do not want to share private information due to the historic oppression they have suffered worldwide (Adebajo et al., 2014). Currently, there is a growing need for research and public policy developers to close the gaps in the information on MSM, while respectfully addressing them, assuring not to violate their rights and giving them a confidential space to share accurate information (Stahlman et al., 2017; UNAIDS, 2017a). This need is more felt in Latin America and Colombia, where a culture of deep-rooted stigma and discrimination against the MSM and LGBTI populations as well as those living with HIV is still abundant (Lopez Solano, 2017; Tamayo-Zuluaga, Macías-Gil, Cabrera-Orrego, Henao-Pelaéz, & Cardona-Arias, 2015).

The aim of this study was to determine the sexual behaviors, HIV serostatus, and the factors associated with condomless sexual practice with regular and casual partners in MSM from Medellin, Colombia. This information aims to narrow the gaps regarding the sexual behaviors of MSM, necessary to implement successful combined prevention programs and reduce the high HIV rates in this population.

Method

Participants

A cross-sectional study developed in a nonprobabilistic sample of 92 MSM, older than 18 years old, residents in the metropolitan area of Medellin, Colombia, who voluntarily accepted to be part of a cohort to study natural resistance to HIV infection. The study was divided into two phases: (1) the analysis of sexual behaviors, HIV status, and epidemiologic data of all MSM who accepted to participate in the project and (2) the analysis of immunogenetic factors associated with natural resistance to HIV in the MSM who met the inclusion criteria. In this paper, we present the results of the study's first phase. Before the inclusion in the study, each subject was informed of the purposes, procedures, risks, and benefits; then, an informed consent was obtained.

As MSM are a hidden population, we used different methods to sample hard-to-reach populations (Barros, Dias, & Martins, 2015; Gama, Martins, & Dias, 2017), such as snowball sampling, venue sampling, and the calling through flyers and social media (Facebook® and Grindr®). Likewise, we developed a community engagement approach to generate a relationship of trust with the object population. The data collection was carried out by individual and confidential interviews. The recruitment of subjects and data collection were carried out from 2015 to 2018. We included 92 MSM

from a reference population of 115,021 MSM from Medellín city (Berbersi Fernández et al., 2019).

LGBTI community-based organizations and the MSMs leaders were identified in Medellín through a collaboration with the Colombian project "*Global Fund to Fight AIDS-ENterritorio* (COL-H-ENterritorio)." Together with the *Corporación Stonewall, Alianza Social LGBTI de Medellín y Antioquia*, and *Consejo Consultivo LGBTI de Medellín*, focus groups with MSM were addressed to identify their perceptions, knowledge, and needs in regard to HIV and STIs (Vagenas et al., 2017). Moreover, the MSM leaders helped us to identify and contact the managers of commercial venues such as gay bathhouses and nightclubs in the downtown Medellín area. We provided educational talks, scientific support, individual counseling and free HIV testing for MSM from LGBTI community-based organizations, customers of commercial venues, and the participants in this study. This strategy allowed us to establish a relationship of trust with LGBTI organizations and the MSM population.

Procedure

The data were collected through the use of a structured survey and in-depth interviews. All of the interviews were held one-on-one and took approximately 3 h on average (2.5–5.5 h). A confidential and judgment-free space was provided for the participants to give them the possibility to share their experiences and personal history. Each individual was tested for HIV infection, and a structured survey was applied, which had dichotomous, polytomous and open-ended questions to characterize their sociodemographic conditions and sexual behaviors. Likewise, the previous history of STI was collected by the participants' self-report during this survey.

For the interview, a regular sexual partner was defined as one with whom a man had been having an affectionate and ongoing sexual relationship. In contrast, a casual sexual partner was defined as one with whom a man had sex only once, or repeatedly over time, however without any emotional attachment. We built a detailed description of each participant's sexual behavior, with the calculation of the number of sexual partners, the number of sexual intercourses with each partner, and the number of unprotected sexual intercourses in the last three months. Likewise, the approximated number of lifetime sexual partners was calculated.

The interviewer used autobiographical memory recalling techniques (Holland & Kensinger, 2010; Lolich & Azzollini, 2017) to guide the participant in the recall of his lifetime sexual partners' number. A timeline of his sexual life was built, doing a chronological journey year after year, from the age of sexual debut to the present, using anchor points to generate accurate counts of the number of partners (Mitchell et al., 2007). Across each year of the lifetime, the participant was asked to recall the number of partners before or after a significant life event that happened in that year, such as age, school, work, university, etc. Another anchor point was the count of long-term partners, from which the "gaps" were reviewed by counting short-term partnerships. Some participants had a "known" number because they use to have a list of sexual partners (like a diary) or remembered a previous count they had done. In those cases, the "known" number was revised upward to reflect changes since that time. To control for interviewer bias, only the researcher leader who had taken a previous training with MSM leaders to learn their social language, habits, and perceptions about sexuality carried out all in-depth interviews. Likewise, privacy during the interview and confidentiality of the data were guaranteed to control for responder bias.

Measures

Sociodemographic and Behavioral information

The applied structured survey and in-depth interview were built with the recommendations from *The Survey Resources Network (SRN) Question Bank* and the *Biobehavioural Survey Guidelines For Populations At Risk For HIV* (Mercer, 2010; WHO—World Health Organization, Centers for Disease Control and Prevention (CDC), UNAIDS, & FHI 360, 2017). A question bank was built based on previous literature and mentioned recommendations, including open-ended questions such as "Tell me about your alcohol use"; closed-ended questions, such as "Are you in a sexual relationship?" Presumptive questions, like "Do you have sex with men or women or both?" And direct questions about specific behaviors, as "How often do you use condom with you regular partner (always-sometimes-never)?" The survey was validated in appearance and content by a panel of researcher experts in the LGBTI and HIV fields. Finally, a pilot study was developed at the start of the study with the 10% of desired sample to verify the survey and interviewer performance.

HIV Test

All of the participants were initially tested for HIV using a third-generation rapid test (SD HIV-1/2 3.0 Bio Line, Abbot®; sensitivity: 100%; specificity: 99.8%), to detect anti HIV1/2 antibodies in whole blood. All positive tests were confirmed by a reference laboratory, using a fourth-generation ELISA to detect anti-HIV1/2 antibodies and HIV-1 p24 antigen.

Statistical Analysis

Participants were classified into three groups according to the number of sexual partners in the last three months (0–4,

5–10 and > 10), to compare the sociodemographic conditions and sexual behaviors. This classification was based on the results of two of the most important studies on MSM in the U.S., the EXPLORE (Koblin et al., 2006) and InvolveMENt (Kelley et al., 2012) studies. To describe the demographic and sexual behaviors, absolute and relative frequencies were calculated with 95% confidence intervals for the qualitative variables, and summary measures for the quantitative variables. Bivariate and multivariate logistic regressions were used to explore the associated factors with condomless sexual practice (CSP), defined as the infrequent use (sometimes) or not use (never) of condom in any anal intercourses (insertive unprotected anal intercourse [UIAI] or unprotected receptive anal intercourse [URAI]) with regular or casual partners.

For the bivariate analysis, the chi-square, Fisher's exact, and Mann–Whitney U tests were used since the data did not follow a normal distribution, evaluated by the Shapiro–Wilk test. Multivariable analyses were developed by logistic regression models, controlling for potential confounding and interacting factors, and evaluating the additive effect of four psychosocial conditions: socioeconomic status, education level, drug use, and age of sexual debut. All analyses were performed using SPSS software version 25. A p value < .05 was considered statistically significant.

Results

We recruited 92 MSM, with an average age of 27 years (SD = 8; Me = 25, IQR 22-31). The majority of subjects identified themselves as homosexual (85.9%) and 14.1% as bisexual or pansexual; no participants identified as heterosexual. The predominant MSM were single (93.5%), students (38.0%), and belonged a medium–low socioeconomic status (middle: 60.9%; low: 28.3%). More than half of participants (60.9%) had started or finished undergraduate university education (Table 1). When we compare the three groups classified by the number of sexual partners in the last three months, similar distributions were observed regarding sexual orientation, marital status, occupation, socioeconomic status, or level of education. However, the group of MSM who had > 10 sexual partners showeda higher average age (31 ± 6 SD) than the other groups(26 ± 8 SD and 25 ± 11 SD) (Table 1).

From all the MSM studied, 88% practice a receptive sexual role (72.8% versatile role, and 15.2% receptive role exclusively). More than a third had unprotected intercourse less than a month ago (35.9%), while 31.5% and 20.7% had their last unprotected intercourse between 1 to 5 or more than 6 months ago, respectively. Only 15.2% and 33.7% always use a condom with their regular and casual sexual partners, respectively. Interestingly, only one MSM reported using a condom to practice oral sex (fellatio). When we asked for the reasons for not using condoms, the most common answers were trust in the partner, decreased pleasure, and unavailability of condoms. With regard to the use of condoms, the MSM adduce reasons such as fear of STIs, have some STIs, and self-care. The majority (55.4%) expressed never had sex with a person living with HIV; however, none claimed to have known the serostatus of all their sexual contacts. Although 65.2% reported that at least once in their lives they had used drugs, 100% denied using injectable drugs. Only 7.6% percent had received blood transfusions, 27.2% had at least one tattoo and 34.8% at least one piercing. Half of the individuals reported having had a history of previous STIs; the most common reported infection was gonorrhea (n = 21) followed by syphilis (n = 15), condylomas (n = 14), and hepatitis B (n = 1). The mean of age at their sexual debut was 16 (± 5 SD). Regarding the number of sexual partners and unprotected intercourse in the last three months, we found an average of 28 (± 54 SD) and 14 (± 44 SD), respectively; likewise, half of the participants reported having at least 58 different lifetime sexual partners (Me = 58; IQR 20-404). Thirteen percent of the subjects had never had an HIV test, and four subjects were positive for HIV testing in the study, yielding a estimated prevalence of 4.3% in this cohort (Table 2).

Interesting differences were found between the group of > 10 sexual partners in the last three months and the other two MSM groups regarding their sexual behaviors and HIV status. The group of > 10 sexual partners reported withmore frequency to have had sex with people living with HIV (PLWH) (66.7% vs. 25.0% and 35.3%; p = .011) and STIs (74.1% vs. 41.7% and 40.4%; p = 0.014), as well as higher median of sexual intercourses (Me = 27, IQR 18-45 vs.Me = 9, IQR 5-13 and Me = 5, IQR 2-10; p < .001) and unprotected sexual intercourses in the last three months (Me = 10, IQR 2-24 vs. Me = 4, IQR 1.5-7.5 and Me = 1, IQR 0-6; p = .007), and higher number of lifetime partners (Me = 786, IQR 147-3035 vs. Me = 60, IQR 38-388 and Me = 22 IQR 9-88; p < .001) than the other groups of MSM. Although this group was the only one that did not present subjects who had never had an HIV test and presented more frequency ofHIV test in the previous six months (59.3% vs. 41.7% and 36.5%; p = .042), it is noteworthy that the total of the subjects diagnosed with the virus in this study belonged to this set of MSM (100%; p = .019) (Table 2). Since these findings show that MSM who belong to the > 10 partners group have very high-risky sexual behaviors, we analyzed the concomitance of one or more of those behaviors in each MSM belonging to this group. We found that there is a marked stratification of the group depending on the number of risky practices: the greater the number of risky behaviors, the lower the number of individuals who practice them (Fig. 1).

Table 1 Sociodemographic characteristics of total and grouped men who have sex with men

Variable		Total[a]		Number of sexual partners in the last 3 months[a]						χ^2 test *p* value
				0–4		5–10		>10		
		n	% (95.0% CI)	n	% (95.0% CI)	n	% (95.0% CI)	n	% (95.0% CI)	
Qualitative variables										
Sexual orientation	Homosexual	79	85.9 (78.8–93.0)	44	84.6 (73.1–92.4)	10	83.3 (56.0–96.4)	24	88.9 (73.2–96.8)	.910
	Bisexual–pansexual	13	14.1 (7.0–21.2)	8	15.4 (7.6–26.9)	2	16.7 (3.6–43.6)	3	11.1 (3.2–26.8)	
Marital status	Single	86	93.5 (88.4–98.5)	49	94.2 (85.4–98.3)	11	91.7 (67.2–99.10)	25	92.6 (78.3–98.4)	.728
	Cohabiting	4	4.3 (0.2–8.5)	2	3.8 (0.8–11.8)	1	8.3 (0.9–32.8)	1	3.7 (0.4–16.0)	
	Divorced	1	1.1 (−1.0–3.2)	0	–	0	–	1	3.7 (0.4–16.0)	
Occupation	Student	35	38.0 (28.1–48.0)	22	42.3 (29.6–55.8)	4	33.3 (12.5–61.2)	9	33.3 (17.9–52.1)	.808
	Teacher	6	6.5 (1.5–11.6)	2	3.8 (0.8–11.8)	1	8.3 (0.9–32.8)	3	11.1 (3.2–26.8)	
	Health worker	8	8.7 (2.9–14.5)	4	7.7 (2.7–17.3)	2	16.7 (3.6–43.6)	2	7.4 (1.6–21.7)	
	Other[+]	43	46.7 (36.5–56.9)	24	46.2 (33.1–59.2)	5	41.7 (18.0–68.8)	13	48.1 (30.3–66.4)	
Socioeconomic status	Low	26	28.3 (19.1–37.5)	17	33.3 (21.6–46.9)	2	16.7 (3.6–43.6)	6	22.2 (9.8–40.2)	.373
	Middle	56	60.9 (50.9–70.8)	28	54.9 (41.3–68.0)	8	66.7 (38.8–87.5)	20	74.1 (55.7–87.6)	
	High	9	9.8 (3.7–15.9)	6	11.8 (5.10–	2	16.7 (3.6–43.6)	1	3.7 (0.4–16.0)	
Level of education	High school	12	13.0 (6.2–19.9)	7	13.5 (6.2–24.6)	2	16.7 (3.6–43.6)	3	11.1 (3.2–26.8)	.471
	Vocational education	24	26.1 (17.1–35.1)	10	19.2 (10.3–31.4)	4	33.3 (12.5–61.2)	10	37.0 (20.9–55.8)	
	Undergraduate education	56	60.9 (50.9–70.8)	35	67.3 (53–78.9)	6	50.0 (24.3–75.7)	14	51.9 (33.6–69.7)	

Variable	Total			Number of sexual partners in the last 3 months									Kruskal–Wallis *p* value
				0–4			5–10			>10			
	M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	
Quantitative variables													
Age	27 ± 8	25 (22–31)	18–52	26 ± 8	24 (20–28)	18–52	25 ± 11	22 (18–28)	18–50	31 ± 6	32 (25–35)	23–42	.015*

[a]It is presented the percentage of the column for each variable, taking as n the total respective valid data.

[+]Other category includes engineer, finance executive, operator, messenger, musician and waiter. 95,0% CI: 95% confidence interval. * Statistically significant differences

Table 2 Sexual behaviors and HIV status of total and grouped men who have sex with men

Variable		Total[a]		Number of sexual partners in the last 3 months[a]						χ^2 test *p* value
				0-4		5-10		>10		
Qualitative variables		n	% (95.0% CI)	n	% (95.0% CI)	n	% (95.0% CI)	n	% (95.0% CI)	
Sexual role	Insertive	11	12.0 (5.3–18.6)	5	9.6 (3.8–19.8)	3	25.0 (7.6–52.9)	3	11.1 (3.2–26.8)	.364
	Receptive	14	15.2 (7.9–22.6)	10	19.2 (10.3–31.4)	0	–	4	14.8 (5.2–31.5)	
	Versatile	67	72.8 (63.7–81.9)	37	71.2 (57.9–8.1)	9	75.0 (47.1–92.4)	20	74.1 (55.7–87.6)	
Last unprotected sexual intercourse	< 1 month	33	35.9 (26.1–45.7)	15	34.1 (21.4–48.8)	4	36.4 (13.7–65.2)	14	56.0 (36.8–73.9)	.400
	1 to 5 months	29	31.5 (22.0–41.0)	16	36.4 (23.4–51.1)	5	45.5 (20.0–73.0)	7	28.0 (13.5–47.3)	
	Use of drugs									
	Transfu-sion									
Protected sexual intercourse with regular partners										
Protected sexual intercourse with casual partners										
Have had sex with PLHIV										

≥ 6 months	Never	28	30.4 (21.0–39.8)	17	35.4 (23.1–49.5)	2	18.2 (4.0–46.7)	9	39.1 (21.4–59.4)	.270
19	Sometimes	40	43.5 (33.3–53.6)	20	41.7 (28.5–55.8)	8	72.7 (43.5–91.7)	12	52.2 (32.5–71.3)	
20.7 (12.4–28.9)	Always	14	15.2 (7.9–22.6)	11	22.9 (12.8–36.2)	1	9.1 (1.0–35.3)	2	8.7 (1.9–25.1)	
13	Never	6	6.5 (1.5–11.6)	3	6.0 (1.7–15.2)	1	8.3 (0.9–32.8)	2	7.4 (1.6–21.7)	.165
29.5 (17.7–44.0)	Sometimes	52	56.5 (46.4–66.7)	24	48.0 (34.6–61.6)	8	66.7 (38.8–87.5)	20	74.1 (55.7–87.6)	
	Always	31	33.7 (24.0–43.4)	23	46.0 (32.7–59.7)	3	25.0 (7.6–52.9)	5	18.5 (87.4–35.9)	
2										
18.2 (4.0–46.7)	No	51	55.4 (45.3–65.6)	33	64.7 (51.1–76.7)	9	75.0 (47.1–92.4)	9	33.3 (17.9–52.1)	.011*
4	Yes	39	42.4 (32.3–52.5)	18	35.3 (23.3–48.9)	3	25.0 (7.6–52.9)	18	66.7 (47.9–82.1)	
16.0 (5.7–33.7)	No	31	33.7 (24.0–43.4)	20	38.5 (26.2–52.0)	3	25.0 (7.6–52.9)	8	29.6 (15.1–48.2)	.570
	Yes	60	65.2 (55.5–74.9)	32	61.5 (48–73.8)	9	75.0 (47.1–92.4)	19	70.4 (51.8–84.9)	
	No	84	91.3 (85.5–97.1)	50	96.2 (88.2–99.2)	11	91.7 (67.2–99.1)	23	85.2 (68.5–94.8)	.221
	Yes	7	7.6 (2.2–13.0)	2	3.8 (0.8–11.8)	1	8.3 (0.9–32.8)	4	14.8 (5.2–31.5)	
Tattoo	No	65	70.7 (61.3–80.0)	36	69.2 (55.9–80.5)	10	83.3 (56.4–96.4)	19	73.1 (54.3–87.1)	.613
	Yes	25	27.2 (18.1–36.3)	16	30.8 (19.5–44.1)	2	16.7 (3.6–43.6)	7	26.9 (12.9–45.7)	
Piercing	No	58	63.0 (53.2–72.9)	31	59.6 (46.1–72.1)	10	83.3 (56.4–96.4)	17	65.4 (46.3–81.3)	.300
	Yes	32	34.8 (25.1–44.5)	21	40.4 (27.9–53.9)	2	16.7 (3.6–43.6)	9	34.6 (18.7–53.7)	
Have had a prior	No	45	48.9 (38.7–59.1)	31	59.6 (46.1–72.1)	7	58.3 (31.2–82.0)	7	25.9 (12.4–44.3)	.014*
	Yes	46	50.0 (39.8–60.2)	21	40.4 (27.9–53.9)	5	41.7 (18.0–68.8)	20	74.1 (55.7–87.4)	
STI										

Table 2 (continued)

Variable		Total[a]		Number of sexual partners in the last 3 months[a]						χ^2 test *p* value
				0-4		5-10		>10		
		n	% (95.0% CI)	n	% (95.0% CI)	n	% (95.0% CI)	n	% (95.0% CI)	
Last HIV test	< 6 months	41	44.6 (34.4–54.7)	19	36.5 (24.5–50.1)	5	41.7 (18.0–68.8)	16	59.3 (40.6–76.1)	.042*
	6 to 12 months	21	22.8 (14.2–31.4)	13	25.0 (14.8–37.9)	1	8.3 (0.9–32.8)	7	25.9 (12.4–44.3)	
	> 1 year	18	19.6 (11.5–27.7)	9	17.3 (8.9–29.2)	5	41.7 (18.0–68.8)	4	14.8 (5.2–31.5)	
	Never	12	13.0 (6.2–19.9)	11	21.2 (11.8–33.6)	1	8.3 (0.9–32.8)	0	0–	
HIV testing	Positive	4	4.3 (0.2–8.5)	0	–	0	–	4	14.8 (5.2–31.5)	.019*

Variable	Total[a]			Number of sexual partners in the last 3 months[a]									Kruskal–Wallis *p* value
				0–4			5–10			>10			
	M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	
Quantitative variables													
Sexual debut	16 ± 5	16 (14–18)	6–22	16 ± 4	16 (14–17)	6–39	15 ± 5	16 (11–19)	8–25	16 ± 5	15 (14–19)	9–36	.920
Number of sexual intercourses in the last 3months	28 ± 54	10 (4–26)	0–360	19 ± 57	5 (2–10)	0–360	26 ± 55	9 (5–13)	2–183	43 ± 43	27 (18–45)	5–174	<.001*
Number of sexual intercourses withoutprotection in the last3 months	14 ± 44	3 (1–12.5)	0–359	15 ± 56	1(0–6)	0–359	11 ± 22	4 (1.5–7.5)	0–71	16 ± 22	10(2–24)	0–104	.007*
Number of lifetime sexual partners	624 ± 1600	58 (20–404)	3–11,570	84 ± 197	22 (9–88)	3–1224	334 ± 591	60 (38–388)	26–1842	1851 ± 2686	786 (147–3035)	10–11,570	<.001*

PLHIV: People living with HIV, M: Mean, SD: Standard deviation, Me: Median, Min: Minimum, Max: Maximum, IR: Interquartile range, [a] It is presented the percentage of the column for each variable, taking as n the total respective valid data. + Fisher's exact test, 95% CI: 95% confidence interval

As URAI and UIAI are the riskier factors to acquire HIV, and we found that our population shows different behaviors regarding the use of condoms with its regular and casual partners, we analyzed the associated factors with having CSP with both types of partners. In the bivariate analysis, drug use (p = .038), the number of sexual intercourses in the last three months (p = .035), and the number of lifetime partners (p = .039) were identified as associated factors with CSP with regular partners (Table 3). Meantime, the age of sexual debut (p = .037) and the numbers of sexual partners in the last three months (p = .010) were the associated factors with having CSP with occasional partners (Table 4).

