INSTITUTO DE PESQUISA CLÍNICA EVANDRO CHAGAS
DOUTORADO EM PESQUISA CLÍNICA EM DOENÇAS INFECCIOSAS

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DIFERENCIAÇÃO ENTRE SOROCONVERSÃO RECENTE E DE LONGO TERMO NA INFECÇÃO PELO HIV-1: IMPLICAÇÕES NOS ESTUDOS SOBRE DINÂMICA DA EPIDEMIA, DIVERSIDADE VIRAL, VIGILÂNCIA DA RESISTÊNCIA AOS ANTIRRETROVIRAIS E PATOGÊNESE

Rio de Janeiro
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CARLOS AUGUSTO VELASCO DE CASTRO

Tese apresentada com vistas à obtenção do Título de Doutor em Ciências na área de concentração: Pesquisa Clínica em Doenças Infecciosas.

Orientadores: Dra. Mariza Gonçalves Morgado
Dra. Beatriz Grinsztejn

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VELASCO de CASTRO, Carlos Augusto

Diferenciação entre soroconversão recente e de longo termo na infecção pelo HIV-1: implicações nos estudos sobre dinâmica da epidemia, diversidade viral, vigilância da resistência aos antirretrovirais e patogênese.

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Andrea Doria

Legião Urbana

"Às vezes parecia
Que, de tanto acreditar
Em tudo que achávamos tão certo
Teríamos o mundo inteiro e até um pouco mais
Faríamos floresta do deserto
E diamantes de pedaços de vidro
Mas percebo agora
Que o teu sorriso
Vem diferente
Quase parecendo te ferir

Não queria te ver assim
Quero a tua força como era antes.
O que tens é só teu
E de nada vale fugir
E não sentir mais nada

Às vezes parecia
Que era só improvisar
E o mundo então seria um livro aberto
Até chegar o dia em que tentamos ter demais
Vendendo fácil o que não tinha preço
Eu sei, é tudo sem sentido
Quero ter alguém com quem conversar
Alguém que depois
Não use o que eu disse
Contra mim

Nada mais vai me ferir
É que eu já me acostumei
Com a estrada errada que eu segui
E com a minha própria lei
Tenho o que ficou
E tenho sorte até demais
Como eu sei que tens também"

Aos meus pais, Zilda e Augusto,
e a minha esposa Flávia.

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LISTA DE ABREVIATURAS

3TC: Lamivudina

ABC: Abacavir

Ag p24: antígeno p24

AI: Avidity Index

AIDS: Síndrome da Imunodeficiência Adquirida

APJ: co-receptor no sistema nervoso central

APOBEC3G: apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G

ART: antirretroviral

ARV: associated retrovirus ou retrovírus associado à AIDS

AZT: Zidovudina

BED-CEIA: ensaio imunoenzimático de captura com antígenos dos subtipos B, D e E

C2-V3: fragmento do gene *env* entre a região conservada 2 e a variável 3.

CA: Capsídeo viral

CCR2: co-receptor. Participa do processo de entrada do vírus na célula hospedeira

CCR3: co-receptor. Participa do processo de entrada do vírus na célula hospedeira

CCR5: co-receptor da família das β quimiocinas Participa do processo de entrada do vírus na célula hospedeira

CCR8: co-receptor. Participa do processo de entrada do vírus na célula hospedeira

CCR9: co-receptor. Participa do processo de entrada do vírus na célula hospedeira

CDC: “Centers for Disease Control and Prevention” - Centro de Controle de Doenças

cDNA: DNA complementar

COAS: Centro de Orientação e Apoio Sorológico

CRF: Forma Recombinante Circulante

CTA: Centro de Testagem e Aconselhamento

CXCR4: co-receptor da família das α quimiocinas Participa do processo de entrada do vírus na célula hospedeira

D4T: Estavudina

DDI: Didanosina

DNA: Ácido Desoxirribonucléico

dNTP: desoxirribonucleotídeos trifosfatados

DST: Doenças Sexualmente Transmissíveis

env: gene que codifica as glicoproteínas do envelope viral

ETR: Eritricitabina

FIOCRUZ: Fundação Oswaldo Cruz

FRS: Federação das Repúblicas Soviéticas

gag: gene que codifica as proteínas estruturais

Gp: glicoproteína

gp120: glicoproteína de 120 kD.

gp160: glicoproteína de 160 kD.

gp41: glicoproteína de 41 kD.