Multivariable logistic regression analyses showed that the CSP with regular partners was not associated with the analyzed variables. However, the CSP with casual partners was associated with the number of sexual partners. The number of sexual partners in the last 3 months generates a 5% risk of not using a condom with casual partners (OR, 1.058; 95% CI, 1.00–1.11) (Table 5). In addition, as the HIV infection behaves as a syndemic model, we analyzed whether the previously reported psychosocial conditions that increase the risk of HIV in Colombian MSM had an additive interaction in our studied population. As in the initial bivariate analysis, the age of sexual debut and drug use were associated with having USP with casual and regular partners; however, interaction or synergy between those variables and socioeconomic status and level of education were not found.

Discussion

Globally, it has been demonstrated the need to improve the research and medical intervention processes in key populations to guaranty the protection of their rights, avoid their re-victimization and contribute to a successful HIV response that looks the subjects in their integrity and gives them ethic, integral, and humanized attention (Rhodes & Wong, 2016; Sullivan et al., 2012; UNAIDS, 2017b). Those processes must provide spaces for trust, without prejudice, judgment, and discrimination, to identify personal and col- lective conditions susceptible to intervention (UNAIDS, 2017a). In this sense, an adequate process of community engagement has great importance in the development of biomedical research, to reduce the gap between research- ers and communities (HIV/AIDS Network Coordination & Community Partners, 2014). A successful process of researching on key populations implies an approach in which the researcher has to had a position of listening and respect, allowing the object population to establish a relationship of trust and credibility in the research team.

In this study, we used a combination of community engagement strategies and sampling methods that allowed us to reach a difficult-to-access population in a space of confidence and trust. It was not an easy task in a region where MSM are still stigmatized and discriminated. Although in the last years Latin America was advancing in the inclusion and sexual diversity policies, there are still prejudices, stigmatization, and discrimination against the LGBTI population, with a constant violation of their rights (SinViolencia LGBTI Red Regional de Información sobre

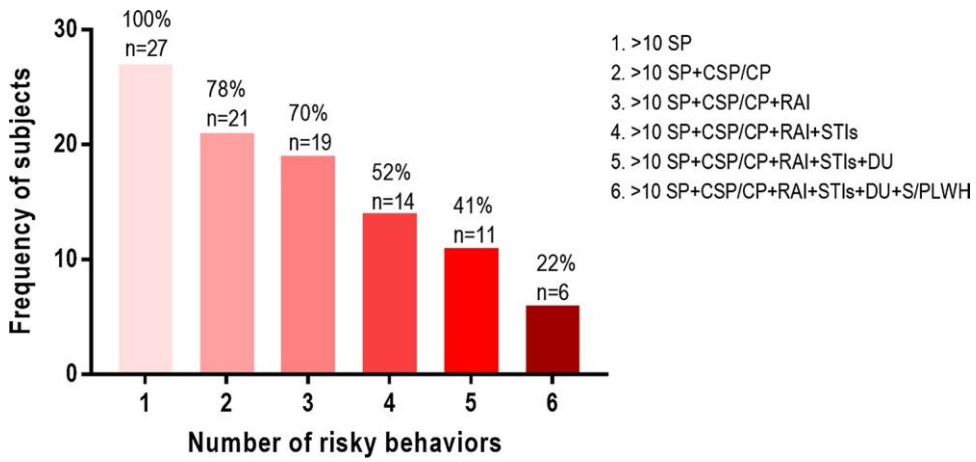

Fig. 1 Subjects who have >10 sexual partners in last three months and concomitantly have other risky behaviors. The figure shows the number of subjects belonging to the MSM group with >10 sexual partners in the last three months and their concomitance or not with other risky behaviors. The concomitance of one or more risky behavior was grouped in the following order, successively and cumulatively: >10 sexual partners in the last three months (>10 SP); condomless sexual practice with casual partners (CSP/CP); receptiveanal intercourse (exclusively receptive or versatile role ; RAI) ; previ-ous history of STIs (STIs); drug use and (DU); and having sex with people living with HIV (S/PLWH)

Table 3 Factors associated with condomless sexual practice with regular partners in the last three months

Variable		No			Yes			χ^2 test *P* value
		n	% (95%CI)		n	% (95%CI)		
Unprotected sexual intercourse with regular partners								
Qualitative variables[a]								
Sexual role	Insertive	2	14.3 (3.1–38.5)		8	11.8 (5.7–21.0)		.317
	Receptive	4	28.6 (10.5–4.5)		9	13.2 (6.8–22.8)		
	Versatile	8	57.1 (31.9–79.7)		51	75.0 (63.8–84.1)		
Have had sex with PLHIV	No	10	71.4 (45.5–89.5)		37	54.4 (42.6–65.9)		
	Yes	4	28.6 (10.5–54.5)		31	45.6 (34.1–57.4)		.241
Drug use	No	8	57.1 (31.9–79.7)		19	27.9 (18.4–39.4)		.038*[+]
	Yes	6	42.9 (20.3–68.1)		49	72.1 (60.6–81.6)		
Transfusion	No	14	100		62	91.2 (82.7–96.2)		.313[+]
	Yes	0	0		6	8.8 (3.8–17.3)		
Tattoo	No	12	85.7 (61.5–96.9)		46	67.6 (56.0–77.9)		
	Yes	2	14.3 (3.1–38.5)		22	32.4 (22.1–44.0)		.151[+]
Piercings	No	10	71.4 (45.5–89.5)		43	63.2 (51.4–74.0)		
	Yes	4	28.6 (10.5–54.5)		25	36.8 (26.0–48.6)		.399[+]
Have had a prior STI	No	10	71.4 (45.5–89.5)		37	54.4 (42.6–65.9)		
	Yes	4	28.6 (10.5–54.5)		31	45.6 (34.1–57.4)		.24
Last HIV test	< 6 months	6	42.9 (20.3–68.1)		30	44.1 (32.8–56.0)		1.000
	6 to 12 months	3	21.4 (6.4–46.9)		14	20.6 (12.3–31.3)		
	> 1 year	3	21.4 (6.4–46.9)		14	20.6 (12.3–31.3)		
	Never	2	14.3 (3.1–38.5)		10	14.7 (7.8–24.5)		
Variable		M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	Mann–Whitney *p* value
Quantitative variables								
Sexual debut		16 ± 3	17 (15–18)	9–22	16 ± 5	16 (14–18)	6–39	.438
Number of different sexual partners in the last 3 months		4 ± 5	2 (1–4)	1–18	12 ± 17	4 (2–16)	0–90	.082
Number of sexual intercourses in the last 3 months		10 ± 12	4 (2–18)	1–39	31 ± 58	12 (5–27)	0–360	.035*
Number of lifetime sexual partners		161 ± 355	25 (11–52)	3–1224	734 ± 1795	87 (22–505)	5–11,570	.039*

PLHIV: People living with HIV, Me: Median, SD: Standard deviation, Min: Minimum, Max: Maximum, IR: Interquartile range

* Statistically significant differences

** Has never been tested for HIV before to this study

[a] It is presented the percentage of the column for each variable, taking as n the total respective valid data

+Fisher's exact test, 95% CI: 95% confidence interval

Table 4 Factors associated with condomless sexual practice with casual partners in the last three months

Variable		No			Yes			χ^2 test *p* value
		n	% (95.0%)		n	% (95%CI)		
Condomless sexual intercourse with casual partners								
Qualitative variables[M]								
Sexual role	Insertive	4	12.9 (4.5–27.8)		7	12.1 (5.6–22.2)		.629
	Receptive	6	19.4 (8.5–35.6)		7	12.1 (5.6–22.2)		
	Versatile	21	67.7 (50.3–82.1)		44	75.8 (63.8–85.4)		
Have had sex with PLHIV	No	18	58.1 (40.6–74.1)		32	56.1 (43.2–68.5)		.862
	Yes	13	41.9 (25.9–59.4)		25			
Drug use	No	10	32.3(17.9–49.7)		21	36.2 (24.7–49.0)		.709
	Yes	21	67.7 (50.3–82.1)		37	63.8 (51.0–75.3)	43.9 (31.5–56.8)	
Transfusion	No	30	96.8 (85.9–99.6)		52	89.7 (79.9–95.6)		.226[+]
	Yes	1	3.2 (0.4–14.1)		6	10.3 (4.4–20.1)		
Tattoo	No	24	77.4 (60.7–89.3)		40	70.2 (57.5–80.8)		.32
	Yes	7	22.6 (10.7–39.3)		17	29.8 (19.2–42.5)		
Piercing	No	23	74.2 (57.1–87.0)		34	59.6 (46.7–71.6)		.172
	Yes	8	25.8 (13.0–42.9)		23	40.4 (28.4–53.3)		
Have had a prior STI	No	17	54.8 (37.5–71.3)		26	44.8 (32.5–57.6)		.368
	Yes	14	45.2 (28.7–62.5)		32			
Last HIV test	< 6 months	10	32.3 (17.9–49.7)		29	50.0 (37.4–62.6)		.336
	6 to 12 months	8	25.8 (13.0–42.9)		13	22.4 (13.2–34.3)	55.2 (42.4–67.5)	
	> 1 year	9	29 (15.4–46.3)		9	15.5 (8.0–26.4)		
	Never	4	12.9 (4.5–27.8)		7	12.1 (5.6–22.2)		
Variable		M ± SD	Me (IR)	Min–Max	M ± SD	Me (IR)	Min–Max	Mann–Whitney *p* value
Quantitative variables								
Sexual debut		17 ± 4	16 (15– 18)	6–25	16 ± 5	15 (13–17)	7–39	.037*
Number of different sexual partners in		5 ± 8	2 (1–5)	0–36	15 ± 25	5 (2–21)	0–145	.010*
Number of sexual intercourses in the las		26 ± 67	9 (4–18)	0–360	30 ± 46	11 (5–28)	1–183	.33
Number of lifetime sexual partners		579 ± 2079	39 (11–192)	3–11,570	665 ± 1308	95 (22–672)	5–5400	.089

the last 3 months

t

3 months

PLHIV: People living with HIV, Me: Median, SD: Standard deviation, Min: Minimum, Max: Maximum, IR: Interquartile range

[M]It is presented the percentage of the column for each variable, taking as n the total respective valid data

[*]Statistically significant differences

[**]Has never been tested for HIV before to this study

[+]Fisher's exact test, 95% CI: 95% confidence interval.

Table 5 Logistic regression analysis on condomless sexual practice with regular and casual partners

Variable (associated factor)	Beta coefficient	OR	95% CI	Adjusted OR	95% CI
Condomless sexual intercourse with regular partners in the last 3 months					
Drug use	1.066	3.439*	1.05–11.23	2.905	0.78–10.79
Number of sexual intercourses in the last 3 months	0.032	1.080	0.98–1.19	1.033	0.97–1.10
Number of lifetime sexual partners	0.000	1.001	0.99–1.00	1.000	0.99–1.00
Condomless sexual intercourse with casual partners in the last three months					
Sexual debut	−0.050	0.957	0.87–1.05	0.951	0.86–1.05
Number of different sexual partners in the last 3 months	0.056	1.055*	1.00–1.11	1.057*	1.00–1.11

*$p < .05$

Violencias LGBTI en América Latina y el Caribe, 2019). Between 2014 and 2019, there were 1292 homicides of LGTBI people in the region, and Colombia was the second country with the highest number of cases (43% = 542/1292) (Colombia Diversa & Caribe Afirmativo, 2018). This panorama enforces in the region the need remarked by UNAIDS to guarantee the ethical compromise and the use of adequate community engagement strategies to involve key populations into research studies and intervention programs (UNAIDS, 2017a).

We reached a difficult-to-access population to describe in deep their sexual behaviors. However, the fact that 100% of the participants openly identified themselves as homosexual, bisexual, or pansexual shows that we were unable to reach the most hidden groups of this MSM, such as heterosexual men who have sex with men, with transgender women or both.

No differences were found between the MSM groups regarding sociodemographic conditions, except for age. These findings are different to the well-established evidence that lower educational, economic, and social conditions are related to the practice of risky sexual behaviors and to the acquisition of HIV infection (Igulot & Magadi, 2018; Pellowski, Kalichman, Matthews, & Adler, 2013), for example, having a great number of sexual partners as a consequence of the economic need to practice transactional sex. All MSM in this study have had access to high school and the majority to vocational/undergraduate programs, showing in general, a good access to education; in addition, all the groups of MSM had the same socioeconomic status. This shows that contrary to the economic, social, or educational conditions, there could be other conditions related to making the decision to practice risky sexual behaviors in our population. Some studies suggest that psychosocial factors such as emotional issues, depression, and other mental health conditions contribute to the decision to assume sexual risky behaviors in MSM (Klein, 2014; Sandfort, Yi, Knox, & Reddy, 2013). To address this question in our population, we compared the psychoneuroimmunological conditions of two groups of MSM from this cohort, who exhibited very different sexual behaviors (manuscript in preparation).

The overall estimated prevalence of HIV in this cohort was 4.3%, which is higher than the prevalence in Colombian general population (0.4%) and lower than the previously reported in Colombian MSM (17%) (UNAIDS, 2019). However, since all of the new diagnosis was in the group MSM with > 10 partners, the specific estimated prevalence in this group is 14,8%, which reflects a better correlation with the national prevalence in MSM, and, in turn, is more high than the prevalence in Latin America (12.5%) (UNAIDS, 2019). The majority of MSM in all the groups practice a recep- tive role that, in combination with the low rates of condom use they showed, demonstrate they could be exposed to HIVand STIs (Patel et al., 2014). Although now it is known that PLWH who have undetectable viral load do not transmit the virus by sexual way (Eisinger, 2019), here we considered having sex with PLWH as a risky behavior in our population; since all of the MSM who reported had had sex with PLWH, they accepted to not to know whether their contacts PLWHV were undetectable; indeed, there were stories of MSM who did not know whether their partners PLWH were taking treatment and others who realize that their sexual contact was a PLWH, time after their sexual intercourse.

In this cohort, the main risky behaviors related to HIV acquisition were the sexual ones, since nobody uses injectable drugs, and there are low proportions of MSM who have had blood transfusions, tattoo, and piercings. Although addressing blood transfusions, tattoo, and piercings as risky situations to acquire HIV could be appreciated as stigmatizing, we take into account them because despite the Colombian protocols, in 2018, there were three cases of HIV transmission by blood transfusion (Instituto Nacional de Salud (INS), 2019), and, in 2017, there were 14 and three cases of HIV transmission by tattoos and by application of piercings, respectively (Instituto Nacional de Salud (INS), 2018).

The cross-sectional design of this study does not allow us to calculate the risk of seroconversion in the study participants. However, our population show—and exceed—the risky sexual behaviors associated with HIV acquisition. In the EXPLORE study, the authors found that having more than four sexual partners in the last 6 months was a factor

associated with seroconversion (Koblin et al., 2006). Likewise, the InvolveMENt study found a high risk of exposure to HIV in those MSM who had > 10 sexual partners in the last 12 months or more than ten encounters with URAI in the last 12 months (Kelley et al., 2012). In the study car- ried out in MSM from London by Aghaizu et al. (2016), the individuals with the highest risk of HIV acquisition were those who reported URAI with one or more casual partners, who did not practice serosorting exclusively in the last year, MSM with the largest number of occasional sexual partners, and those who have been diagnosed with an STI in the last year (Aghaizu et al., 2016). On the other hand, in a study conducted in African MSM from Malawi, Namibia, and Botswana, it was found that the individuals with high risk of infection were those with a previous history of STI, older than 25 years, and who reported not always using the condom in their sexual intercourses (Baral et al., 2009). Finally, in a study of HIV prevalence and associated factors in Colombian MSM, it was found that being between 25 and 39, or older than 40 years, has encounters with casual sexual partners in saunas, and previous STIs were all associated factors to acquire HIV (Rubio Mendoza et al., 2015). In comparison with those previous findings, the majority of our studied population showed several risky behaviors, but also, the MSM belonging to the group of > 10 partners in last three months are at extreme risk of HIV infection, showing behaviors that exceed by far the factors associated with seroconversion in the other MSM cohorts. Taking into account the extremely high risk of this group and their negative serostatus, plus the high prevalence of HIV in Colombian MSM, it is interesting to study whether there are individuals who have biological factors associated with HIV natural resistance, which is our second goal in the study of this cohort.

We observed that MSM showed different practices between regular and casual partners, similar to previous studies. Kong, Laidler, and Pang (2012) found that in MSM from China condom use is different according to the type of partners, because the condom use decreased when the affective distance with a partner increased. Meanwhile, Hicks, Kogan, Cho, and Oshri (2017) found that no individual risk factors were associated with regular partner inconsistent condom use; however, impulsivity and anger/hostility positively predicted inconsistent condom use with a casual partner.

In this MSM population, the number of sexual partners in the last three months generates a 5% risk of not using a condom with casual partners. These results differ from those in the study made by Pines et al. (2016) in MSM from Los Angeles, U.S., where the factors associated with noncondom use were reporting high level of school education and methamphetamine use, specifically during receptive anal intercourses with casual partners. Nevertheless, our results are similar to other studies in which the number of sexual partners and relationship status are associated with unprotected sex across emerging adulthood (Ashenhurst, Wilhite, Harden, & Fromme, 2017), and having multiple partners is correlated with an increase in unprotected anal intercourse among MSM (Cheng et al., 2014).

It is striking that not interactions and synergy were found between socioeconomic status, education level, use of drugs, and the age of sexual debut despite that those psychosocial conditions have been associated with an increased risk of HIV in Colombian MSM. Mustanski et al. (2007) demonstrated the cumulative effect of drug use, psychological distress, and violence in the increased probability to having multiple anal sex partners, unprotected anal sex, and an HIV-positive status among young MSM form Chicago. Likewise, Alvarado et al. (2020) found a higher likelihood of transactional sex in Colombian MSM when they had three or four of the next psychosocial conditions: child sexual abuse (sex debut below 13 years old), recent history of forced sex, drug use, and alcohol drinking. The lack of syndemic model in our study could be explained by the low size of our population. Despite the lack of syndemic interactions between the evaluated psychosocial conditions, the findings of this study together with the previous studies in MSM reinforce the theory about how the HIV infection is driven by the combination of behavioral and sociostructural conditions and not only by the biological factors. For example, here the drug use and the number of sexual partners were initially associated with having USP; several studies have demonstrated that the drug use and seeking for sexual partners are related to psychological distress (Dyer, Regan, Pacek, Acheampong, & Khan, 2015). Moreover, the low income can influence the frequency of USP when this practice is asked in the transactional sex, and at the same time, people that are forced to practice transactional sex because of a low income commonly have lower levels of education, use drugs, and present higher levels of psychological issues. In this sense, how the interactions between those factors can have an additive effect on the risk HIV acquisition and transmission is evident.

Although the methodological approach of this study is quantitative, the tools that we used from the qualitative research methods, such as the MSM's focus groups and the in-depth interviews, allowed us to identify some important anecdotes related with the knowledge, perceptions, and practices regarding HIV, in our cohort of MSMs. We observed a common knowledge about HIV and STIs in the MSM, for example, the routes of transmission and symptoms; however, a marked tendency was found to not recognize the oral sex as a route of HIV transmission. We found a repeated perception of HIV infection as a chronic disease and the possibility of lead an "almost normal life" for PLWH who are in treatment. However, the fear of the discrimination and stigmatization to which they would be subjected if they acquired HIV was a constant concern among individuals. We also observed that some MSMs showed trust in the advances of science, mainly

regarding the effectiveness of antiretroviral treatment. Nevertheless, this trust allowed them to have a feeling of tranquility in the face of risky sexual behaviors that put them in exposure to the HIV, losing sight of the probability of acquiring other STIs. Finally, although PrEP has not yet been approved in Colombia (UNAIDS, 2019), we asked MSM their knowledge about this prevention strategy, and we observed that only few MSM showed some grade of knowledge about it. Moreover, it was interesting for us to hear that two MSM expressed that some of their friends are taking PrEP.

The results of this study show that the group of MSM with > 10 partners (29.3%) are at extremely great risk of HIV infection and provide new information about their sexual behaviors, which may be susceptible to intervention. Colombia has made progress in its HIV prevention policies and, according to UNAIDS, is one of the only four countries in Latin America that is self-evaluating its national policies regarding HIV infection (UNAIDS, 2019). However, our data and the national high prevalence of HIV in MSM confirm the needs to improve our strategies against HIV infection. While other countries have national combination programs, in Colombia only until now there is a plan for the implementation of these programs in some cities, and the use of PrEP was started just like a pilot study. It is necessary that the government commits to give continuity and enhance the efforts and goals that Colombia has achieved with the accompaniment of Global Fund to Fight AIDS.