Gpr1: co-receptor. Participa do processo de entrada do vírus na célula hospedeira

Gpr15: co-receptor. Participa do processo de entrada do vírus na célula hospedeira

hA3G: APOBEC3G humana

HAART: Terapia Anti-retroviral Altamente Ativa

HIV: Vírus da Imunodeficiência Humana

HIV – 1: Vírus da Imunodeficiência Humana tipo 1

HIV – 2: Vírus da Imunodeficiência Humana tipo 2

HIVDR: resistência as drogas antirretrovirais

HLA: Antígeno Leucocitário Humano

HSH: Homens que fazem sexo com homens

HTLV: Human T-Limphotropic Virus Type ou Vírus T-linfotrópico Humano

HTLV III: Human T-Limphotropic Virus Type III ou Vírus T-linfotrópico Humano tipo 3

ICAM: Inter-Cellular Adhesion Molecule 1

IC: intervalo de confiança

IF: Inibidor de Fusão

IFN γ: intrerferon gama

IL-10: interleucina 10

IN: Integrase

INI: Inibidor da Integrase

IOC: Instituto Oswaldo Cruz

IP: Inibidor da Protease

IPEC: Instituto de Pesquisa Clínica Evandro Chagas

ITRN: Inibidor da Transcriptase Reversa análogo de Nucleosídeo

ITRNN: Inibidor da Transcriptase Reversa Não análogo de Nucleosídeo

LAV: lymphadenopthy-associated virus ou vírus associado à linfadenopatia

LS – EIA: ensaio imunoenzimático menos sensível

LTNP: Long Term Non Progressors ou não progressores de longo termo

LTR: long terminal repeats ou Longas sequências terminais repetitivas

MA: Matriz

mRNA: RNA mensageiro

NC: nucleocapsídeo

nef: gene que codifica a proteína nef

nef - negative factor ou fator negativo da expressão viral

NK: “natural killer”

ORF: Quadro aberto de leitura

p7: proteína do nucleocapsídeo

p10: proteína protease

p17: proteína matriz

p24: proteína do capsídeo viral

p31: proteína integrase

p66/51: proteínas da transcriptase reversa

p7/6: proteínas do nucleocapsídeo

PCR: Polymerase Chain reaction ou Reação em cadeia de Polimerase

PN-DST/AIDS: Programa Nacional de Aids

pol: gene que codifica as enzimas que atuam na replicação viral (polimerase,integrase e transcriptase reversa)

PR: Protease

R5: vírus que expressam fenótipo relacionado com o co-receoptor CCR5

RDC: República Democrática do Congo

rev: gene regulatório de expressão viral póstranscripcional

RNA: Ácido Ribonucléico

RNAt: RNA transportador

RT: Reverse Transcriptase ou - Transcriptase Reversa

sCD8: CD8 solúvel

sCD14: CD14 solúvel

sCD25: CD25 solúvel

SIV: Simio Immunodeficiency Virus ou Vírus da Imunodeficência Símia

SIV_{CPZ}: Vírus da Imunodeficência Símia (Chipanzé)

SIV_{GOR}: Vírus da Imunodeficência Símia (Gorila)

SIV_{SM}: Vírus da Imunodeficência Símia (Sooty Mangabey)

sTNF – RII: receptor tipo II solúvel de TNF

STRL33: co-receptor. Participa do processo de entrada do vírus na célula hospedeira

SU: superfície

TAM: Mutação Análoga de Timidina

TAR: elemento de resposta à trans-activação

TDF: Tenofovir

T_H0: T Helper 0

T_H1: T Helper 1

T_H2: T Helper 2

tat: gene regulatório de expressão viral transcripcional e pós transcripcional

TM: transmembranar

TNF α: fator de necrose tumoral alfa

Trim5α: fator que previne a infecção cruzada entre diferentes espécies

UDI: Usuário(s) de Drogas Injetáveis

UNAIDS: United Nations Programme on HIV/ AIDS

URF: formas recombinantes únicas

vif: virion infectivity factor ou fator de infectividade viral

vpr: Viral protein R ou proteína acessória da partícula viral

vpu: Viral protein U ou gene regulatório de expressão viral

X4: vírus que expressam fenótipo relacionado com o co-receoptor CCX4

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RESUMO

A diferenciação sorológica entre casos incidentes e prevalentes de infecção pelo HIV tem sido descrita como uma importante ferramenta para subsidiar a análise das tendências da epidemia de HIV/AIDS. Considerando a importância de tal abordagem para avaliar eventuais tendências na diversidade viral, na resistência primária aos antirretrovirais nas infecções recentes e no monitoramento de grupos em risco, avaliamos indivíduos que buscaram a testagem para o HIV de novembro de 2004 a dezembro de 2008 em CTAs localizados em 4 cidades do estado do Rio de Janeiro (Rio de Janeiro, Nova Iguaçu, Duque de Caxias e São Gonçalo).

As amostras soropositivas foram testadas para a diferenciação entre infecções recentes e de longo termo utilizando-se o teste BED-CEIA. Um subconjunto destas também foi avaliado pelo método de indice de avidez (IA) e por marcadores imunológicos - linfócitos $TCD4^+$ e/ou β2 microglobulina, a fim de propor um algoritmo com maior especificidade para a detecção de amostras de indivíduos recém infectados pelo HIV-1. Amostras de soroconvertidos recentes (SR) e um subconjunto dos soroconvertidos de longo termo (SLT) foram selecionados para os protocolos de biologia molecular, sendo sequenciado um fragmento do gene *pol* da região que codifica a protease e a transcriptase reversa.

Nossos resultados destacam que os HSH continuam a ser uma população altamente vulnerável, tendo prevalência até onze vezes maior que os homens heterosexuais entre os SR. A aplicação de uma zona cinzenta e a avaliação do estado imune contribuiram para uma redução significativa da presença de amostras indeterminadas quando os testes BED-CEIA e de IA foram aplicados, sendo o de IA mais específico.

Embora não tenha sido observado tendência de mudança importante no padrão de subtipos de HIV-1 quando comparou-se RS e LTS no primeiro ano, ao longo do estudo revelou-se um incremento da proporção de amostras recombinantes, bem como uma crescente proporção de amostras relacionadas com CRFs em comparação com URFs, o que alinha-se com dados recentes publicados pela OMS-UNAIDS. Em nosso estudo esta tendência se deveu ao aumento entre os homens, sendo este aumento mais pronunciado entre os heterossexuais do que entre os HSH. Entre estes genomas recombinantes foi encontrado pela primeira vez no Rio de Janeiro amostras relacionados ao subtipo K, assim como uma detecção crescente de amostras com recombinação envolvendo os subtipos A e G entre os indivíduos caracterizados como recém infectados. Nossos dados revelam que os níveis de resistência primária aos antirretrovirais permaneceram relativamente estáveis ao longo do tempo, mas em um patamar preocupante. Entre os HSH a taxa de resistência foi o dobro da registrada em homens heterosexuais, o que sugere uma distribuição desigual em relação a resistência primária em determinados subgrupos. Novos estudos devam ser realizados com foco em populações específicas de forma a contribuir para o monitoramento da dinâmica da epidemia nas mesmas.

ABSTRACT

The serological differentiation between incident and prevalent cases of HIV infection has been described as an important tool to detect trends in HIV/AIDS. Considering the importance of such approach to assess possible trends in viral diversity and primary resistance to antiretroviral drugs in recent infections and monitoring of groups at risk, we evaluate individuals who sought testing for HIV from November 2004 to November 2008 in VCTs located in four cities in the state of Rio de Janeiro (Rio de Janeiro, Nova Iguaçu, Duque de Caxias and Sao Goncalo).

The seropositive samples were tested to differentiate between recent and long-term infections using the BED-CEIA test and a subset was also evaluated by the avidity test (AI) and immunological markers, lymphocytes $TCD4^+$ and/or β2 microglobulin, aiming to propose an algorithm with more specificity for the detection of individuals newly infected with HIV-1. Samples of recent seroconverted (SR) and a subset of long-term seroconverted (LTS) were selected for molecular protocols, where sequences were obtained from the fragment of the *pol* gene region which encodes the protease and reverse transcriptase.