There were limitations in the study. The little size in our population, mainly in the stratified analysis by three groups, could lead us to have a type II error. Moreover, the limited size, convenience-chosen sample and the fact that all the included subjects identified themselves as MSM evidence that this is not a representative sample of MSM from Medellín. There is still the need to continue applying strategies to reach the most hidden population of MSM in the city, to study and better understand their sexual behaviors. In this study, only one Afro-Colombian man and nobody from Indigenous people were enrolled, which do not let us make an analysis by ethnicity. This is an important limitation since there is evidence that black race MSM show a greater risk to acquire HIV because of their social conditions (Kelley et al., 2012). In addition, the study also relied on self-reported STIs history rather than evaluated biologically, which can subestimate STIs prevalence in the studied population. Finally, although it was an achievement to calculate the number of lifetime sexual partners and we used recommended tools to have accurate information, because of the great number of partners in some individuals, this account must be understood as an approximated number.

Conclusion

The study participants showed a higher estimated prevalence of HIV than general Colombian population, especially those who have a higher number of sexual partners in the last three months and engaged in several risky sexual behaviors. Here, the number of sexual partners in the last 3 months generates a 5% risk of not using a condom with casual partners. This study demonstrates that Colombian MSM continue to have a high risk of HIV infection/transmission and reinforce the need to implement adquate combination prevention programs that include interventions to address the social disparities, provide PrEP, and guarantee access to treatment for people living with HIV.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Universidad de Antioquia (Act No.007, May 22, 2014).

Informed Consent Before the inclusion in the study, each subject was informed of the purposes, procedures, risks, and benefits and then an informed consent was obtained.

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Conclusions

In this phase of study, we were able to reach MSM who exhibited very risky sexual behaviors, most of them exceeding those which have been associated as risk factors for HIV acquisition in international cohorts of MSM[1–3]. Having into account those risky sexual behavior showed by the participants, the high Colombian MSM's prevalence of HIV (17%), the elevated probability of HIV transmission by anal sex, and the fact that no one in this cohort was using PrEP, it seems possible to found seronegative MSM who exhibit associated factors to HIV-1 natural resistance, mainly in those who exhibited the riskiest sexual behaviors.

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Chapter 3

Genetic analysis

Study phase II

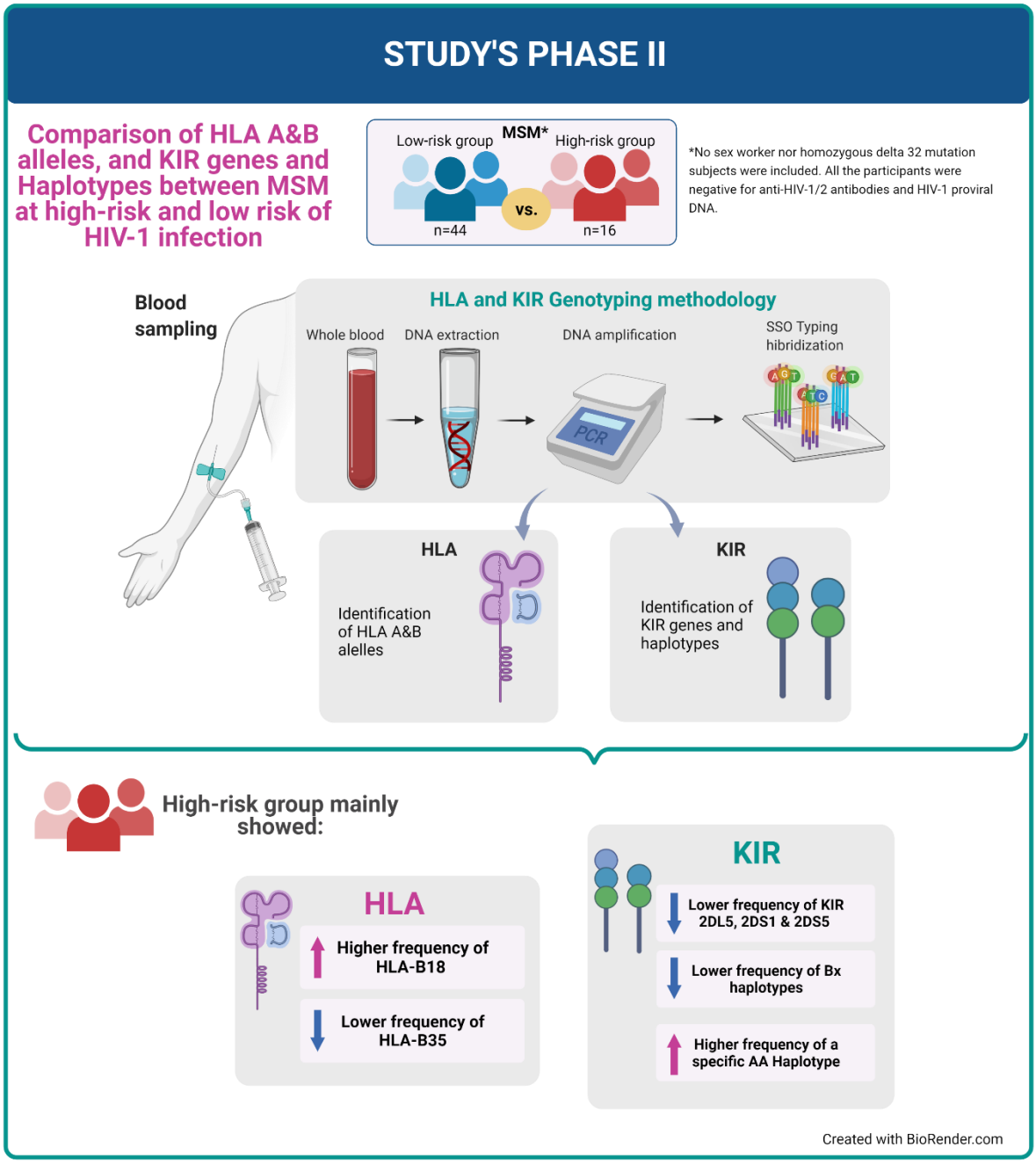
"Science increases our power to the extent that it reduces our pride." Herbert Spencer

“La ciencia aumenta nuestro poder en la medida en que reduce nuestra soberbia”. Herbert Spencer

Introduction

The present chapter shows the second part of Study's Phase II, which encompasses comparing the genetic profile between seronegative MSM at low and high risk of HIV-1 infection. It was found that the MSM in the high-risk group showed protective HLA alleles and KIR genes & haplotypes. The results of this comparison were compiled in a research article that was submitted to The Journal of Infectious Diseases (JID).

Graphic abstract



HLA alleles and KIR genes & Haplotypes in MSM at high risk of HIV-1 infection

Running title:

HLA Alleles and KIR genes in MSM at risk of HIV

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Abstract

Background: Understanding the immune response against HIV-1 and the natural resistance exhibited by HESN brings the possibility of proposing new prevention and cure strategies. Several studies suggest an important role of HLA and KIR genes in the protection of HIV-1 infection. Moreover, there is a significant gap in the knowledge about these genetic factors in Latino American seronegative MSM at high risk of infection. **Objective:** This study aimed to analyze the HLA and KIR genes in a cohort of Colombian seronegative MSM at low and high risk of HIV-1 infection. **Results:** The high-risk group showed a higher frequency of the protective HLA-B*18 allele (25% vs. 4.5%; p=0.036), and a lower frequency of the HLA*B35 (6.3% vs. 36.4%; p=0.047), which has been previously associated with susceptibility to HIV-1 infection. Likewise, this group exhibited an increased profile of inhibitory KIR genes, with a low frequency of 2DL5 (6.7% vs. 47.6%; p=0.019), 2DS1 (13.3% vs. 50%; p=0.022), 2DS5 (6.7% vs. 40.5%; p=0.037) genes and Bx haplotypes (53.3% vs. 81%; p=0.019), and a higher frequency of one AA haplotype (40% vs. 7%; p=0.007). **Conclusion:** MSM at high risk showed genetic factors associated with protection against HIV-1 infection.

Keywords: HIV, HIV-Exposed seronegative individual, Men who have sex with men, HLA, KIR.

Background

The genetic background of each individual can influence the immune response against HIV-1. The presence of specific HLA (human leukocyte antigen), and KIR (Killer-cell immunoglobulin-like receptor) genes confer resistance/susceptibility to HIV-1 infection[1]. However, the genotypes associated with HIV-1 protection vary between different populations[2]. The comprehension of immune response to HIV-1 infection and the natural resistance that exhibits the HIV-exposed seronegative individuals (HESN) remains a meaningful way to understand better the determining mechanisms in the transmission and infection establishment[3], which in turn, opens the possibility to design new prevention and cure strategies.

The HLA-I alleles have been associated with a decreased risk of infection in HESN cohorts and infection control in controller HIV-1 positive individuals, while others are related to increased susceptibility[4–6]. Some of the HLA-I alleles associated with natural resistance to HIV-1 are A*24, A*25, A*32, B*13, B*14, B*18, B*27, B*57, B*58[4,7–10]. In contrast, the B*35 allele is frequently related to susceptibility and rapid progression to AIDS[11,12]. In turn, the activating KIR genes, mainly the KIR3DS1, and its combination with the HLA-Bw4 group are commonly associated with protection against HIV-1 in HESN cohorts[13,14]. Interestingly, inhibitory KIR genes such as KIR3DL1, and the KIR2DL2/KIR2DL3 heterozygosity in the absence of HLA-C1 have been also associated with protection against HIV-1[15,16]. Most of the information about KIR genes and their role in HIV-1 protection comes from Caucasian, African,

and Asian populations, but a significant gap exists about Latin-American individuals[8,17].

The HLA and KIR genes play an important role in the response against HIV-1 and other infections. The types of KIR and HLA genes influence the NK cell responsiveness against HIV-1 through the combination of activating and inhibitory signals[18,19]. Likewise, the HLA genes modulate the specific response against the virus by the CD4+ and CD8+ T cells[1,20]. Thus, the susceptibility or control to HIV-1 infection could be mediated by the genetic background of each individual. This study aimed to determine the HLA and KIR genes, and KIR haplotypes associated with HIV protection in a cohort of Colombian seronegative men who have sex with men (MSM) at low and high risk of HIV infection.

Methods

Study design and Population

It is a cross-sectional study with sixty subjects from a cohort of seronegative MSM from Medellin-Colombia at different risks of HIV acquisition, as we previously reported[21]. The high-risk group (n=16) and low-risk group (n=44) were MSM with more than 14, and 4 or fewer sexual partners in the last three months, respectively. All individuals met the following inclusion/exclusion criteria: no one was a sex worker nor taking PrEP; all of them were negative for anti-HIV-1 antibodies, HIV-1 proviral DNA, and delta 32 mutation in the *CCR5* gene in a homozygous state. According to the Helsinki declaration, the study was performed and approved by the

Ethics Committee of Universidad de Antioquia's School of Medicine (Act No.007, May 22th, 2014).

HLA and KIR genotyping

Peripheral blood was obtained from each subject to extract Genomic DNA, which concentration was adjusted to 40ng/ul. The PCR amplification of the HLA-A and B alleles was carried out using Rapid HLA-A, B, SSO typing kit (Immucor-Lifecodes System, Inc Stanford, CT). The hybridization of the PCR products was carried out by liquid phase using Luminex® beads, which recognize the polymorphism of the second and third exon on HLA class I molecules. Reading was performed in a Luminex® IS 200 System, xPONENT® 3.1 Software (Luminex Corporation, Austin, TX), and final HLA typing was analyzed with the Match IT! ™ DNA software (Immucor-Lifecodes System, Inc Stanford, CT).

The KIR genes typing was performed according to the manufacturer's instructions by PCR-SSOP (Polymerase Chain Reaction-Sequence Specific Oligonucleotide Probes) using the SSO LabType® kit (One Lambda, San Diego, CA, USA) after evaluating the purity and adjusting the concentration at 20 ng/µl of genomic DNA for the final volume of 20µl per PCR reaction. The amplified product was hybridized with microspheres bound to probes specific to the KIR gene. The resulting products were analyzed using the Luminex® IS 200 System flow cytometer (Luminex Corporation, Austin, TX) and final KIR typing analyzed with the HLA Fusion™ Software (One Lambda, San Diego, CA, USA). The results were compared by

matching the pattern of positive and negative beads for the specific genes with the information in the product worksheet. The following fourteen KIR genes and two pseudogenes were studied: KIR3DL3, KIR2DS2, KIR2DL3, KIR2DL2, KIR2DL5B, KIR2DS3, KIR2DL1, KIR2DL4, KIR3DL1, KIR3DS1, KIR2DL5A, KIR2DS5, KIR2DS1, KIR2DS4, KIR3DL2, KIR2DP1 and KIR3DP1. The KIR haplotypes and genotypes were obtained using the Allele Frequency Net Database http://allelefrequencies.net/default.asp[22].

Statistical Analysis

Differences between groups were assessed using the Chi-squared test for categorical variables and parametric or non-parametric tests (according to the normality test) for quantitative variables. The association of each gen with HIV susceptibility or protection was measured by the odds ratio (OR) with a 95% confidence interval (CI). Crude and adjusted ORs and 95% CI were calculated for each independent variable. Analyses were carried out using SPSS, version 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp). The Linkage disequilibrium (LD) and haplotype analysis were performed using Haploview software (Copyright (c) 2003-2006 Broad Institute of MIT and Harvard (http://sourceforge.net/projects/haploview/).

Results

Sociodemographic and sexual behavior data of MSM

Sociodemographic data and sexual behaviors of both MSM groups were previously reported[21,23]. Briefly, most of the subjects in both groups identified themselves as gay/homosexual males, were single, and had access to vocational/professional education. Despite being seronegative and not having HIV-1 proviral DNA, the MSM in the high-risk group showed very risky sexual behaviors, associated with HIV-1 seroconversion in MSM cohorts[21]. This group showed a higher number of lifetime sexual partners (Me 1,078 vs. 26; $p<0.001$), unprotected intercourses in the last three months (Me 10 vs. 2; $p=0.001$), and a higher percentage of individuals reporting never using a condom with their regular partners (93.8% vs. 75%; $p=0.001$) compared to the low-risk MSM group. Moreover, despite the high frequency of casual partners in their lifetime and the last three months before the study enrollment (14 or more partners), 81.3% of the MSM in the high-risk group informed never to use condoms with their casual partners, and 87.5% reported previous sexually transmitted infections.

The high-risk MSM group exhibited a higher frequency of protective HLA alleles

The HLA A and B alleles were determined in both groups at an intermedium resolution. The MSM in the high-risk group showed a higher frequency of HLA-B*18 (25.00% vs. 4.50%; $p=0.036$; OR=0,143 [95% CI, 0.023-0.877]), while exhibited a lower frequency of HLA-B*35 (6.30% vs. 36.40%; $p=0.047$; OR=8.571 [95% CI, 1.034-71.081]). No differences were found between the groups regarding the frequencies

of HLA-A alleles or the HLA-Bw4 alleles (Table 1). The HLA-A and B genotypes can be reviewed in Supplement 1.

Table 1. Frequency of HLA alleles in MSM groups

HLA-A (n=60)	All MSM		High-risk (n=16)		Low-risk (n=44)		p-Value	OR (95% CI)
	Freq	%	Freq	%	Freq	%		
1	5	8.33	1	6.3	4	9.1	0.726	1.500 (0.155-14.522)
2	26	43.33	9	56.3	17	38.6	0.227	0.490 (0.154-1.561)
3	12	20	4	25	8	18.2	0.561	0.667 (0.170-2.614)
11	5	8.33	2	12.5	3	6.8	0.488	0.512 (0.077-3.388)
23	5	8.33	2	12.5	3	6.8	0.488	0.512 (0.077-3.388)
29	2	3.33	1	6.3	1	2.3	0.466	0.349 (0.021-5.932)
30	7	11.67	3	18.8	4	9.1	0.312	0.433 (0.086-2.195)
68	10	16.67	3	18.8	7	15.9	0.794	0.820 (0.184-3.648)
HLA-B (n=60)	**All MSM**		**High-risk (n=16)**		**Low-risk (n=44)**		**p-Value**	**OR (95% CI)**
	Freq	**%**	**Freq**	**%**	**Freq**	**%**		
7	6	10	3	18.8	3	6.8	0.19	0.317 (0.057-1.766)
8	5	8.33	0	0	5	11.4	0.999	662758909.9 (0.000-.)
18	6	10	4	25	2	4.5	**0.036**	0.143 (0.023-0.877)
35	17	28.33	1	6.3	16	36.4	**0.047**	8.571 (1.034-71.081)
38	4	6.67	1	6.3	3	6.8	0.938	1.098 (0.106-11.385)
39	4	6.67	1	18.8	3	6.8	0.19	0.317 (0.057-1.766)
44	10	16.67	5	31.3	5	11.4	0.078	0.282 (0.069-1.154)
52	2	3.33	1	6.3	1	2.3	0.466	0.349 (0.021-5.932)
55	2	3.33	1	6.3	1	2.3	0.466	0.349 (0.021-5.932)
58	5	8.33	2	12.5	3	6.8	0.488	0.512 (0.077-3.388)
60	2	3.33	1	6.3	1	2.3	0.466	0.349 (0.021-5.932)
61	12	20	1	6.3	11	25	0.14	5.000 (0.591-42.334)
62	3	5	1	6.3	2	4.5	0.79	0.714 (0.060-8.460)
64	2	3.33	1	6.3	1	2.3	0.466	0.349 (0.021-5.932)
65	5	8.33	2	12.5	3	6.8	0.488	0.512 (0.077-3.388)
Bw4	45	75	12	75	33	75	0.971	1.025 (0.270-3.895)

The more frequent HLA-A and -B alleles are shown. The alleles A*26, 32, 33 and 34 & B*13, 14, 27, 41, 45, 49, 50, 51, 53, 57, 60, 63, 64, 71, and 72 also were detected in the studied subjects, but they are not presented due their lower frequencies.

MSM in the high-risk group exhibited protective KIR genes & haplotypes

According to the DNA availability, the presence of 14 KIR genes and 2 pseudogenes was determined in 57 of the sixty MSM. The MSM in the high-risk group exhibited lower frequencies of the KIR genes 2DL5 (6.70% vs. 47.60%; p=0.019; OR=12,727 [95% CI, 1.532-105.736]), 2DS1 (13.30% vs. 50.00%; p=0.022; OR=6.500 [95% CI, 1.303-32.17]), and, 2DS5 (6.70% vs. 40.50%; p=0.037; OR=9.520 [95% CI, 1.142-79.332]) (Table 2).

Table 2. Frequency of KIR genes in MSM groups.

KIR (n=57)	All MSM		High-risk (n=15)		Low-risk (n=42)		p-Value	OR (95% CI)
	Freq	%	Freq	%	Freq	%		
2DL1	47	82.46	14	93.3	33	78.6	0.224	0.262 (0.030-2.268)
2DL2	30	52.63	6	40	24	57.1	0.258	2.000 (0.602-6.642)
2DL3	41	71.93	12	80	29	69	0.422	0.558 (0.134-2.317)
2DL4	48	84.21	13	86.7	35	83.3	0.762	0.769 (0.141-4.192)
2DL5	21	36.84	1	6.7	20	47.6	**0.019**	12.727 (1.532-105.736)
2DP1	50	87.72	13	86.7	37	88.1	0.885	1.138 (0.196-6.600)
2DS1	23	40.35	2	13.3	21	50	**0.022**	6.500 (1.303-32.417)
2DS2	31	54.39	5	33.3	26	61.9	0.063	3.250 (0.939-11.243)
2DS3	11	19.3	3	20	8	19	0.936	0.941 (0.214-4.139)
2DS4	45	78.95	13	86.7	32	76.2	0.4	0.492 (0.095-2.562)
2DS5	18	31.58	1	6.7	17	40.5	**0.037**	9.520 (1.142-79.332)
3DL1	48	84.21	13	86.7	35	83.3	0.762	0.769 (0.141-4.192)
3DL2	55	96.49	14	93.3	41	97.6	0.458	2.929 (0.172-49.996)
3DL3	46	80.7	13	86.7	33	78.6	0.499	0.564 (0.107-2.970)
3DP1	49	85.96	13	86.7	36	85.7	0.927	0.923 (0.165-5.162)
3DS1	23	40.35	3	20	20	47.6	0.071	3.636 (0.894-14.785)
3DL1/Bw4*	35	61.4	9	60	26	61.9	0.897	1.083 (0.324-3.619)
3DS1/Bw4*	16	28.07	2	13.3	14	33.3	0.154	3.250 (0.642-16.440)
3DS1 or 3DL1/Bw4*	38	66.67	9	60	29	69	0.525	1.487 (0.438-5.051)

*Presence of KIR3DLS1 or KIR3DL1 and their combination with the HLA alleles belonging to the Bw4 group.

Based on KIR-gene content and the *Allele Frequency Net Database* imputing results, the KIR genetic profiles of all MSM were classified into AA haplotypes, which exclusively carry genes of the A haplotype (22.8%), and Bx haplotypes, which include the A and B haplotypes (AB) or only the B-haplotype (BB) (73.6%)[22,24]. It was impossible to identify the KIR haplotype in two individuals (3.6%) by the Allele Frequency Net Database imputation. These two haplotypes were compared between

both groups of MSM, and it was observed that the high-risk group exhibited a lower frequency of Bx haplotypes (53.30% vs. 81.00%; p=0.019; OR=0.202 [95% CI, 0.53-0.766]). Complementary, a higher but not statistically significant frequency of AA haplotypes was detected in the same group (46.70% vs. 14.30%; p=0.999).