Our results highlight that MSM continue to be a highly vulnerable population, with prevalence up to eleven times higher than among heterosexual men between the RS samples. In fact, prevention interventions focused on this group should be continuously implemented. The application of a gray zone area and the evaluation of the immune status contributed to a significant reduction in the presence of indeterminate samples when both BED-CEIA and AI tests were applied, and the AI showed to be more specific than the BED-CEIA. Although no trend has been observed in the pattern of HIV-1 subtypes when RS and LTS in the first year were compared, an increase in the proportion of recombinant samples as well as an increasing proportion of samples related to CRF compared with URFs - which aligns with recent data published by WHO-UNAIDS- were detected throughout the study. In our study this trend was due to the increase among men, and even more pronounced among heterosexual men than among MSM. Among the recombinant genomes, samples related to subtype K, were found for the first time in Rio de Janeiro and a growing proportion of samples with recombination involving subtypes A and G among individuals characterized as newly infected was detected. Overall levels of primary resistance to antiretroviral drugs are a matter of concern, although remained relatively stable over time. Among MSM, the resistance rate was twice as high as among heterosexual men, suggesting an uneven distribution in relation to primary resistance in certain groups. Further studies should be implemented in order to monitor the dynamics of the epidemic in key populations.

1 – INTRODUÇÃO:

1.1 – O agente etiológico da AIDS e seu histórico:

No presente ano, 2011, a descoberta da Síndrome da Imunodeficiência Adquirida (AIDS) completa a terceira década. A AIDS foi descrita no ano de 1981, acometendo homossexuais do sexo masculino, anteriormente saudáveis, apresentando pneumonia causada por *Pneumocystis carinii,* intensa candidíase de mucosa além de múltiplas infecções virais [1]. Os Centros de Controle e Prevenção de Doenças (CDC) conduziram investigação epidemiológica e laboratorial a respeito do crescente número de casos de sarcoma de Kaposi, pneumonia por *Pneumocystis carinii* e outras infecções oportunistas graves em homossexuais masculinos dos Estados Unidos, evidenciando-se 159 casos destas patologias em apenas 5 meses. Todos os casos, com exceção de um, ocorreram em homens e 92% destes eram homens que faziam sexo com homens (HSH) [2].

Um caso similar foi reportado em 1982, em um usuário de drogas injetáveis de 28 anos, que relatava ter múltiplas parcerias heterossexuais e nenhum contato homossexual. O quadro clínico relatado era de linfadenopatia, candidíase oral e sarcoma de Kaposi [3]. Este caso revelou ainda uma alteração nas populações de linfócitos T do sangue periférico. Este quadro sugeriu que fatores não limitados aos HSH estariam implicados na transmissão e desenvolvimento da síndrome até então pouco clara, e que a população sob risco poderia ser mais ampla do que aquela sugerida inicialmente.

Estudos começaram a sugerir a transmissão por via sexual [4], auto-administração de drogas intravenosas [5] e transfusões sanguíneas [6]. Os registros médicos, materiais de biópsias realizadas anteriormente ao óbito dos pacientes e, principalmente, materiais obtidos em autópsias, foram revistos e patologias micro e macroscópicas foram relatadas. Reichert e cols. sugeriram em 1983 [7] que a síndrome poderia apresentar três tipos de manifestações – morfológicas, de profunda depleção linfóide, infecciosas; usualmente por

múltiplos patógenos oportunistas [8, 9] e neoplasias não usuais, mais freqüentemente sarcoma de Kaposi ou linfomas de alto grau de morbi-mortalidade [7]. Patógenos oportunistas foram relatados, inclusive acometendo órgãos não usualmente descritos para estes agentes – ex. citomegalovírus afetando o coração, as meninges, o cérebro e nervos periféricos [8].

A etiologia viral desta até então nova patologia foi evidenciada pelo isolamento do vírus por pesquisadores do Instituto Pasteur, em Paris na França, sendo o agente então denominado de "Limphadenopathy-Associated Virus" (LAV) pelo grupo de Luc Montanier [10]. Em 1984, foi denominado de "Human T-Limphotropic Virus Type III" (HTLV-III), por Robert Gallo e equipe [11]. Ainda em 1984, Levy e cols. [12] isolaram um retrovírus a partir de pacientes com AIDS de diferentes grupos de risco e o denominaram vírus associado à linfoadenopatia (ARV).

Nos 2 anos seguintes, estes três retrovírus (LAV, HTLV-III e ARV) foram reconhecidos e evidenciados como sendo o mesmo vírus, membro do gênero *Lentivirinae*, família *Retroviridae*, sub-família dos Lentivírus e, de acordo com seus caracteres biológicos e genéticos, verificou-se que eram distintos do HTLV. No ano de 1986, o Comitê Internacional de Taxonomia dos Vírus recomendou que o vírus da AIDS fosse designado separadamente como "Human Imunodeficiency Virus" (HIV), [13, 14]. Naquele mesmo ano, foi identificado um segundo retrovírus em um paciente africano, cuja análise molecular mostrou semelhança em cerca de 50% de sua seqüência genômica com o HIV-1, sendo então denominado Vírus da Imunodeficiência Humana tipo 2 (HIV–2) [15].

1.2 – Epidemiologia do HIV/AIDS:

Desde o início da epidemia, estima-se que 65 milhões de pessoas foram infectadas pelo HIV. Estimativas globais indicam que mais de 29 milhões de pessoas morreram em decorrência da AIDS. O HIV continua sendo um problema global de saúde com uma estimativa de 34 milhões de pessoas vivendo com HIV no final de 2010 (figura 1).

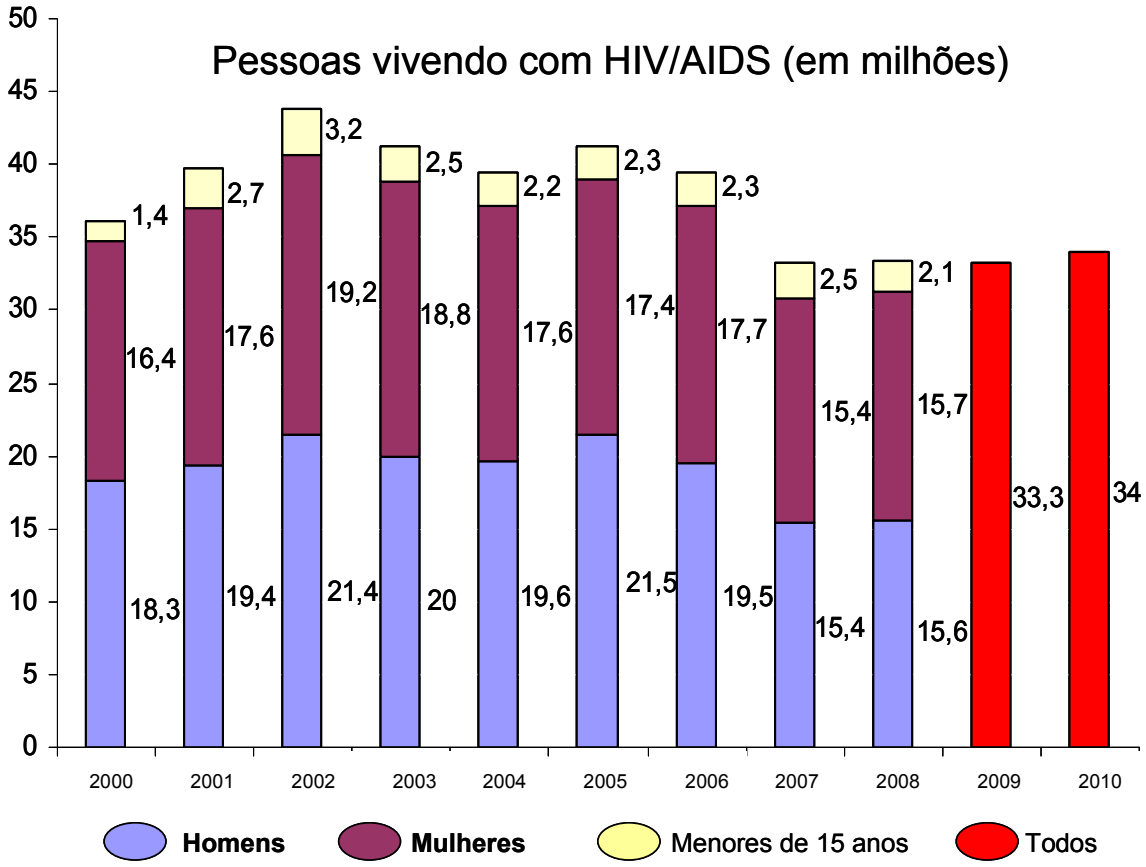

Figura 1: Estimativa do número de pessoas vivendo com HIV/AIDS entre 2000 e 2010. Gráfico realizado a partir dos Boletins epidemiológicos da UNAIDS nos anos de 2001 a 2011, obtidos em (http://www.unAIDS.gov).