The diversity of AA and Bx Haplotypes was analyzed in the 55 individuals in which their haplotypes were imputed, identifying forty-one different multi loci haplotypes in all subjects, of which four haplotypes were AA and 35 were Bx. By reviewing the frequencies of the different haplotypes in each MSM group, it was observed that only four haplotypes were shared between both groups (two AA and two Bx, supplement 2). Furthermore, an AA haplotype (No.1) was the most frequent in all subjects (15.8%), and it was the haplotype that marked the most significant difference between the groups, being statistically more frequent in the high-risk group than in the low-risk group (40% vs. 7%; p=0.007; OR=0.115 [95% CI, 0.24-0.551]; precisely, this haplotype is characterized by the absence of the 2DL5, 2DS1 and 2DS5 genes (supplement 2).

A higher frequency of linkage disequilibrium in KIR genes was observed in the low-risk MSM than high-risk MSM group. However, this difference may be influenced by the small sample size of this study, mainly in the high-risk MSM group. This group showed a strong linkage disequilibrium of 2DL3-2DS2; 2DL2-2DS2; 2DL5-2DS2, 2DL3 and 2DL2; 2DS1-2DS2, 2DL3 and 2DL5; 2DS4-2DL1 genes. Likewise, the low-risk MSM group exhibited a significative linkage disequilibrium of 2DL2-2DS2 and 2DL3; 2DS3-2DL2; 2DL1-2DS2; 3DS1-3DL1; 2DS5-3DL1, 3DS1; 2DS1-2DL5, 2DL1 and 3DL1 genes. The significative linkage disequilibrium r2 values are described in Table 3.

Table 3. Linkage Disequilibrium of KIR genes in MSM groups

Gene	3DL3	2DS2	2DL3	2DL2	2DL5	2DS3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DS5	2DS1	2DS4	3DL2
3DL3	*	-	-	-	-	-	-	0,488	0,412	-	-	-	-	-	0,261	-
2DS2	-	*	**0,5**	0,75	0,5	-	-	-	-	-	-	-	-	0,308	-	-
2DL3	-		*	**0,078**	**0,083**	-	-	-	-	-	-	-	-	**0,041**	-	-
2DL2	-	0,75	**0,375**	*	0,375	-	-	-	-	-	-	-	-	-	-	-
2DL5	-	-	-	-	*	-	-	-	-	-	-	-	-	0,615	-	-
2DS3	-	-	-	0,006	-	*	-	-	-	-	-	-	-	-	-	-
2DP1	0,464	-	-	-	-	0,4643	*	-	-	-	-	-	-	-	-	-
2DL1	1	0,077	-	-	-	-	0,464	*		-	-	-	-	-	1	-
3DP1	1	-	-	-	-	-	0,464	1	*	-	-	-	-	-	0,235	-
2DL4	-	-	-	-	-	-	0,464	0,179	-	*	-	-	-	-	-	-
3DL1	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-
3DS1	-	-	-	-	-	-	-	-	-	-	**0,018**	*	-	-	-	-
2DS5	-	-	-	-	-	-	-	-	-	0,011	0,286	0,005	*	-	-	-
2DS1	-	-	-	-	0,615	-	-	**0,024**	-	-	0,087	0,011	0,011	*	-	-
2DS4	1	-	0,018	-	-	-	-	1	1		0,087	-	-	-	*	-
3DL2	-	0,143	-	-	-	-	-	-	-	-	-	-	-	-	-	*

This table shows the Linkage Disequilibrium (LD expressed as r2) of KIR genes in both groups of MSM. The top and bottom sides show the LD in high-risk and low-risk MSM groups, respectively. Only the significative r2 are presented. The bold values represent a negative value of Linkage Disequilibrium. The KIR genes order corresponds to the physical order previously reported[25].

Finally, the previously reported association of the combination KIR3DL1/S1/HLA-Bw4 with natural protection to HIV-1 infection was also analyzed. However, no differences were detected between MSM groups regarding those combinations (Table 2).

Discussion

It is noteworthy that high-risk MSM group remains seronegative despite their risky sexual behaviors, which far exceed those associated with seroconversion in other MSM cohorts[21,26–28]. This situation suggests that these individuals could have protective factors against HIV-1 infection. Moreover, the protection against HIV-1

cannot be explained by the CCR5 delta 32 mutation, because it was not found in the subjects (one of our exclusion criteria for study enrollment).

A significative higher frequency of the HLA-B*18 allele was found in the high-risk MSM group. Several HLA alleles, most of them belonging to HLA-B are found in higher proportions in HESN compared to the general population and people living with HIV (PLWHIV), suggesting association with the natural resistance to HIV-1[1]. Farquhar *et al.* reported that HLA-B*18 allele was associated with a lower rate of HIV-1 mother-to-child transmission in Kenyan HIV+ mothers and their children; likewise, no one of the HLA-B*18+ uninfected newborns were infected through breast milk[29]. This allele was also associated with HIV-1 natural resistance in serodiscordant couples from India, more frequent in HESN than their HIV-1 positive partners and healthy controls[30].

Conversely to HLA-B*18, we showed a lower HLA-B*35 frequency in the high-risk MSM group, suggesting that this allele could be associated with HIV-1 protection. This result is consistent with previous studies showing that HLA-B*35 is associated with increased susceptibility to HIV-1 infection[31,32]. A study in India found higher frequencies of this allele in the HIV+ positive individuals than in healthy controls[33]. This allele has been associated with a significant risk of mother-to-child HIV-1 transmission. Arnaiz-Villena et al. demonstrated a higher frequency of this allele in HIV-1-infected children compared with the HIV-1-exposed but uninfected[34]. Furthermore, there is evidence that HLA-B*35+ individuals have a rapid disease progression to AIDS[32,34,35]. In a multicentric study, Carrington *et al.* observed an

association of the B*35 allele with rapid progression to AIDS in several cohorts of HIV+ infected individuals from the USA[36].

Although the Bw4 group of HLA-I and the heterozygosity of HLA have been associated with HIV-1 protection, we did not find differences in these factors or other protector alleles between the MSM groups[4,7,37]. The protective effect against HIV-1 infection of some HLA molecules, including several HLA-B, has been related to a more remarkable ability to recognize pivotal HIV-1 antigens and induce a robust cytotoxic T lymphocyte (CTL) response against the virus[1,38,39].

A lower frequency of 2DL5, 2DS1 and 2DS5 KIR genes was identified in the high-risk MSM group. Likewise, this group exhibited a lower frequency of KIR-Bx haplotypes and a significative increased frequency of one KIR-AA haplotype, which marked the most significant difference between both groups of MSM. The 2DL5, 2DS1 and 2DS5 genes are characteristically present in the Bx haplotypes, and they are absent in the AA haplotypes[24], which is consistent with the low frequency of Bx haplotypes and the higher frequency of AA haplotypes observed in the high-risk MSM.

Various studies have suggested the protective role of some KIR genes in the HIV-1 context[8,40]. Unlike our results, the protective effect has been commonly proposed based on the higher frequency of activating KIR genes, such as KIR3DS1, in HESN populations compared to PLHIV[13,37,41]. However, inhibitory genes have also been associated with protection[15]. Paximadis *et al.* reported that inhibitory genes KIR2DL2 and KIR2DL3 were more frequent in HIV+ mothers who did not transmit the virus to their children, compared to those transmitting mothers[42]. A meta-analysis about the influence of individual KIR genes and the genotypes 3DL1/3DS1 in the HIV

acquisition showed a strong protective effect against HIV-1 infection of both 2DL3 and 3DS1S1 polymorphisms in Caucasian, African and Asian HESN individuals[17]. In addition, an association with decreased risk of HIV-1 acquisition was observed by the presence of 2DL5, 2DS1 and 2DS5 genes in all populations[17]. These results differ from ours, where a lower frequency of those genes could be associated with protection in the high-risk MSM group. A lower frequency of 2DL5, 2DS1 and 2DS5 have been also observed in separate cohorts of Caucasian, African and Asian HESN[17].

The KIR system is one of the highest polymorphic, and significant gaps remain in its identification and understanding[25]. This situation, plus the broad variability between the methods, sample size, and genetic background of studied subjects generate the controversial role of the KIR genes in the susceptibility/protection to HIV-1 infection. As described above, the low frequency of 2DL5, 2DS1 and 2DS5 in the high-risk MSM group is related to the higher frequency of the AA haplotype. The AA haplotype is characterized by encoding inhibitory KIRs with only one activating receptor (KIR2DS4)[25,43]. Although the Bx haplotype has been more frequently associated with low susceptibility to HIV-1[44], a protective effect of the AA haplotype has been observed in other diseases. Nasa et al. showed that the homozygotic AA haplotype offers protection against classic Hodgkin lymphoma[45]; however, Braun et al. observed a risky effect of this haplotype in individuals infected with *Mycobacterium tuberculosis*[46]. The responsiveness of NK cells depends on the balance of activating and inhibitory signals. However, to be functional, the NK cell must recognize a self-HLA-I through a self-specific KIR receptor; thus, the NK cells that express inhibitory

KIR are licensed, exhibiting a lower threshold for activation and more robust response[47,48]. In this sense, the possible protective effect of the inhibitory KIR genes and AA haplotype in the high-risk MSM group could be explained by the possible greater responsiveness of their NK cells. Indeed, in a portion of this MSM cohort, we previously observed a higher response of NK cells by IFN-γ production and increased cytotoxicity against K562 stimuli by the MSM in the high-risk group[49]. To our knowledge, this is the first report of KIR genes and multi loci haplotypes in Colombian MSM.

Conclusion

Overall, the results of this study show that high-risk MSM group has genetic protective factors against HIV-1 infection, which could partially explain their seronegative status despite their high sexual exposure.

Supplementary Material

Supplement 1. HLA A and B Genotypes in high-risk and low-risk MSM

Subject Code	Risk Group	HLA-A Allele 1	HLA-A Allele 2	HLA-B Allele 1	HLA-B Allele 2
002	High-risk	2	68	49	53
003	High-risk	23	34	65(14)	44
004	High-risk	2	11	38	55
005	Low-risk	11	24	35	35
006	High-risk	24	68	61	53
007	Low-risk	24	24	61	48
008	Low-risk	24	24	62(15)	35
009	Low-risk	1	1	8	58
010	Low-risk	2	24	39	61(40)
011	High-risk	1	30	7	18
012	Low-risk	2	30	27	61(40)
013	Low-risk	2	32	18	35
014	Low-risk	2	68	71(15)	60(40)
015	Low-risk	24	24	7	55
016	Low-risk	1	24	8	8
017	High-risk	29	30	65(14)	60(40)
018	Low-risk	24	26	35	35
019	Low-risk	2	3	61	50
020	Low-risk	1	33	14	57
021	Low-risk	11	24	35	52
022	Low-risk	26	29	38	45
023	Low-risk	3	33	35	57
024	Low-risk	2	23	44	44
025	High-risk	2	3	7	39
026	Low-risk	3	3	18	35
027	High-risk	3	11	64(14)	39
028	Low-risk	3	24	61	51
029	High-risk	2	24	44	7
030	Low-risk	3	33	7	65(14)
031	Low-risk	24	68	35	44
032	Low-risk	2	24	48	58
033	Low-risk	2	32	51	51
034	Low-risk	26	26	8	58
035	Low-risk	24	68	65(14)	61(40)

036	Low-risk	2	_	35	61(40)
037	Low-risk	23	30	8	44
038	Low-risk	26	33	38	41
039	Low-risk	2	3	44	51
040	Low-risk	24	24	61	35
041	High-risk	30	68	13	18
042	High-risk	2	24	62(15)	44
043	High-risk	2	3	18	52
044	High-risk	2	24	18	35
045	High-risk	23	23	44	58
046	Low-risk	3	30	7	8
047	Low-risk	2	24	35	61(40)
048	Low-risk	2	24	35	39
049	Low-risk	2	68	39	39
050	Low-risk	11	24	35	51
051	High-risk	2	3	53	58
052	High-risk	2	24	39	44
053	Low-risk	23	23	64(14)	64(14)
054	Low-risk	3	30	27	35
055	Low-risk	2	24	35	35
056	Low-risk	2	24	62(15)	44
057	Low-risk	2	68	61(40)	61(40)
058	Low-risk	2	68	72(15)	72(15)
059	Low-risk	24	33	65(14)	61(40)
060	Low-risk	1	24	63(15)	35
061	Low-risk	24	68	38	57

Supplement 2. KIR multi loci haplotypes and their frequency in high-risk and low-risk MSM

																		All		High-risk		Low-risk	
No.	3DL3	2DS2	2DL3	2DL2	2DL5	2DS3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DS5	2DS1	2DS4	3DL2	Type	%	S.D.	%	S.D.	%	S.D.
1	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	1	AA	15,8	4,9	40	13,1	7,1	4
2	1	1	1	1	0	0	0	1	1	1	1	1	0	0	1	1	Bx	7	3,4	6,7	6,7	7,1	4
3	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	1	AA	3,5	2,5	6,7	6,7	2,4	2,4
4	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	Bx	3,5	2,5	6,7	6,7	2,4	2,4
5	1	0	1	0	1	0	1	1	1	1	1	1	0	0	1	1	Bx	1,8	1,8	6,7	6,7	0	0
6	0	0	1	1	0	0	0	1	1	0	0	1	0	0	0	1	Bx	1,8	1,8	6,7	6,7	0	0
7	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	Bx	1,8	1,8	6,7	6,7	0	0
8	1	1	1	1	0	0	0	1	1	1	1	0	0	0	1	1	Bx	1,8	1,8	6,7	6,7	0	0
9	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	0	Bx	1,8	1,8	6,7	6,7	0	0
10	1	1	0	1	0	1	0	0	1	1	1	1	0	1	1	1	Bx	1,8	1,8	6,7	6,7	0	0
11	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	Bx	3,5	2,5	0	0	4,8	3,3
12	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	Bx	3,5	2,5	0	0	4,8	3,3
13	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
14	1	1	1	1	0	0	0	1	1	1	1	1	0	0	1	1	Bx	3,5	2,5	0	0	4,8	3,3
15	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	Bx	1,8	1,8	0	0	2,4	2,4
16	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	Bx	1,8	1,8	0	0	2,4	2,4
17	1	1	0	1	0	0	1	1	0	0	0	0	1	0	0	1	Bx	1,8	1,8	0	0	2,4	2,4
18	1	1	1	1	0	0	0	0	1	1	1	1	0	0	1	1	Bx	1,8	1,8	0	0	2,4	2,4
19	1	1	0	1	1	0	1	0	0	1	1	1	0	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
20	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	NA	1,8	1,8	0	0	2,4	2,4
21	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
22	1	1	0	1	0	0	0	1	1	1	1	1	0	0	1	1	Bx	1,8	1,8	0	0	2,4	2,4
23	0	0	1	0	1	0	0	1	0	0	0	1	1	0	0	1	Bx	1,8	1,8	0	0	2,4	2,4
24	1	0	0	0	0	0	0	1	1	1	1	1	0	0	1	0	AA	1,8	1,8	0	0	2,4	2,4
25	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	1	Bx	1,8	1,8	0	0	2,4	2,4
26	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	1	NA	1,8	1,8	0	0	2,4	2,4
27	0	0	1	0	0	0	0	1	1	0	1	1	0	0	1	1	AA	1,8	1,8	0	0	2,4	2,4

28	0	0	0	1	1	0	0	1	1	1	1	1	1	1	0	1	Bx	1,8	1,8	0	0	2,4	2,4
29	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	1	Bx	1,8	1,8	0	0	2,4	2,4
30	1	1	0	1	1	1	0	1	1	1	1	0	1	1	0	1	Bx	1,8	1,8	0	0	2,4	2,4
31	1	0	1	0	1	0	0	1	0	1	1	1	0	0	1	1	Bx	1,8	1,8	0	0	2,4	2,4
32	0	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
33	1	0	1	0	1	1	1	1	1	1	1	0	1	1	0	1	Bx	1,8	1,8	0	0	2,4	2,4
34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
35	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	Bx	1,8	1,8	0	0	2,4	2,4
36	0	1	0	1	0	0	1	0	0	1	1	0	0	1	0	1	Bx	1,8	1,8	0	0	2,4	2,4
37	0	1	1	0	0	0	0	1	1	1	1	1	0	0	1	1	Bx	1,8	1,8	0	0	2,4	2,4
38	1	1	1	0	1	0	1	1	1	1	0	1	1	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
39	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
40	1	1	0	1	1	0	1	0	0	1	1	1	1	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4
41	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	Bx	1,8	1,8	0	0	2,4	2,4

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Conclusions

The MSM in the high-risk group remains seronegative despite the extremely sexual exposure. Compared with the low-risk MSM group, the donors in the high-risk group exhibited genetic factors associated with HIV-1 protection in HESN cohorts. This group showed a higher frequency of protective HLA-B*18 allele and a lower frequency of HLA*B35, which is associated with susceptibility to HIV-1 infection. Regarding KIR genes, the MSM in the high-risk group exhibited an increased profile of inhibitory KIR genes and haplotypes, which are associated with HIV-1 protection in other HESN populations. These results demonstrate that the MSM in the high-risk group has genetic factors that could protect them from developing HIV-1 infection despite the sexual exposure.

Chapter 4

Immunologic analysis

Study phase II

“Science is but a perversion of itself unless it has as its ultimate goal the betterment of humanity”.

Nikola Tesla

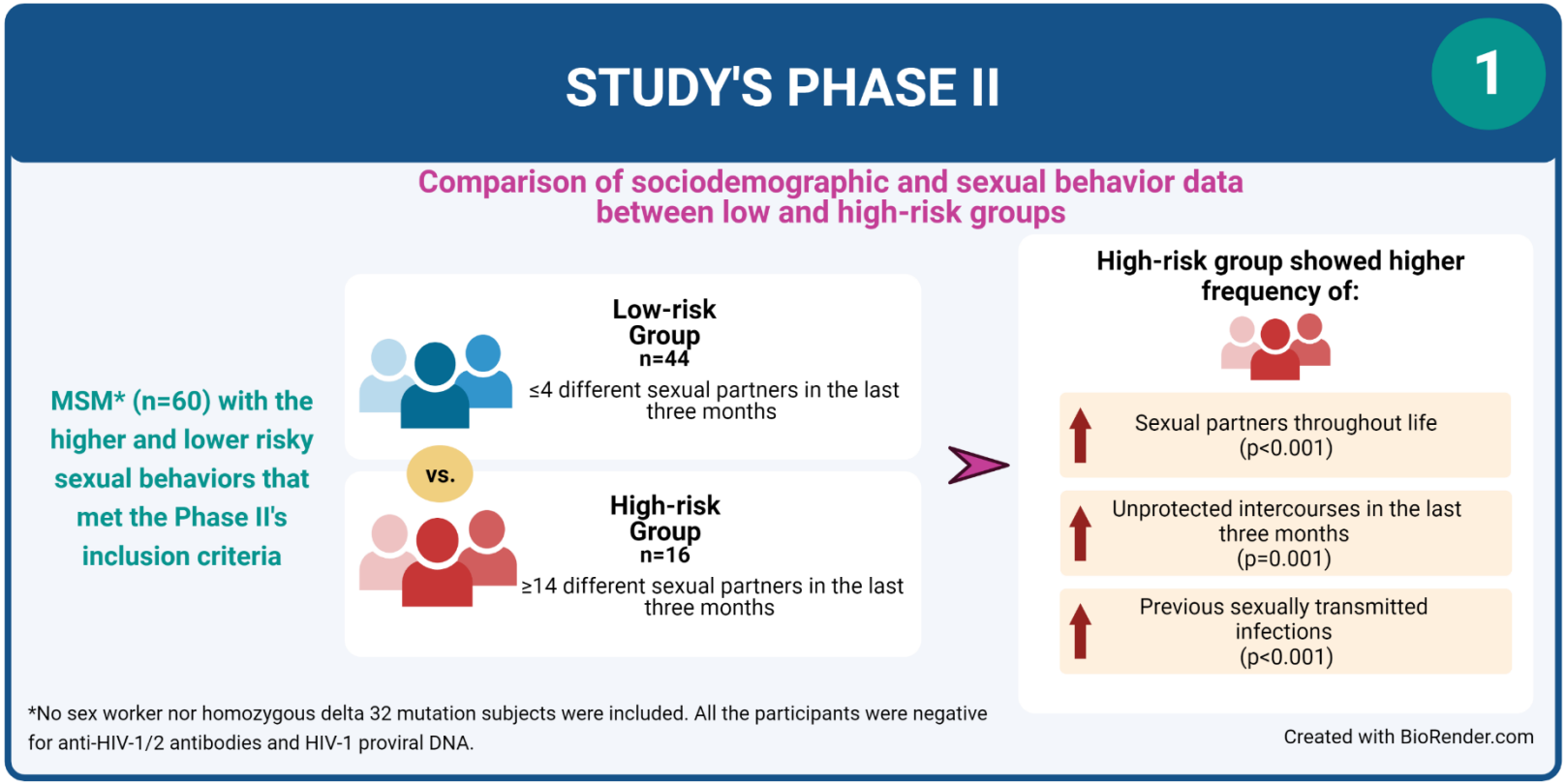
“La ciencia no es sino una perversión de sí misma a menos que tenga como objetivo final el mejoramiento de la humanidad”.

Nikola Tesla

Introduction

In this chapter, the reader will find the first part of Study's Phase II, corresponding to the comparison of the immunological profile between seronegative MSM at low and high-risk of HIV infection owing to their sexual behaviors. It was found that the high-risk group showed a quiescent immunological profile, with low activation of T and NK cells, but also with preservation of their responsiveness to polyclonal stimulus, and a higher expression of Serpin A1 by the PBMCs. The results of this comparison were compiled in a research article submitted to the PlosOne journal.

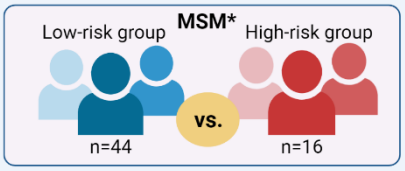
Graphic abstract



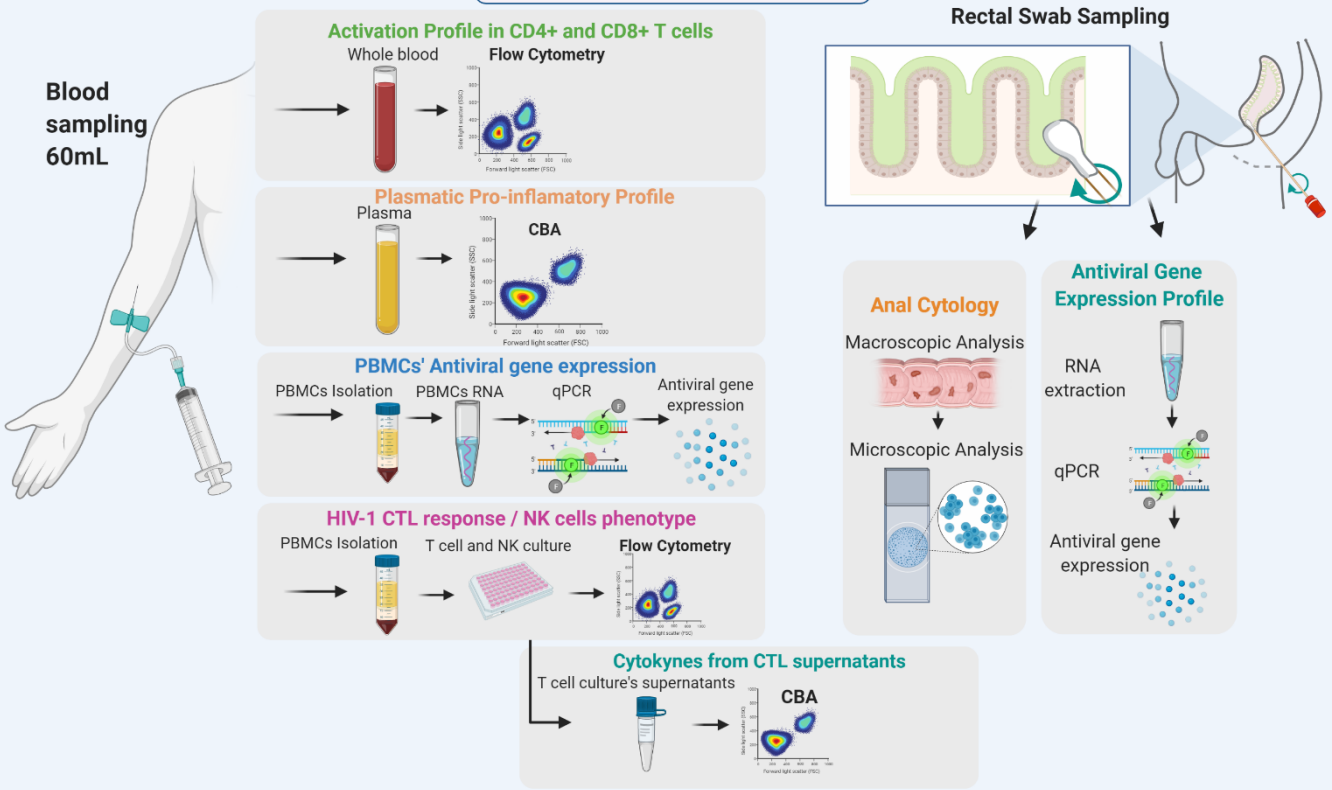
STUDY'S PHASE II

2

Comparison of immune profiles between low and high-risk groups



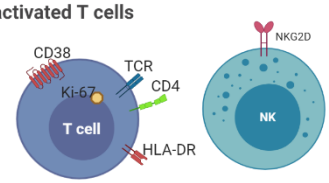
*No sex worker nor homozygous delta 32 mutation subjects were included. All the participants were negative for anti-HIV-1/2 antibodies and HIV-1 proviral DNA.



High-risk group mainly showed:

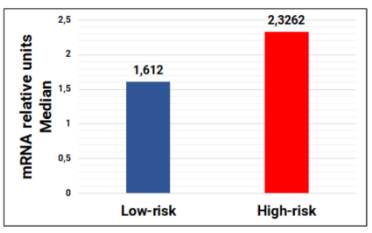
Quiescent NK and T Cell Profile

Lower % of T cells expressing CD38 and Ki67 and higher % of inactivated T cells



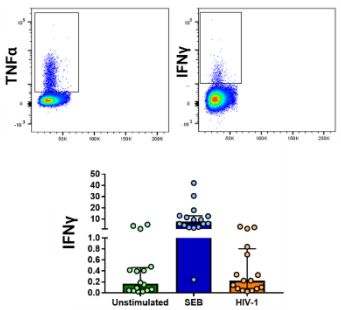
Lower % of NK cells expressing NKG2D

Higher Serpin A1 mRNA Expression in PBMCs



Strong T cell response to polyconal stimulus

IFNγ and TNFα expression upon SEB stimulus



Staphylococcus aureus Enterotoxin B

Created with BioRender.com

Seronegative MSM at high-risk of HIV-1 acquisition show Immune quiescent profile with normal immune response against common antigens

Short title:

Immune profile of MSM at high risk of HIV-1 acquisition

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¶ These authors contributed to study design, subject recruitment, lab assays, data collection and analysis, interpretation, and writing of first and subsequent drafts of the paper.

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Abstract

HIV infection still represents a major public health problem worldwide, and a vaccine remains elusive. The study of HIV-exposed seronegative individuals (HESN) brings important information about the natural resistance to HIV, allows a better understanding of the infection and opens doors for new preventive and therapeutic strategies. Among HESN groups there are some men who have sex with men (MSM) with high-risk sexual behaviors, who represent an adequate cohort for the study of HESN because of their major exposure to HIV in the absence of infection. This study aimed to compare the immunological profile of Colombian seronegative MSM with different risk sexual behaviors. Sixty MSM at high-risk (n=16) and low-risk (n=44) of HIV-1 acquisition were included. No sex worker nor homozygous delta 32 mutation subjects were included. All the participants were negative for anti-HIV-1/2 antibodies and HIV-1 proviral DNA. The high-risk MSM presented a higher frequency of sexual partners in the last 3 months previous to the study's enrollment (Median 30 vs. 2), lifetime sexual partners (Median 1708 vs. 26), and unprotected anal intercourse (Median 12.5 vs. 2) than low-risk MSM. This group also showed a quiescent profile of T cells and NK cells, with a significantly lower percentage of CD4+CD38+, CD4+HLADR-CD38+, CD4+Ki67+ T cells, NKG2D+ NK cells (CD3-CD16+CD56+), a significantly higher percentage of CD4+HLADR-CD38- and a tendency to show a higher percentage of

CD8+HLADR+CD38- T cells, than the low-risk group. Likewise, they showed higher mRNA levels of Serpin A1 from PBMCs. The results suggest that this cohort of MSM could be HESN individuals and their resistance would be explained by a quiescent profile of T cells and NK cells, and increased expression of Serpin A1. It is necessary to continue the study of MSM at high-risk of exposure to HIV-1 to better understand the natural resistance to HIV.

Keywords

HIV exposed seronegative, Natural resistance to HIV, Men who have sex with men, Quiescent immune profile.

Introduction

HIV-1 infection remains to be a big health issue and priority worldwide, despite the availability of highly effective antiretroviral therapy (1). Several efforts to find a vaccine have been done but it continues elusive (2). Some people -HIV-exposed seronegative individuals (HESN)- exhibit a natural resistance to HIV-1, who persist without an established infection besides their exposure to the virus (3). The comprehension of HESN's biological phenomena is an advantage to better understand the HIV-1 infection and brings new options for therapeutic and preventive strategies design (3,4). Since the first reports of natural resistance to HIV-1 (5,6), the mechanisms that can explain this phenomenon in HESN individuals are a major concern for HIV researchers, and still current now.

The main factors associated with HIV natural resistance are the homozygous deletion of 32bp in CCR5 co-receptor or Δ32 mutation, present in near to 1% of the global population (7), and recently described, the heterozygous single nucleotide deletion in the stop codon of the nuclear import factor Transportin 3 gene (*TNPO3*) (8). However, not all HESN subjects have these protective genotype and other immunological and genetic factors have been associated with HIV-1 natural resistance in HESN cohorts, such as the overexpression of antimicrobial peptides in the

mucosa, increased activity of NK cells, a combination of certain HLA-KIR genotypes, and HIV-1-specific CTL (cytotoxic T cells) responses (9–13).

In the context of HIV-1 exposure the presence of activated cells and inflammation at the site of HIV entry increase the risk of infection, as reported in people with clinical or subclinical STIs and mucosal inflammatory process (14–17). The immune quiescence, characterized by a low activation profile of target cells, has been proposed as a protection mechanism against HIV infection and it has been found in several HESN cohorts, including men who have sex with men (MSM) (18–22). In previous studies in the Pumwani cohort, a well-studied HESN cohort of African female sex workers, reduced susceptibility to HIV-1 infection was related to a lower frequency of activated CD4+CD69+ T cells and an elevated number of Treg cells (23). Low expression of genes implicated in T cells receptor signaling and HIV host-dependent factors also have been described together with lower levels of secreted cytokines at basal state, but no after stimulation, showing an important difference between quiescence and immunosuppression (24,25). Contrariwise, other studies have shown that the immune activation state is related to protection. Biasin *et al.* reported in 2000 an increased expression of proinflammatory cytokines and chemokine receptors in cervical biopsies of HESN women (26). Increased memory and activated T cells have been also described

(27,28). These results show that the role of cell activation in HIV-1 protection is not clear.

Since the first reports of resistance in 1989 (6) to the present, MSM have represented an adequate cohort for the study of HESN individuals. The high prevalence of HIV in this population (1) plus the higher probability to acquire the infection through anal sex (29), put MSM who practice risky sexual behaviors, such as anal sex without a condom and having multiple partners, at extreme risk of HIV exposure. Therefore, the study of seronegative MSM with high-risk behaviors and the possible finding of HESN individuals in this population represents an important opportunity to better understand HIV natural resistance.

The HIV prevalence in Colombian MSM is ~43 times higher than in the general population [17% vs. 0.4%] (1), and pre-exposure prophylaxis (PrEP) has not yet been approved in the country. Therefore, Colombian seronegative MSM that practice high-risk sexual behaviors are a very interesting population to address the mechanisms underlying HIV natural resistance. However, engaging this population in Latin America is not easy because most MSM do not want to share private information or even being identified due to the historic oppression they have suffered. This study aimed to compare the immunological profile of Colombian HIV seronegative MSM.

Material and methods

Study population

Sixty subjects were included from a cohort of MSM from Medellin-Colombia who signed informed consent and accepted to participate in the study. The recruitment of participants was carried out by the combination of several methods to sample hard-to-reach populations and sociodemographic data and sexual behaviors were defined by structural surveys and in-depth interviews, as we previously reported (30). The subjects were classified into two groups according to the frequency of sexual partners in the last three months before the inclusion of the study. The high-risk group (n=16) was defined as MSM with more than 14 sexual partners, and the low-risk group (n=44) were MSM with four or fewer sexual partners in the last three months, respectively. All individuals met the following inclusion/exclusion criteria: no one was a sex worker nor taking PrEP; all of them were negative for anti-HIV-1 antibodies, HIV-1 proviral DNA, and delta 32 mutation in the *CCR5* gene in a homozygous state. The study was performed according to the Helsinki declaration and was approved by the Ethics Committee of Universidad de Antioquia's School of Medicine (Act No.007, May 22th, 2014).

Biological Samples

Peripheral blood and anal mucosa samples were obtained from each subject; the whole blood was used to obtain plasma and serum, to extract DNA, as well as for PBMCs isolation. The anal mucosa sample was used for cytology analysis and mRNA extraction and collected using an optimized protocol: the cytobrush was inserted 5 cm beyond the anal verge, close to the anal wall, rotated slowly while were withdrawn to capture cells. Then, the sample was spread onto slides for cytology analysis and the cytobrush' head was cut and put into a vial with RNAlater reagent. A second cytobrush was inserted to obtain more mucosal sample, and this head was put as well into RNAlater reagent (Invitrogen). The samples were preserved at 4°C. Then, the cytobrush heads were removed and the cell pellet was obtained by centrifugation; TRIzol Reagent (Zymo) was added to lyse the cells. The samples were preserved at -80°C until RNA extraction. The anal sampling and cytology analysis was done by qualified staff from the reference lab "Laboratorio Clínico VID".

T cells basal activation profile

Fresh whole blood was stained for 25 min at room temperature in the dark with the monoclonal antibodies anti-CD4-PerCp-Cy5.5 clone OKT4, anti-CD8-eFluor 450 clone OKT8, anti-HLA-DR-FITC clone LN3, anti-CD69-APC clone FN50, anti-CD38-PE-Cy7 clone HIT2, and fixable viability dye-eFluor 506 (Thermo Fisher Scientific, Wilmington, DE, United States). Erythrocytes were lysed (BD FACS Lysing Solution, BD Biosciences, San Jose, CA, United

States) according to the manufacturer's instructions, next, the cells were permeabilizated and stained with anti- Ki-67-PE clone B56 (BD Biosciences, San Jose, CA, United States) and anti-CD3-Alexa eFluor 700 clone UCHT1 (Thermo Fisher Scientific, Wilmington, DE, United States) for 25 min at 4ºC in the dark. The cells were fixed with 1% formaldehyde, acquired using a BD LSRFortessa™ flow cytometer and, analyzed in FlowJo software (Becton–Dickinson, San Diego, CA, USA).

HIV-1-specific T cells responses

PBMCs were isolated by density gradient with Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, United States), washed with Phosphate-Buffered Saline (PBS) (Lonza, Rockland, ME, United States), and suspended in RPMI medium (Lonza, Rockland, ME, United States) supplemented with fetal bovine serum (FBS) at 10% (Gibco, Grand Island, NY, United States) and penicillin/streptomycin at 1% (Thermo Fisher Scientific, Wilmington, DE, United States). The cells were culture in the presence of 3µg/mL brefeldin A, 2uM monensin, 500µg anti-CD28 clone CD28.2, 500µg anti-CD49d clone 9F10 (Thermo Fisher Scientific, Wilmington, DE, United States), and stimulated overnight with a pool of peptides from HIV-1 subtype B consensus Gag (National Institutes of Health, AIDS Research and Reference Reagents Program). *Staphylococcus* enterotoxin B (SEB) was used as the positive control. After the overnight stimulus, the supernatants were collected and stored at -80°C until the cytometric bead array (CBA)

assay, and the cells were permeabilizated and stained with monoclonal antibodies CD3-Alexa eFluor 700 clone UCHT1, CD4-eFluor 660 clone OKT4, CD8-eFluor 450 clone OKT8, TNF-α-PerCp-Cy5.5 clone Mab11, fixable viability dye-eFluor 506 (Thermo Fisher Scientific, Wilmington, DE, United States), IFN-γ-brilliant Violet 711 clone 4S.B3 (BioLegend INC, San Diego, CA), Granzyme B-FITC clone GB11 and, MIP1-β-PE clone D21-1351 (BD Biosciences, San Jose, CA, United States), during 25min at 4°C in the dark. The cells were fixed with 1% formaldehyde, acquired using a BD LSRFortessa™ flow cytometer and, analyzed in FlowJo software (Becton–Dickinson, San Diego, CA, USA).

NK-cell profile

PBMCs were stimulated with IL-12 and IL-15 (20µg/mL) for 48 hours; 3µg/mL brefeldin A and 2uM monensin were added 24 hours post culture. Cells were stained with monoclonal antibodies CD16-Alexa Fluor 647 clone 38G (BioLegend INC, San Diego, CA), CD56- PE-Cy5 clone CMSSB, NKG2D-PerCP-eFluor710 clone 1D11, and fixable viability dye-eFluor 506 (Thermo Fisher Scientific, Wilmington, DE, United States) for 25 min at room temperature in the dark. Then, the cells were permeabilized and stained with CD3-Alexa eFluor 700 clone UCHT1 (Thermo Fisher Scientific, Wilmington, DE, United States), IFN-γ -Brilliant Violet 711 clone 4SB3 (BioLegend INC, San Diego, CA), Granzyme-FITC clone GB11 and, Perforin-PE (BD Biosciences, San Jose, CA, United States), during 25min at 4°C in

the dark. Finally, the cells were fixed with 1% formaldehyde and acquired using a BD LSR Fortessa™ flow cytometer and analyzed in FlowJo software (Becton–Dickinson, San Diego, CA, USA).

mRNA quantification by real-time RT-PCR from PBMC and mucosal samples

Total RNA was purified using the Direct-zol RNA Miniprep kit (Zymo Research), treated with DNase and, retrotranscribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Wilmington, DE, United States). PCR reactions were performed using the Maxima SYBR Green qPCR master mix kit (Fermentas). The specific primers and PCR conditions are shown in Tables 1 and 2 in S1 Text. Real-time RT-PCR was performed in a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Wilmington, DE, United States). The data are expressed as mRNA relative units of each gene normalized against the constitutive gene PGK (Phosphoglycerate kinase) using the formula $1.8^{-[\Delta Ct]}$, where 1.8 corresponds to the mean PCR efficiency of 90%.

Cytokines quantification by CBA

The levels of IL-1β, IL-6, IL-8, IL-12p70, TNF-α, and IL-10 were analyzed in plasma and CTL culture supernatants. The concentration of cytokines was determined by BD™ cytometric bead array (CBA) Human Inflammation Kit (Becton–Dickinson, San Diego, CA, USA), according to the manufacturer's

instructions. The data were analyzed in BD LSR Fortessa™ flow cytometer and FlowJo software (Becton–Dickinson, San Diego, CA, USA).

Results

The MSM from the high-risk group show risky sexual behaviors and remain HIV seronegative

The median age was 24.5 and 32 for subjects in the low and high-risk groups, respectively. In both groups, most of subjects identified themselves as gay/homosexual males, were single, and had access to vocational/professional education. In comparison with the low-risk group, the MSM in the high-risk group showed more risky sexual behaviors, including a higher number of sexual partners in the last three months ($p<0.001$), higher number of lifetime sexual partners ($p<0.001$), more unprotected intercourses in the last three months ($p=0.001$), and lower frequency of protected intercourses with their regular partners ($p=0.001$). In addition, in the high-risk group 81.3% practice receptive or versatile role; 81.3% never use condoms with their casual partners, 87.5% reported a previous history of STI, and 100% seek casual sex at bathhouses and clubs. The sociodemographic and sexual behavior data of MSM groups are described in Table 1.

Table 1. Sociodemographic and sexual behavior data of low and High-risk groups of MSM.

Variable	Low-risk (n= 44)	High-risk (n=16)	p-value
Sociodemographic data			
Age Median (IQR)	24.5 (20-29)	32 (26.5-36.7)	**0.002**[a]
	Low-risk n (%)	**High-risk n (%)**	**p-value**
Gender identity			
Male	43 (97.7)	16 (100)	NA
Queer	1 (2.3)	-	
Level of education			
High School	3 (6.8)	1 (6.3)	**<0.001**[b]
Vocational/Undergraduate education	41.1 (93.1)	15 (93.8)	
Occupation			
Student	15 (34.1)	-	**0.026**[b]
Student and Employed	5 (11.4)	-	
Employed Bachelor	17 (38.6)	11 (68.8)	
Unemployed Bachelor	1 (2.3)	1 (6.3)	
Employed/other not professional services	6 (13.6)	4 (25)	
Marital Status			
Single	42 (95.5)	14 (87.5)	NA
Cohabiting	2 (4.5)	2 (12.5)	
Sexual Behavior			
Sexual orientation			
Bisexual	4 (9.1)	3 (18.8)	NA
Gay/Homosexual	38 (86.4)	13 (81.3)	
Pansexual	1 (2.3)	-	
Age of sexual debut			
6-9 years old	4 (9.2)	1 (6.3)	0.36[a]
10 -14 years old	8 (18.2)	5 (31.3)	
15 – 17 years old	18 (40.9)	5 (31.3)	
18 and older	14 (31.8)	5 (31.3)	
Type of sexual partners			
Men and Women	-	1 (6.3)	NA
Only Men	44 (100)	15 (93.8)	
Sexual role			
Insertive	3 (6.8)	3 (18.8)	NA
Receptive	10 (20.5)	1 (6.3)	
Versatile	31 (70.5)	12 (75)	

Protected sexual intercourse with regular partners			
Always	9 (20.5)	-	**0.001**[b]
Never	33 (75)	15 (93.8)	
Protected sexual intercourse with casual partners			
Always	22 (50)	3 (18.8)	0.29[b]
Never	20 (45.5)	13 (81.3)	
Casual sex (bathhouses, clubs, seeking sex by social media)			
Yes	9 (20.5)	16 (100)	NA
No	35 (79.5)	-	
Last unprotected sexual intercourse			
Never	6 (13.6)	1 (6.3)	
<1 month	12 (27.3)	2 (12.5)	
1 – 2 months	9 (20.5)	3 (18.8)	NA
3-5 months	6 (13.6)	3 (18.8)	
6 – 11 months	4 (9.1)	-	
≥1 year	7 (15.9)	2 (12.5)	
Reported Previous STIs	14 (31.8)	14 (87.5)	0.6[b]
Type of reported STIs			
Hepatitis B	-	2 (14.3)	
Gonorrhea	5 (35.7)	7 (50)	
Syphilis	3 (21.4)	6 (42.9)	NA
Herpes	1 (7.1)	1 (7.1)	
PVH	7 (50)	2 (14.3)	
Not specified	-	2 (14.3)	
Frequency of HIV positive sexual partners	16 (36.6)	9 (56.2)	0.16 [b]
Number of sexual partners in the last 3 months			**<0.001**[a]
Median (IQR)	2 (1-4)	31 (23-45)	
Number of unprotected sexual intercourses in the last 3 months			**0.001**[a]
Median (IQR)	2 (0-2)	10 (6-24)	
Number of lifetime sexual partners			**<0.001**[a]
Median (IQR)	26 (11-97)	1078 (900-4100)	

[a] Mann–Whitney U test

[b] Chi-squared test

NA: not applicable, not analyzed

Seronegative MSM at high-risk of HIV-1 infection show a low T cell activation profile and a higher expression of Serpin A1

To explore the basal activation profile of CD4+ and CD8+ T cells, the percentage of cells expressing CD38, HLA-DR, CD69, and Ki-67 molecules was quantified by flow cytometry (Fig 1 in S1 Text). The high-risk group showed a low activation profile in T cells with lower percentage of CD4+CD38+ (p=0.002), CD4+HLADR-CD38+ (p=0.027), and CD4+Ki67+ cells (p=0.048). Likewise, this group had a higher percentage of CD4+HLADR-CD38- (p=0.013). Although it was not statistically significant, there was a tendency in the high-risk group to present a lower percentage of CD8+HLADR+CD38- cells (p=0.058). No differences were found in the expression of CD69 between both groups (Fig 1). To identify whether high-risk group exhibit differences in the basal levels of plasmatic cytokines and soluble factors, the concentrations of IL-6, IL-8 IL-10, IL-12p70, and TNF-α were quantified by CBA, and the mRNA expression of Elafin, Serpin A1, MIP1-β, RANTES, IL-1β, IL-18, IL-22, Caspase 1, and FoxP3, in PBMCs through qPCR. Compared with the low-risk group, the MSM in the high-risk group exhibit higher mRNA levels of Serpin A1 (p=0.018) and a tendency to express more MIP1-β (0.27 vs. 0.10; p=0.06). No differences were found regards the other soluble factors evaluated (Tables 2 and 3).

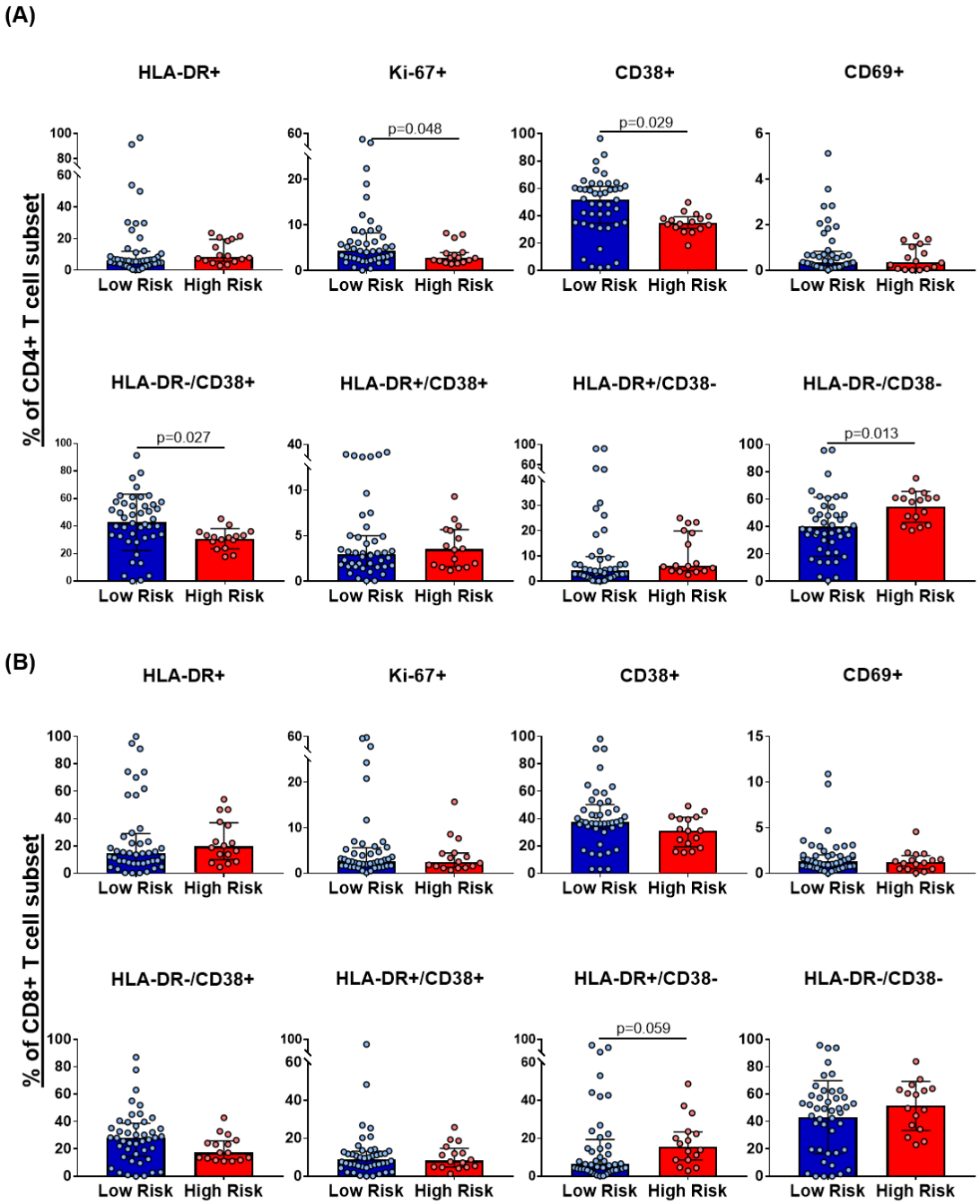

Fig 1. CD4+ and CD8+ T cells basal activation profile in both groups of MSM.

Comparison of the percentage of **(A)** CD4+ and **(B)** CD8+ T cells expressing HLA-DR, Ki-67, CD38, and CD69 activation markers between the groups. High-risk group n=16, low-risk group n=44. All data are reported after background correction. The comparison was realized with the Mann-Whitney U test or Student T-test according to the data distribution.

Table 2. Plasmatic levels of cytokines in both groups of MSM.

Cytokine	Low-risk (n=44) pg/mL Median (IQR)	High-risk (n=16) pg/mL Median (IQR)	U Mann Whitney p-value
IL-6	13.9 (12.8-15.0)	14.0 (12.9-14.9)	0.95
IL-8	24.3 (23.0-26.2)	26.1 (24.4-29.1)	0.08
IL-10	15.7 (15.4-15.9)	15.5 (15.4-15.6)	0.23
IL-12	17.4 (17.0-19.0)	17.1 (16.8-17.5)	0.10
TNF-α	0.0 (0.0-4.7)	0.0 (0.0-3.9)	0.61

Table 3. mRNA gene expression in PBMCs of both groups of MSM.

Gene	Low-Risk (n=29) mRNA relative units Median (IQR)	High-Risk (n=9) mRNA relative units Median (IQR)	U Mann-Whitney p-value
Caspase 1	0.285 (0.0634-0.174)	0.330 (0.174-0.534)	0.49
Elafin	0.0003 (0.0001-0.0006)	0.0005 (0.0001-0.0012)	0.51
IL-1β	0.334 (0.167-0.855)	0.334 (0.167-0.855)	0.79
IL-18	0.004 (0.002-0.007)	0.004 (0.003-0.008)	0.82
Serpin A1	1.612 (0.785-2.750)	2.326 (1.390-3.456)	**0.01**
FoxP3	0.005 (0.001-0.012)	0.005 (0.001-0.011)	0.76
IL-22	0.0005 (0.0003-0.0012)	0.0004 (0.0003-0.0006)	0.74
MIP1-β	0.130 (0.059-0.237)	0.245 (0.102-0.349)	0.06
RANTES	1.223 (0.473-1.839)	1.258 (0.604-2.370)	0.15

The data are expressed as mRNA relative units of each gene (median and interquartile range -IQR) normalized against the constitutive gene PGK.

There are no differences in the T cells response to HIV-1 Gag peptides between both groups of MSM

The intracellular expression of Granzyme B, MIP1-β, TNF-α, and IFN-γ by CD4+ and CD8+ T cells was measured after stimulus with a pool of peptides from HIV-1 subtype B consensus Gag or SEB (Fig 2 in S1 Text). Likewise, the levels of IL-10, IL-12, IL-1b, IL-6, IL-8, and TNF-α in the culture's supernatants were quantified by CBA. All the 60 MSM showed a strong specific response to SEB, however, the HIV-1 specific response only was detected in some subjects and there was not a defined pattern of response by risk group. No differences were found in the magnitude nor index of T cell polyfunctionality between the MSM groups in response to HIV-1 Gag peptides (Fig 2). Finally, no statistical differences were found between the groups in the levels of IL-10, IL-12, IL-1β IL-6, IL-8, and TNF-α in the supernatants of HIV-1 stimulated cells (Table 3 in S1 Text).

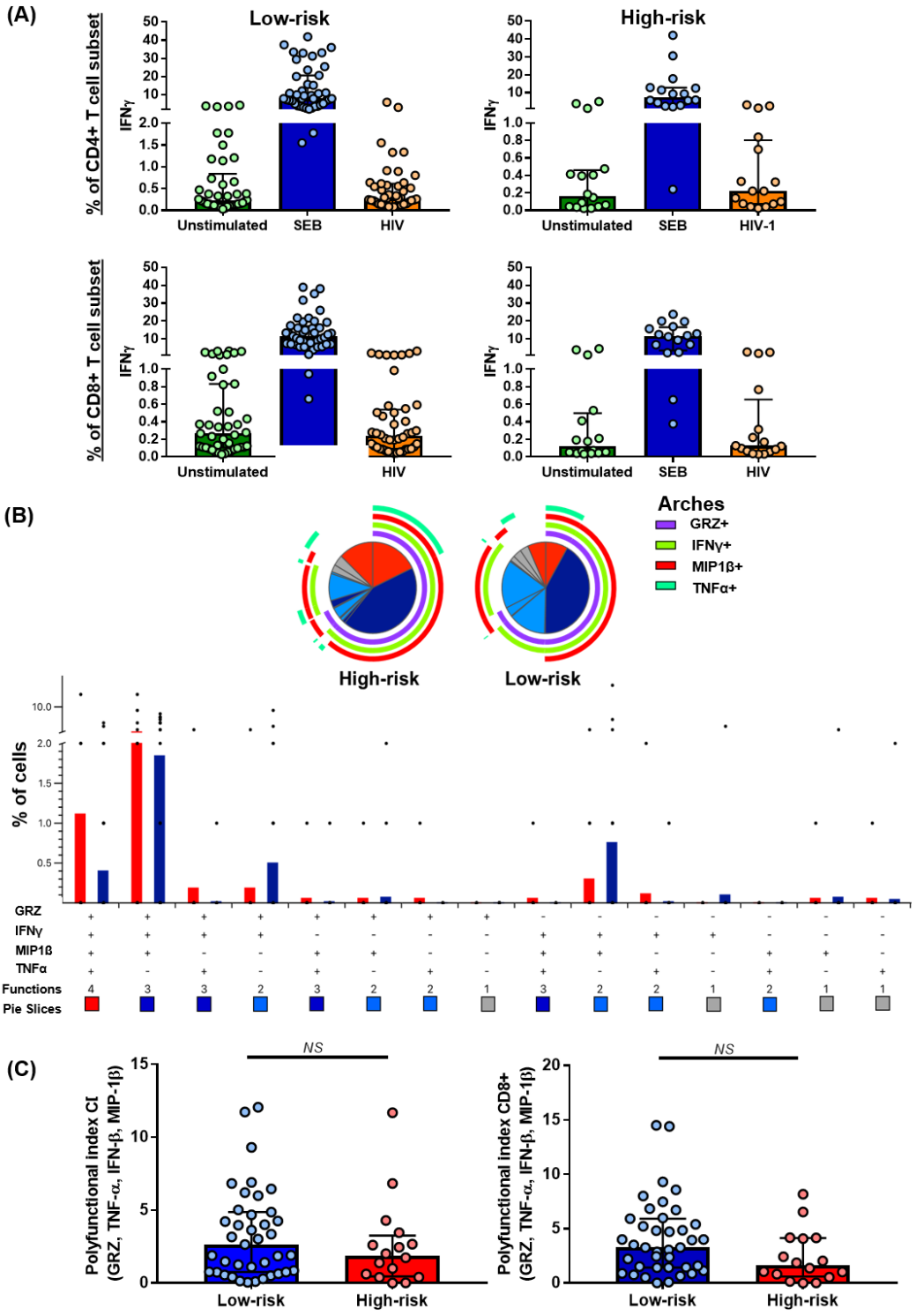
(A)
Low-risk
High-risk
% of CD4+ T cell subset
% of CD8+ T cell subset
IFNγ
Unstimulated
SEB
HIV
HIV-1
(B)
Arches
GRZ+
IFNγ+
MIP1β+
TNFα+
High-risk
Low-risk
% of cells
GRZ
IFNγ
MIP1ß
TNFα
Functions
Pie Slices
(C)
NS
Polyfunctional index CI (GRZ, TNF-α, IFN-β, MIP-1β)
Polyfunctional index CD8+ (GRZ, TNF-α, IFN-β, MIP-1β)
Low-risk
High-risk

Fig 2. T cells response against common pathogens (SEB) and a pool of HIV-1 Gag peptides.
(A) IFN-γ expression by unstimulated CD4+ and CD8+ T cells or after SEB and HIV-1 Gag peptides stimulus. **(B)** Comparison of the polyfunctional profiles of Gag-specific responses in CD4+ T cells from both MSM groups. The slices of the pies correspond to the proportions of Gag-specific CD4+ T cells expressing 1 (grey), 2 (light blue), 3 (dark blue), or 4 (red) functions, until n simultaneous parameters (n+1 dimensions) calculated using Boolean gating; the results are presented as mean. In the permutation analysis carried out in the SPICE platform only data higher than 0.1% were included (after background subtraction). **(C)** INDEX of polyfunctionality (pINDEX) of CD4+ and CD8+ T cells from both MSM groups based on the proportions of cells producing intracellular combinations of Granzyme B, MIP1-β, TNF-α, and IFN-γ; the pINDEX was calculated with Funky Cells software (31); the comparison was realized with the Mann-Whitney U test. For all the panels: high-risk group n=16, low-risk group n=44.

Seronegative MSM at high-risk of HIV-1 infection show a low expression of NKG2D on NK cells

To explore the different NK cells subpopulations in both MSM groups, the CD56 and CD16 expression was analyzed in the $CD3^-$ cells from fresh peripheral blood (Fig 3A in S1 Text). Both groups of MSM showed a similar distribution of NK cells subpopulations. Then, the expression of IFN-γ, Perforin, Granzyme B, and NKG2D was assessed in total NK cells from PBMCs after being stimulated for 48 hours with the combination of IL-12 and IL-15 (Fig 2 in S1 Text). The high-risk group showed a lower percentage of total NK cells expressing NKG2D (p=0.021). No differences were found in the magnitude nor polyfunctional index in those cells (Fig 3).

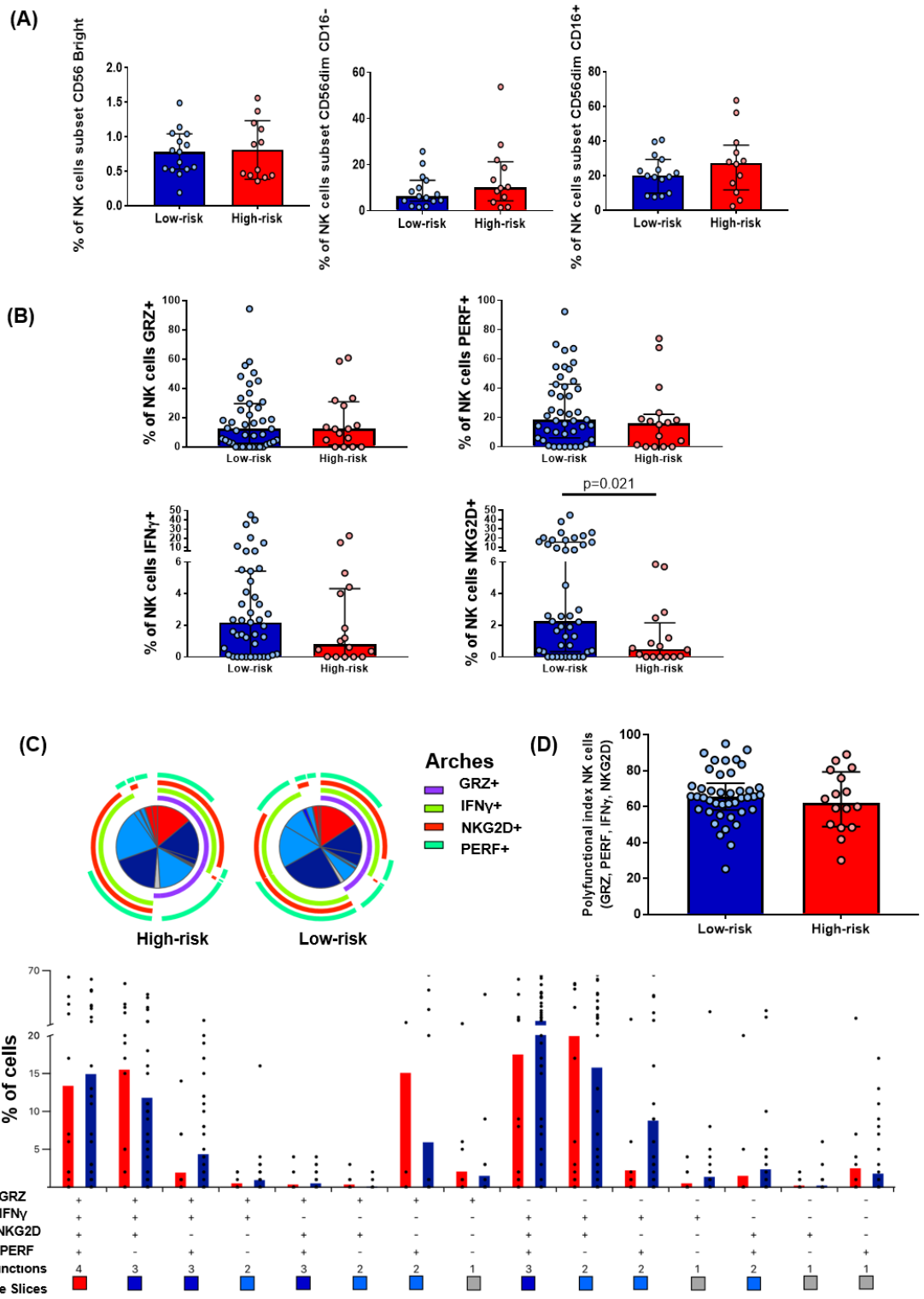
(A)
% of NK cells subset CD56 Bright
% of NK cells subset CD56dim CD16-
% of NK cells subset CD56dim CD16+
Low-risk
High-risk
(B)
% of NK cells GRZ+
% of NK cells PERF+
% of NK cells IFNγ+
% of NK cells NKG2D+
p=0.021
(C)
Arches
GRZ+
IFNγ+
NKG2D+
PERF+
High-risk
Low-risk
(D)
Polyfunctional index NK cells (GRZ, PERF, IFNγ, NKG2D)
% of cells
GRZ
IFNγ
NKG2D
PERF
Functions
Pie Slices

Fig 3. NK cells subset distribution, NKG2D expression, and polyfunctional index.

(A) Comparison between both MSM groups of NK cells subsets (CD56bright, CD56dim CD16+, CD56dim CD16-) from fresh peripheral blood. The comparison was realized with the Mann-Whitney U test. Low-risk n=15, high-risk n=12. **(B)** Comparison of percentage of total NK cells (CD3-CD56+CD16- and CD3-CD56+CD16+ PBMCs) expressing Granzyme B, Perforin, IFN-γ or NKG2D between both groups of MSM. The comparison was realized with the Mann-Whitney U test. Data are presented after background subtraction. Low-risk n=43, high-risk n=14. **(C)** Comparison of the polyfunctional profiles of total NK cells (CD3-CD56+CD16- and CD3-CD56+CD16+ PBMCs) from both MSM groups. The slices of the pies correspond to the proportions of total NK cells expressing 1 (grey), 2 (light blue), 3 (dark blue), or 4 (red) functions, until n simultaneous parameters (n+1 dimensions) calculated using Boolean gating; the results are presented as mean. In the permutation analysis carried out in the SPICE platform only data higher than 0.1% were included (after background subtraction). **(D)** INDEX of polyfunctionality (pINDEX) of total NK cells from both MSM groups based on the proportions of cells expressing Granzyme B, Perforin, IFN-γ and NKG2D; the pINDEX was calculated with Funky Cells software; the comparison was realized with the Mann-Whitney U test. Low-risk n=43, high-risk n=14.

There are no differences in the expression of antiviral genes in anal mucosa between both groups of MSM

To explore the macroscopic and microscopic state of anal mucosal tissue and the expression of the antiviral genes *HPN1, HBD2, HBD3, SLPI, RNAse7, TRIM5α,* and *APOBE3G*, an anal sampling was made. In both groups, most MSM presented no symptoms, a healthy anus, and a low frequency of intraepithelial lessons. No statistical differences were found regarding symptoms or the macro and microscopic state of anal mucosa between both groups (Fig 4). Quantification of the mRNA expression of the antiviral genes was no possible due to the low quantity of mRNA obtained, so a qualitative comparison of the detected genes was made between both groups. There were no statistical differences in the detection of the antiviral genes *HPN1, HBD2, HBD3, SLPI, RNAse7, TRIM5α,* and *APOBE3G* (Table 4 in S1 Text).

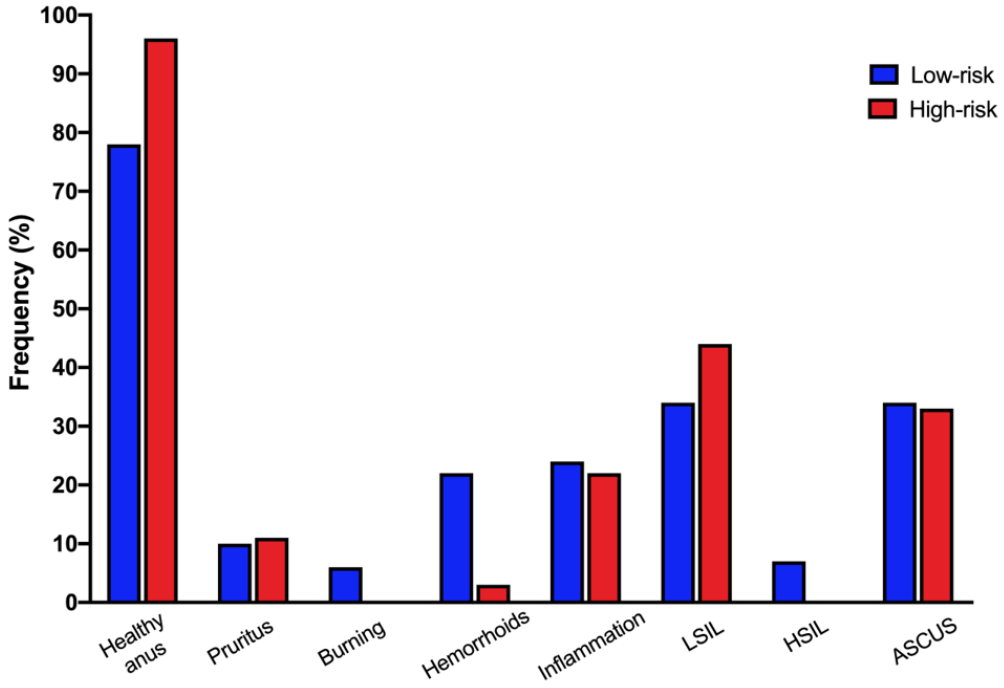

Fig 4. Symptoms, macroscopic and microscopic findings in anal mucosa tissue of both MSM groups.

Comparison of symptoms, macroscopic, and microscopic findings in the anal mucosa tissue of both MSM groups. Data represent the percentages of people presenting each condition; there were no statistical differences between both groups through Chi-square test. Low-risk n=29, high-risk n=9. ASCUS: atypical squamous cells of uncertain significance; LISIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

Discussion

This study reached a population with challenging access given the conditions of the vulnerability of the LGBTI population in our country (32) and the inclusion criteria that directed the recruitment of individuals with very high-risk sexual behaviors in the absence of sex work. These results and our previous study showed that MSM individuals included in the high-risk group are at extreme risk of HIV infection, showing behaviors that exceed by far the factors associated with seroconversion in the other MSM cohorts, who remain seronegative (30). Although no correlations were observed between the immunological variables and the sexual behaviors of MSM, significant differences were found between both groups regarding immune factors that have been previously associated with resistance to HIV-1 and that limit its transmission/acquisition.

The MSM group at high-risk of HIV infection showed a low activation profile of T and NK cells. The lower T cells activation profile has been described previously in HESN, which has low expression of T cell activation markers (33) and higher percentages of Treg cells (34). The study of Camara *et al.* (35) showed that HESN of serodiscordant couples had a lower percentage of CD4+ T cells expressing CD38 than control subjects. A similar low percentage of CD4+ T cells positive for CD38 has been described in MSM HESN from an Amsterdam cohort (33), in HESN from female sex workers of the Pumwani cohort in Kenya (36), as well as in Colombian elite

controllers (37). Moreover, it has been described that the low percentages of T cells expressing activation molecules are related to a lower susceptibility of HIV-1 infection *in vitro*, and the persistent HIV-1 seronegative status is associated with lower T cell activation (33,38).

Our group has previously described that elite controllers exhibit a lower percentage of NK and T cells expressing activation molecules than HIV-1 progressors (39), which, together with the aforementioned studies, point to a protective quiescent cell profile characterized by a low activation of immune cells without the loss of functionality. These findings are similar to those in this cohort, where a low activation profile of T cells with a strong response to common pathogens and HIV-1 peptides was observed in some individuals. Although there were not found other studies reporting low expression of NKG2D in HESN nor elite controllers, Muntasell *et al*. demonstrated in a model of CMV infection that the exposure to the virus resulted in a decreased expression of NKG2D, which selectively limited the ability of NK cells to kill target cells expressing high levels of NKG2D ligands, while preserving the expression of other NK activation molecules and the NK cytotoxic potential (40). Likewise, we previously found that MSM at high-risk from this cohort showed higher cytotoxic capacity and IFN-γ production in response to K562 cell-stimuli compared to MSM at low-risk of HIV-1 infection (41). Those findings reinforce the theory that the quiescent immune profile is a protective factor associated with the control

of HIV-1 and is not limited to T cells. To our knowledge, this is the first evidence of low expression of NKG2D by NK cells associated with natural resistance to HIV-1 infection in HESN.

A higher expression of Serpin A1 in PBMCs was found in the high-risk group. High levels of Serpin A1 have previously been described in the genital mucosa of HESN female sex workers (42), genital and oral mucosa of HESN serodiscordant couples, and GALT tissue of HIV-1 elite controllers (9). The anti-HIV function of Serpin A1 in the mucosa is well understood, as it prevents damage to the tissue integrity, thus avoiding the inflammatory response and transmigration of the virus to other tissues (43). Although less discussed, the anti-HIV effect of Serpin A1 in peripheral blood is significant considering that this serine protease: (i) inhibits neutrophil elastase, which promotes the entry of HIV-1 into the cell through its binding to gp120, and, through the cleavage that makes SDF-1 (CXCL12) and CXCR4, facilitating the binding of HIV-1 with this receptor; (ii) inhibits the formation of gp120; and (iii) inhibits the processing of p55 to p24 by protease (44). This anti-HIV effect has been previously demonstrated by the absence of HIV infection in whole blood compared to the presence of infection in lymphoid nodules under the same *in vitro* conditions (45), as well as in the easier replication of the virus in whole blood of individuals with inherited Serpin A1 deficiency, compared to the lower infection rate in whole blood of healthy controls (46). To our knowledge, this is the first

evidence of increased expression of Serpin A1 by PBMCs in seronegative MSM cohorts at high risk of HIV-1 infection.

The limitations of our study rely upon small sample size, no probabilistic sampling, and the cross-sectional nature of it, which do not allow us to infer and extrapolate our findings to other populations, as well as to build an explicative and causality model of HIV-1 resistance in MSM at high-risk of HIV-1 infection.

Taken together, this suggests that Colombian MSM at high-risk could be HESN individuals and natural resistance against HIV-1 could be a combination of quiescent T and NK cells profiles and increased expression of Serpin A1 by PBMCs. It is necessary to continue the study of MSM at high-risk of exposure to HIV-1 to better understand their natural response to the virus and improve the prevention and therapeutic strategies against HIV-1 infection.

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Conclusions

The participants in the high-risk group exhibited very risky sexual behaviors and were still HIV seronegative. Compared with the low-risk group, those MSM showed immune factors associated with natural resistance to HIV-1 infection, such as the low activation profile of T and NK cells; despite this reduced basal activation, the T cells conserve the capacity to respond to polyclonal stimulus. Moreover, this group showed a higher expression of Serpin A1 by their PBMCs compared to the low-risk group. Took together, these results can suggest that MSM in the high-risk group have immune factors that could protect them from developing HIV-1 infection despite their sexual exposure.

Chapter 5

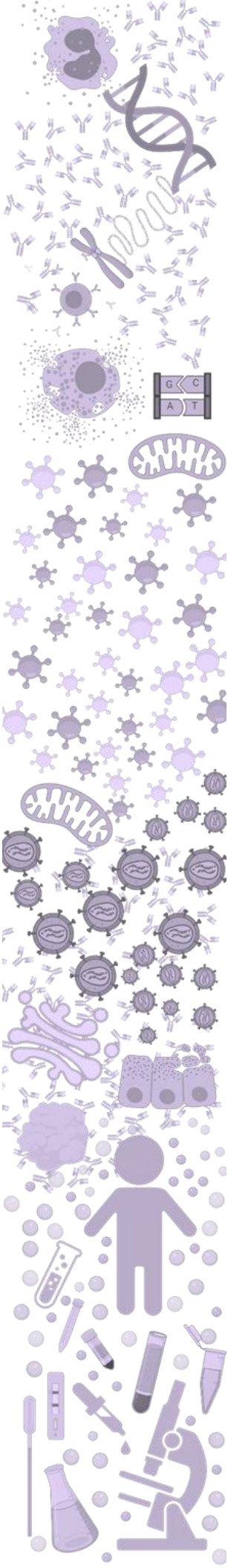
General Discussion and Perspectives

“Science is not only compatible with spirituality; it is a profound source of spirituality”. Carl Sagan

“La ciencia no solo es compatible con la espiritualidad; es una fuente profunda de espiritualidad”. Carl Sagan

Introduction

In this chapter, the reader will find the general discussion, which integrate all the findings across this study: the epidemiological, genetic and immunological data. Then, the limitations of the study are described, and the perspectives to future studies are presented.



General Discussion

The study of the natural resistance to HIV-1 exhibited by HESN allows for a better understanding of the immunopathogenesis of the infection, which, in turn, provides greater possibilities for the design of prevention and cure strategies[1,2]. However, access to individuals that show this natural resistance is limited, given their exceptional nature[3]. In the case of HESN, in addition to their low frequency across different populations[4], there is also the current decrease in exposure to HIV-1 for serodiscordant couples, children of HIV-1 infected mothers, and those exposed through blood transfusions, between others, derived from technological advances in antiretroviral therapy, diagnostic tests, and prevention strategies such as PrEP[5–9]. PLWHIV on treatment and with undetectable viral load do not transmit the virus[9], and the PrEP is nearly 100% effective in reducing sexual transmission of HIV-1[10,11]. Therefore, there is a substantial reduction of HIV-1 exposure for populations with access to prevention strategies, which commonly include the serodiscordant couples and sex workers, two of the most representative HESN populations[3]. Although this situation is quite optimistic regarding infection prevention, the study of natural resistance to HIV-1 has led to more incredible difficulty in identifying and reaching possibly resistant individuals. This was the first challenge we were faced within this study: having the ability to reach individuals at high risk of acquiring the virus, on whom their seronegative status is unsettling because of their high exposure. To meet this challenge, and consider the high prevalence of HIV-1 among Colombian MSM (43 times the prevalence in the general population[12,13]), we aimed

to find seronegative MSM without using PrEP, but with very risky sexual behaviors. We showed in Chapter 1 that the strategies for recruitment of the MSM population let us reach MSM, which exceeded the risky sexual behaviors associated with HIV-1 acquisition in other MSM and sexual workers cohorts[14–19]. Considering the high risk of HIV-1 acquisition and the negative serostatus of the MSM in the high-risk group, we hypothesized that this group exhibits protective mechanisms against HIV-1 infection, which the CCR5 Δ32 mutation cannot explain, the main resistance factor against HIV-1[20–22].

In the search for additional protection mechanisms for the studied MSM population, we found a higher frequency of HLA B18 in those at high risk. We found a higher frequency of the HLAB*18 allele in the MSM of the high-risk group. Various studies have shown that specific HLA genotypes are protective against HIV-1 infection, including the *B18 allele[23]. MacDonald *et al.* found an association of HLA-B*1801 with a decreased risk of HIV-1 seroconversion in female sex workers from Nairobi, Kenya[24]. Likewise, a higher frequency of the B*18 allele was found in female sex workers from Thailand[25]. This allele was also associated with a lower rate of HIV-1 mother-to-child transmission in exposed uninfected babies from Nairobi[26].

Several studies have evidenced that some HESN generate a specific CTL response against HIV-1, and certain HLA-I alleles mediate this response[23,24]. Thus, we carried on additional analysis to assess the CTL response against HIV-1 Gag peptides together with the HLA alleles frequency in both groups of MSM. Although no associations were found between the HLA alleles and CTL response, it is striking that of all HLA B*18 individuals (n=6), 67% showed at least one type of CTL response

(intracellular expression of Granzyme B, MIP1-β, TNF-α, or IFN-γ) by $CD4^+$ or $CD8^+$ T cells against HIV-1 Gag peptides. Reviewing by risk groups, we observed that 100% of HLA-B*18 MSM in the high-risk group showed any CTL response against the HIV-1 Gag peptides by $CD8^+$ T cells, and 75% of them showed any CTL response by $CD4^+$ T cells. Conversely, only 50% of the HLA*B18 MSM in the low-risk group showed any CTL response by $CD8^+$ or $CD4^+$ T cells. However, these differences in the CTL response were not statistically significant, possibly due to the sample size. Similar to those results, HESN and PLWHIV showed a CTL response against HIV-1 led by the HLA-B*18 allele. It was shown that female sex workers from Nairobi exhibited a CTL response to multiple conserved HIV epitopes, but they also generate a strong CTL response to novel epitopes through HLA-A*6802 and HLA-B*18, which in turn, were associated with HIV-1 protection[27]. Likewise, the control of HIV-1 infection by CTL response has been observed on PLWHIV who exhibit the HLA-B*18 allele. It was observed that infected asymptomatic individuals exhibited an HLA-B*18 restricted CTL response against the Nef HIV-1 protein. This response is led against an immunodominant region of Nef, where the 134-144 residues are cognate peptides for HLA-B*18[28,29]. Moreover, HLA-B*18 also recognizes and presents peptides that are restricted to the major homology region (MHR), a highly conserved sequence in the *gag* gene of HIV-1 and other retroviruses[30]. The peptides in the MHR are critically important for HIV-1 particle assembly and production, and the CTL response against them contribute to HIV-1 infection control[31].

We found a lower frequency of the HLA-B*35 allele in the high-risk group. This allele is associated with increased susceptibility to HIV-1 acquisition and disease

progression[32]. In a study from India, a higher frequency of HLA-B*3520 allele was found on PLWHIV than controls[33]. Likewise, a higher frequency of this allele was found in HIV-1 infected Spanish babies than those uninfected[34]. Furthermore, the HLA-B*35 allele has been associated with rapid HIV-1 progression in Caucasians[32]. However, some studies also relate the HLA-B*35 allele with HIV-1 protection[35]. For example, Gambian HESN-sex workers showed CTL response against HIV-1 and HIV-2 driven by HLA-B*35 alleles[36], and Chinese PLWHIV subjects also showed HLA*B35-restricted CTL response associated with protection[37]. Thus, the evidence shows a contrary role of HLA-B*35 that is contingent on specific types of HLA*B35, due to their differential ability to recognize certain HIV peptides and generate a CTL responponse[35]. This allele is classified into HLA*B35-Px and -Py, which differ in the peptides for which they have affinity[35]. -Py alleles (such as B*3501) bind to peptides with a tyrosine residue in position nine and are associated with slow progression to AIDS. Conversely, the -Px alleles (such as B*3503) bind peptides with different residues at position nine with exclusion of tyrosine residues[35]. The HLA*B35-Px alleles are associated with HIV-1 susceptibility and rapid progression to AIDS[35,38]. It has been demonstrated that those alleles have a low maduration rate, and a decreased peptide loading capacity; moreover, their exclusive binding to peptides without tyrosine residues in the nine position, leds to a reduced peptide repertorie and a decreased CTL capacity, which favoreces the infection uncontrol and disease progression[38].

The MSM in the high-risk group showed lower frequencies of 2DL5, 2DS1 and 2DS5 KIR genes, a lower frequency of Bx haplotypes and an increased frequency of the AA

KIR haplotype. Conversely to our results, the 2DL5, 2DS1 and 2DS5 KIR genes were found in higher frequency in HESN babies from Cameroon[39] and associated with reduction of mother-to-child HIV-1 transmission in Kenyan subjects[40]. Nevertheless, the low frequency of 2DL5, 2DS1 and 2DS5 KIR genes is consistent with the low frequency of Bx haplotypes, and the high frequency of AA haplotypes in the high risk-group, since those genes are present and absent in Bx and AA haplotypes, respectively. The AA haplotypes are characterized by mainly carrying inhibitory genes (2DL3, 2DL1, and 3DL1), with one activating gene (2DS4), plus the ambivalent 2DL4 (KIR producing activating signals despite its inhibitory structure)[41,42]. The activating KIR genes are associated with protection against HIV-1 because they have been observed in higher frequencies in HESN than in PLWHIV, under the premise that its activating role enhances the NK cell response[43,44]. However, there is evidence of the association of inhibitory genes/haplotypes with HIV-1 control. Maruthamuthu *et al.* found that the inhibitory receptor 3DL1 and the A haplotype confer protection against the disease's progression in the Indian population[45]. Jennes *et al.* observed that HESN-female sex workers from Côte d'Ivoire had an increased frequency of inhibitory KIR genes in absence of their ligands (HLA-C1 or HLA-Bw4)[46], and Paximadis *et al.* demonstrated that mothers who did not transmit the virus to their children carried the inhibitory 2DL2 and 2DL3 genes[47]. The contradictory results about the effect of KIR genes in HIV-1 protection/susceptibility can be influenced by the high polymorphism and the differences in the genetic background across the studied populations[41]. Two recent meta-analyses about the effect of KIR genes in HIV-1 infection showed that the protective/risky role of each gene could be different

between populations[48,49]. In the overall analysis across the different genetic populations, a protective role of the 2DL3 and 3DS1S1 KIR genes was evidenced in Caucasians; 2DL2, 2DL5 and 2DS3 in Africans; 2DL1, 2DL3 and 3DS1 among East Asians, and, 2DL3 in the Chinese people[48,49]. In general, those results showed a protective role of two activating genes (3DS1 and 2DS3) while four inhibitory genes exhibit this role (2DL1, 2DL2, 2DL3 y 2DL5).

The protective effect of inhibitory KIR genes/A haplotypes may relay in their capacity to exert a potent NK cells activation and function since those genes play a pivotal role in the NK cells licensing[50,51]. Although the function and responsiveness of NK cells depend on the combination of the activating and inhibitory signals, there has been demonstrated that NK cells licensed by inhibitory KIR exhibit a lower threshold of activation and a stronger response[50,51]. Indeed, in this high-risk MSM cohort, we previously observed a higher response of NK cells by IFN-γ production against K562 stimuli[52].

Regarding the immune profile and function, we found that the high-risk MSM exhibited a low basal activation of T and NK cells, with a lower percentage of $CD4^+CD38^+$, $CD4^+HLADR^-CD38^+$, and $CD4^+Ki67^+$; a higher percentage of the double negative $CD4^+HLADR^-CD38^-$ T cells and a lower percentage of $NKG2D^+$ NK cells from peripheric blood. Although a robust immune response has been related to the HIV-1 control[53], there is evidence that the presence of activated cells and inflammation at the site of HIV entry increases the risk of infection, through the recruitment of target cells and damage of epithelial barrer[54]. Likewise, the increased levels of proinflammatory cytokines in blood and the entry tissue have been associated with

a significant risk of HIV transmission and acquisition, as reported in people with clinical or subclinical sexual transmitted infections (STIs) and mucosal inflammatory process[55–57]. Complementary, a low basal immune activation profile has been observed in several HESN populations, generating the theory of "immune quiescence"[58]. This theory proposes that a functional but not activated immune system (in basal conditions) generates an advantage in controlling the HIV-1 infection since with the low activation/inflammation, there are fewer target cells for the virus in localized tissues, as well as a resting cell status, limiting the infection establishment[58]. However, as its name indicates, this quiescence refers at the same time to an intact response capacity, which is exerted when the immunological or infectious challenge is present[58]. Thus, the quiescent profile is characterized by a low activation profile of target cells, low levels of generalized gene transcription, as well as low levels of proinflammatory cytokine and chemokine in the peripheral blood and genital mucosa, and it has been found in several HESN cohorts, including MSM[58–60].

Similarly to our results, in a cohort of seronegative MSM at high risk of infection from Amsterdam, Koning *et al.* observed higher naïve $CD4^+$ and $CD8^+$ T cells ($CD45RO^-$ $CD27^+$) and lower numbers of HLA-DR^+ $CD38^+$ $CD70^+$ $CD4^+$ T cells and fewer proliferating $CD4^+$ and $CD8^+$ T cells ($Ki67^+$) in comparison with HIV-1 susceptible controls[61]. Comparably, lower percentages of activated $CD4^+$ T cells expressing CD38 have been evidenced in serodiscordant couples from Senegal [62] and the Central African Republic[63], in HESN-female sex workers from Kenya[64], and, in Colombian elite controllers[65]. Moreover, a higher frequency of Tregs has been

described in HESN[66]. The lower $CD4^+$ and $CD8^+$ T cell activation have been associated with a reduced *in vitro* susceptibility to HIV-1, and the persistent seronegative status in populations at a high risk of HIV acquisition[61,67].

Although a lower NKG2D expression by NK cells does not have been described in HESN populations, It was demonstrated in a model of CMV infection that the exposure to the virus resulted in a decreased expression of NKG2D, while preserving the expression of other NK activation molecules and the NK cytotoxic potential[68]. Our results showed lower activation of T and NK cells with a robust response against common antigens and HIV-1 Gag peptides by $CD4^+$ and $CD8^+$ T cells; this supports the idea that a quiescent immune profile can play a significant role in the protection against the virus.

Although we did not find significant differences between both groups regarding the CTL response against the HIV-1 Gag peptides, there was a strong response against SEB by all MSM and against Gag peptides in several individuals, including those in the high-risk MSM group. This situation confirms that despite the low activation, T cells of MSM in the high-risk group conserve the ability to respond after stimulus, suggesting that this group exhibits a selective immune quiescence profile with a fully functional capacity. The specific CTL response plays an important role in HIV-1 infection control[69–71]. Though not all the MSM showed this response, the presence in some subjects opens the possibility that this mechanism could help in the natural resistance to HIV-1 infection. A CTL response is expected in infected individuals, but not in individuals without evidence of established infection, such as HESN[69–71]. Nevertheless, a CTL response has been observed in serodiscordant couples from

Spain[72,73], India[74] and Senegal[53]; and Caucasic, African American and Hispanic HESN-heterosexual women[75].

Unexpected HIV-1-specific CTL response would be explained by previous ineffective or “controlled” infection (considering that HESN shows high exposure to the virus) as some HESN populations exhibited a low quantity of naïve T cells, an increased number of activated T $CD8^+$ effector cells[72,76,77]. Another explanation is the broad cross-reactivity, which could correspond to a heterologous response by T cells to other viruses[78,79].

Soluble factors such as cytokines, β-chemokines, α- and β-Defensins, and other molecules with antiviral functions have been associated with the HIV-1 control[80–82]. In this regard, a higher expression of Serpin A1 in PBMCs was found in the high-risk MSM group. This serine protease inhibitor has been associated whit protection against HIV-1 infection. Increased levels of Serpin A1 have been observed in the oral and genital mucosa of Colombian serodiscordant couples[83], Kenyan female sex workers[84], and the GALT tissue from Colombian elite controllers[83]. Serpin A1 plays an important role in the infection control at mucosal tissues, avoiding their damage and the virus transmigration[85]. As we described previously, the protective effect against HIV-1 infection of Serpin A1 in the peripheral blood could be explained by: (i) inhibits neutrophil elastase, which promotes the entry of HIV-1 into the cell through the binding to gp120, and through the cleavage that makes SDF-1 (CXCL12) and CXCR4, facilitating the binding of HIV-1 with this receptor; (ii) inhibits the formation of gp120; and (iii) inhibits the processing of p55 to p24 by protease[86].

Overall, our results support the hypothesis that MSM in the high-risk group have genetic and immunological factors associated with natural resistance to HIV-1 infection, which could help to explain the sustained seronegative status despite their risky sexual behaviors. Thus, in this population, the protection against the HIV-1 infection is mediated by a combination of factors/processes: (1) A quiescent immune profile with low basal activation but also a fully functional capacity of $CD4^+$ T cells and NK cells. This quiescent environment reduces the activated targets limiting the infection establishment[58–60]. (2) A protection exerted by HLA-B*18 through the presentation of essential antigens for the HIV-1 viral cycle such as those of the major homology region (MHR) in the *gag* gene, thus, leading to a highly efficient specific CTL response by $CD8^+$ cells (like that evidenced in some individuals) which limits the infection[27,28,30]. (3) The protection led by the HLA-B*18 is, in turn, complemented by the low frequency of HLA-B*35 alleles (possibly B*35-Px alleles) which are known to recognize and present a reduced repertoire of peptides and are associated with HIV-1 susceptibility[35,38]. (4) A high responsiveness capacity of NK cells drove by the inhibitory KIR genes/AA haplotype, due to the lower threshold of activation and a stronger response that is exhibited by the NK cells that are licensed by this type of KIRs[48,50–52]. Finally (5), An anti-viral protection exerted the Serpin A1 expressed by PBMCs, in three possible ways: (i) inhibiting the neutrophil elastase, since this promotes the entry of HIV-1 into the cell through the binding to gp120, and through the cleavage that makes SDF-1 (CXCL12) and CXCR4, facilitating the binding of HIV-1 with this receptor; (ii) inhibiting the formation of gp120; and (iii) inhibiting the processing of p55 to p24 by protease[85,86]. In summary,

we propose a model of HIV-1 resistance in the high-risk MSM group, consistent in a quiescent immune profile, with a harmonic balance between a low basal activation environment, and full $CD4^+$ T cells and NK cells responsiveness capacity, that is enhanced by specific KIR genes and HLA alleles, and a protective anti-viral effect (direct and indirect) by the Serpin A1(Figure 1).

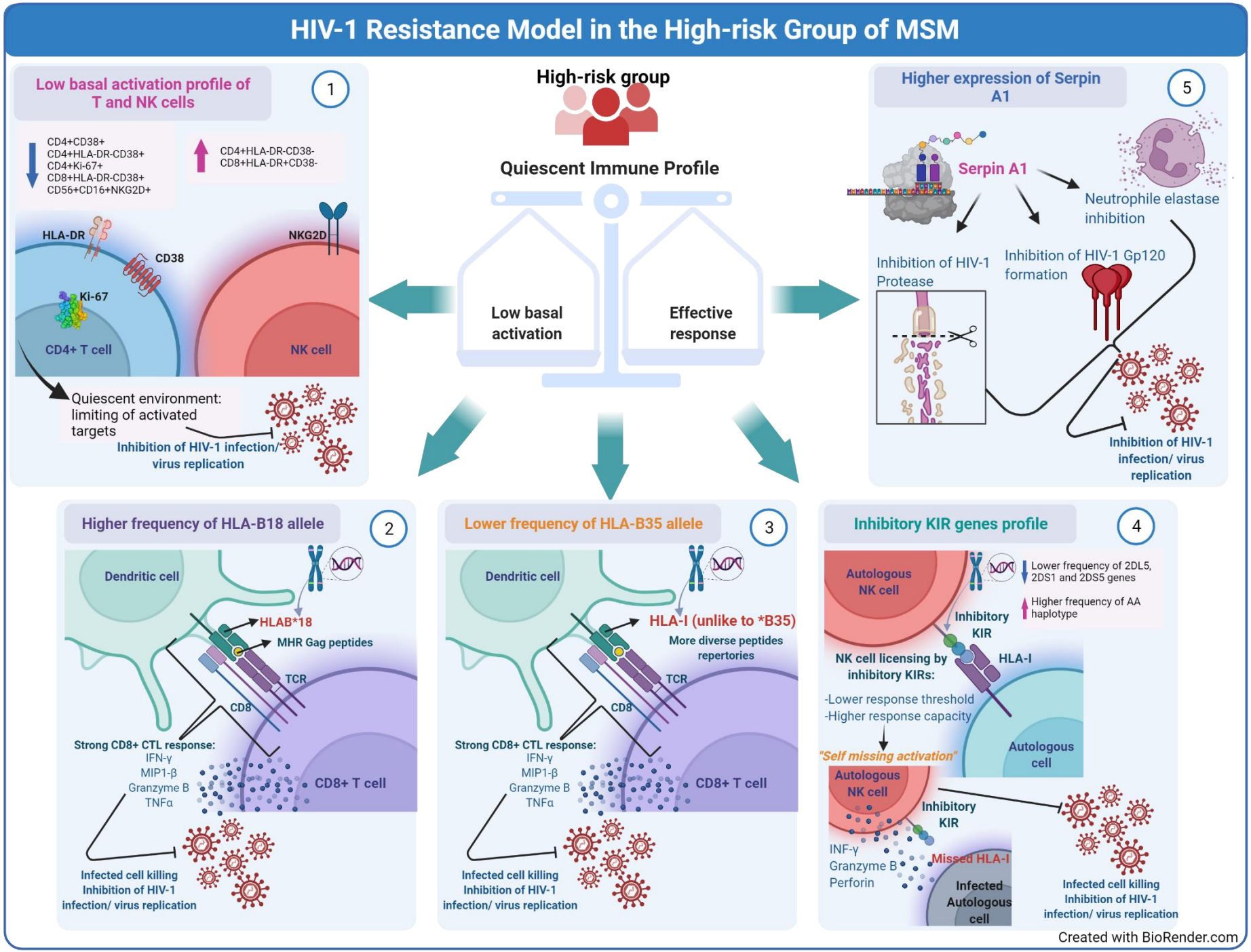

HIV-1 Resistance Model in the High-risk Group of MSM
High-risk group
Quiescent Immune Profile
Low basal activation
Effective response
Low basal activation profile of T and NK cells
1
CD4+CD38+
CD4+HLA-DR-CD38+
CD4+Ki-67+
CD8+HLA-DR-CD38+
CD56+CD16+NKG2D+
CD4+HLA-DR-CD38-
CD8+HLA-DR+CD38-
HLA-DR
CD38
Ki-67
NKG2D
CD4+ T cell
NK cell
Quiescent environment: limiting of activated targets
Inhibition of HIV-1 infection/ virus replication
Higher expression of Serpin A1
5
Serpin A1
Neutrophile elastase inhibition
Inhibition of HIV-1 Protease
Inhibition of HIV-1 Gp120 formation
Inhibition of HIV-1 infection/ virus replication
Higher frequency of HLA-B18 allele
2
Dendritic cell
HLAB*18
MHR Gag peptides
TCR
CD8
Strong CD8+ CTL response:
IFN-γ
MIP1-β
Granzyme B
TNFα
CD8+ T cell
Infected cell killing
Inhibition of HIV-1 infection/ virus replication
Lower frequency of HLA-B35 allele
3
Dendritic cell
HLA-I (unlike to *B35)
More diverse peptides repertories
TCR
CD8
Strong CD8+ CTL response:
IFN-γ
MIP1-β
Granzyme B
TNFα
CD8+ T cell
Infected cell killing
Inhibition of HIV-1 infection/ virus replication
Inhibitory KIR genes profile
4
Autologous NK cell
Lower frequency of 2DL5, 2DS1 and 2DS5 genes
Higher frequency of AA haplotype
Inhibitory KIR
HLA-I
NK cell licensing by inhibitory KIRs:
-Lower response threshold
-Higher response capacity
"Self missing activation"
Autologous cell
Autologous NK cell
Inhibitory KIR
INF-γ
Granzyme B
Perforin
Missed HLA-I
Infected Autologous cell
Infected cell killing
Inhibition of HIV-1 infection/ virus replication
Created with BioRender.com

Figure 1. Proposed model of protection against HIV-1 in the Colombian high-risk MSM group.

We propose a model of HIV-1 resistance in the high-risk MSM group, consistent in a quiescent immune profile, with a harmonic balance between a low basal activation environment, and full CD4+ T cells and NK cells responsiveness capacity, that is enhanced by specific KIR genes and HLA alleles, and a protective anti-viral effect (direct and indirect) by the Serpin A. **1.** A quiescent immune profile with low basal activation but also a fully functional capacity of $CD4^+$ T cells and NK cells. This quiescent environment reduces the activated targets limiting the infection establishment. **2.** The protection exerted by HLA-B*18 through the presentation of essential antigens for the HIV-1 viral cycle such as those of the major homology region (MHR) in the *gag* gene, thus, leading to a highly efficient specific CTL response by $CD8^+$ cells (like that evidenced in some individuals) which limits the infection. **3.** The protection led by the HLA-B*18 is, in turn, complemented by the low frequency of HLA-B*35 alleles (possibly B*35-Px alleles) which are known to recognize and present a reduced repertoire of peptides and are associated with HIV-1 susceptibility. **4**. A high responsiveness capacity of NK cells drove by the inhibitory KIR genes/AA haplotype, due to the lower threshold of activation and a stronger response that is exhibited by the NK cells that are licensed by this type of KIRs. **5.** An anti-viral protection exerted the Serpin A1 expressed by PBMCs, in three possible ways: (i) inhibiting the neutrophil elastase, since this promotes the entry of HIV-1 into the cell through the binding to gp120, and through the cleavage that makes SDF-1 (CXCL12) and CXCR4, facilitating the binding of HIV-1 with this receptor; (ii) inhibiting the formation of gp120; and (iii) inhibiting the processing of p55 to p24 by protease.

Limitations

The small sample size, no probabilistic sampling, and the cross-sectional design do not allow us to infer and extrapolate our findings to other populations, and to build an explicative and causality model of HIV-1 resistance in MSM at high-risk of HIV-1 infection. However, there is important to note the exceptional nature of people who exhibit resistance to HIV-1 infection, which reveals how valuable and highly informative this study is; in addition, this is the first study in Colombian seronegative MSM at high risk of infection with an integrative analysis of the epidemiological, genetic and immune data. The small sample size generates a lower statistical power, which could influence the overestimation of differences between the groups, for example in the frequency of HLA and KIR genes, or the under-identification of some protective factors, which can be elucidated by a bigger sample size. We did not make ancestry analysis, so we could not analyze the frequencies of HLA and KIR genes by ethnic diversity. Likewise, we did not evaluate the expression of HLA and KIR genes, which can variate through the individuals; therefore, differential association with the protection against HIV-1 could be observed when compared with the presence of each gene. We did not analyze the HLA-C alleles, which are also ligands to some KIR, covering possible association between KIR, HLA and the protection against HIV-1. Finally, the cross-sectional design of this study limits the possibility to do a follow-up of the MSM subjects, which avoids the possibility to observe if the protection against HIV-1 infection perdures in the time.

Perspectives

The obtained experience during the development of this study allowed us to establish a cohort of MSM, reaching a “hard-to-reach” population who exhibit extremely risky behaviors and exceptional protective factors. The possible follow-up of this cohort would allow us to continue studying the natural resistance against HIV-1 infection, even in the era of PrEP and HAART. The future studies we propose should be prioritized are:

1. The design of a prospective follow-up of this cohort, that allow us to verify the perduration of the resistant status of each MSM, the changes (or not) in the observed protective factors, and to build an explicative and causality model of HIV-1 resistance in MSM at high-risk of HIV-1 infection.
2. To analyze the KIR and HLA genes in-depth, let us discriminate between the different types of gene/alleles.
3. To analyze the expression of KIR genes, since it has been reported variations in the association with the protection/susceptibility to HIV-1 infection, regarding the level of gene expression.
4. To analyze the oral and anal mucosa to evaluate the expression of antiviral molecules, and the inflammatory profile of this tissue.
5. To analyze the frequency of Tregs both in peripheric blood, anal mucosa and GALT.

6. To analyze the proportion, the activation and functional profiles of $CD4^+$ and $CD8^+$ T cells and NK cells in the GALT tissue, and the lymphoid tissues.
7. To design *in vitro* and *ex vivo* functional assays to assess the CTL and NK response in the context of HLA*B18, other HLA alleles, and inhibitory KIR expression.
8. To design *ex vivo* and *in vitro* assays to evaluate the susceptibility to HIV-1 infection of low activated T and NK cells.

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APPENDIX

List of awards, abstracts, papers, and congress attendances in the frame of this thesis

AWARDS

1. Medal of Decoration *"Recognition of a life dedicated to permanent work in strengthening the local response to HIV/AIDS Category: the current times: Science with humanity"*. Fundación Antioqueña de Infectología & Fundación Ancla. Medellín, Colombia, 2021.

2. Award for Excellence in Social and Solidarity Management-HIV
   Recognition of work in public and social health in HIV, infectious diseases and the LGBTIQ+ population, Universidad Cooperativa de Colombia, Medellín, Colombia, 2021.

3. 3rd Place Oral Modality, Free Papers
   XII Colombian Congress of Allergy, Asthma and Immunology. Cali, Colombia, 2019.

4. 1st Place Award "*Best Poster Award*"
   7th Global Summit on Microbiology Research, EuroScicon. Madrid, Spain, 2018.

5. Nomination for the "*León Zuleta Awards"* from the LGBTIQ+ Population and the Medellín Mayor's Office, Research Modality, for the work carried out in HIV research and its social impact on the city of Medellín and its LGBTIQ+ population. Medellin, Colombia, 2019.

Published Papers

1. Sexual behaviors and factors associated with condomless sexual practice in Colombian MSM at high risk of HIV transmission. **Ana**

Claudia Ossa-Giraldo, John Sebastián Correa, Cristhian, Leonardo Moreno, Yurany Blanquiceth, Lizdany Flórez-Álvareza, Katherin Contreras-Ramíreza, Luis Felipe Higuita-Gutérrez, Juan Carlos Hernández, Wildeman Zapata. Arch Sex Behav (2021). https://doi.org/10.1007/s10508-020-01856-y

2. NK cell activity and CD57+/NKG2Chigh phenotype are increased in men who have sex with men at high risk for HIV. Lizdany Flórez-Álvarez, Yurany Blanquiceth, Katherin Contreras, **Ana Claudia Ossa-Giraldo**, Paula Andrea Velilla, Juan C. Hernandez, Wildeman Zapata. Front Immunol. 2020;11:537044. Published 2020 Sep 11. doi:10.3389/fimmu.2020.537044

Published Abstracts

1. Quiescent profile of T cells from Colombian MSM with extremely high-risk sexual behaviours and HIV-1 specific CTL response. **A Ossa-Giraldo**; Y Blanquiceth; K Contreras; L Florez; J Hernandez and W Zapata. Abstract Supplement HIV Glasgow 2018. Journal of the International AIDS Society 2018, 21(S8):e25187.

2. Frecuencia de alelos HLA y KIR en hombres que tienen sexo con hombres en Medellín, Colombia, con comportamientos sexuales de alto riesgo de infección por el virus de inmunodefciencia humana tipo 1. **Ana Claudia Ossa-Giraldo**, Lizdany Flórez-Álvarez, Yurany Blanquiceth, Katherin Contreras-Ramírez, Carlos A. Peñata, Julián Bustamante-Mira, Nancy D. Marín, Juan Carlos Hernández, Wildeman Zapata. Rev Alerg Mex. 2019;66 Supl 3:1-7º.

3. Immune activation profile of T cells from Colombian MSM with high-risk sexual behaviors and HIV-1 specific cytotoxic T-lymphocytes (CTL) response. **Ana Claudia Ossa Giraldo**, Blanquiceth Y, Contreras K, Flórez L, Hernández J, and Zapata W. Arch Clin Microbiol 2018, Volume 9. DOI: 10.4172/1989-8436-C4-015.

Accepted Papers

1. Accepted paper (written in spanish): ”Comparación psiconeuroinmunológica de hombres que tienen sexo con hombres con diferentes comportamientos de riesgo sexual”. Informes Psicológicos, ISSN: 2422-3271 (en línea)|DOI: 10.18566/infpsic. To be published in 2022.

Submitted Papers

1. Seronegative MSM at high-risk of HIV-1 acquisition show Immune quiescent profile with normal immune response against common antigens. Ana Claudia Ossa-Giraldo, Yurany Blanquiceth, Lizdany Flórez-Álvarez, Katherin Contreras, Mauricio Rojas, Juan C. Hernández, Wildeman Zapata. Manuscript submitted at PlosOne.

2. HLA alleles and KIR genes & Haplotypes in MSM at high risk of HIV-1 infection. Ana Claudia Ossa-Giraldo, Carlos Adrián Peñata, Julián Bustamante Mira, Yurany Blanquiceth, Katherin Contreras-Ramírez, Lizdany Flórez-Álvarez, Julián Bustamante Mira, Juan Carlos Hernández, Wildeman Zapata. Manuscript in preparation to be submitted at JID.

Attendance to Scientific Congress

1. XII Colombian Congress of Allergy, Asthma and Immunology. Cali.
   Co-author oral presentation: “Frequency of HLA and KIR alleles in men who have sex with men (MSM) from Medellín, Colombia, with sexual behaviors of high risk of HIV-1 infection”. Cali, Colombia, 2019.

2. VI National and XVIII Departmental Symposium on HIV and TB. Medellin.
   Oral presentation: “Baseline activation profile of T lymphocytes and specific response to HIV-1 in MSM from Medellín, Colombia”. Medellín, Colombia, 2018.

3. HIV Glasgow 2018 “Drug Therapy”. Glasgow.
   Poster presentation: “Immune activation profile of T cells from Colombian MSM with high-risk sexual behaviors and HIV-1 specific T-lymphocytes (CTL) response”. Glasgow, Scotland, 2018.

4. 7th Global Summit on Microbiology Research, EuroScicon. Madrid.
   Poster presentation: “Immune Activation Profile of T Cells from Colombian MSM with High-Risk Sexual Behaviors and HIV-1 Specific CTL Response”. Madrid, Spain, 2018.