Neste ano, estima-se que ocorreram a cada dois minutos, em torno de sete mortes e dez novas infecções entre adultos e crianças. A taxa de prevalência global mantém-se estável de 2001 para 2010 em 0,8%. O comparativo da última década revela que entre as dez regiões – de acordo com a classificação da UNAIDS, a estabilidade da prevalência foi evidenciada em quatro delas: o Meio Leste e Norte Africano, o Sul e Sudeste Asiático, a América Latina (que, em 2010, contou pela primeira vez com a inclusão do México) e o Oeste e parte Central da Europa. Entretanto, variações regionais podem ser observadas, tendo sido verificado uma diminuição relativa de 10% no Caribe – onde a prevalência passou de 1,0% para 0,9% e de 18% na região que abriga a maior prevalência mundial – a Africa Subsariana, onde a taxa caiu de 5,9% para

5,0%. Em antítese, na região composta pela Europa Oriental e Ásia Central a taxa de prevalência entre os adultos triplicou neste período, de 0,3% para 0,9%. Na América do Norte o aumento relativo foi de 20% com a taxa variando de 0,5% para 0,6%. A região que concentra o menor número absoluto de casos – Oceania, variou de 0,2% para 0,3% e no Leste Asiático que é a região com a menor taxa de prevalência, esta passou de <0,1% para 0,1% [16].

A epidemia de AIDS no Brasil tem ao longo do tempo apresentado importantes mudanças no seu perfil, caracterizando-se pela interiorização, feminilização, juvenilização e pauperização [17, 18]. Dados preliminares do Boletim Epidemiológico, que compreende dados registrados de julho de 2009 a junho de 2010, revelam que um total de 592.914 casos havia sido notificado ao Ministério da Saúde. Em 1984 a razão homem/mulher era de 23:1, passou a 3:1 em 1996, alcançou 1,8:1 em 2003 e em 2005 foi de 1,5:1. No ano de 2009, foram notificados 38.538 novos casos de AIDS [19].

Estudos realizados ou financiados pelo Departamento de DST, Aids e Hepatites Virais mostram que as prevalências de infecção pelo HIV no Brasil se apresentam da seguinte maneira: 0,6% na população de 15 a 49 anos de idade (0,4% nas mulheres e 0,8% nos homens) [20], 0,12% nos jovens do sexo masculino de 17 a 20 anos de idade [21] e 0,28% em mulheres jovens de 15 a 24 anos [20]. Nas populações vulneráveis, as prevalências são mais elevadas e destacam-se aquelas entre usuários de drogas ilícitas (5,9%) [22], HSH (10,5%) [23] e mulheres profissionais do sexo (5,1%) [24].

A análise por regiões demonstra que entre 1980 e junho de 2010 foram identificados 344.150 casos de AIDS na Região Sudeste (58,0% dos casos acumulados no Brasil), 115.598 casos no Sul (19,5%), 74.364 casos no Nordeste (12,5%), 34.057 casos no Centro-Oeste (5,7%) e 24.745 casos na Região Norte (4,2%). Em 2009, a taxa de incidência de AIDS no Brasil foi de 20,1 casos por 100.000 habitantes, sendo 32,4 na Região Sul, 20,4 no Sudeste, 20,1 no Norte, 18,0 no Centro-Oeste e 13,9 no Nordeste [19]. Em 2009 foram identificados, 3.398 casos de AIDS em jovens de 13 a 24 anos de idade; a taxa de incidência foi de 8,3 casos por 100.000 habitantes, sendo 1.875 casos no

sexo masculino (9,1/100.000 habitantes) e 1.523 no feminino (7,5/100.000 habitantes). A razão de sexos, que era de 3,7:1 (37 homens para cada 10 mulheres) em 1990, caiu para 1,1:1 (11 homens para cada 10 mulheres) em 1998, culminando com a inversão dessa razão no ano 2000 (0,9:1 – 9 homens para cada 10 mulheres). Entretanto, entre 2007 e 2009, os jovens do sexo masculino voltam a ter maior participação nos casos de AIDS.

Na região Sudeste, o Rio de Janeiro é o estado com os piores indicadores relacionados à AIDS. Em 2010, a incidência da doença foi de 28,2 para cada 100 mil habitantes, a quinta maior do país e a mais elevada da região. A taxa de mortalidade também é a mais alta do Sudeste. Enquanto no Rio a taxa de óbitos por AIDS é de 10,3 para cada 100 mil, no Brasil é de 6,3. A incidência de AIDS em menores de 5 anos, que há cerca de doze anos era similar à de São Paulo, atualmente registra 5,8 casos em cada cem mil habitantes no Rio de Janeiro enquanto em São Paulo registraram-se dois casos [19].

1.3 – A origem do HIV:

O HIV é um retrovírus, atualmente classificado na família *Retroviridae* e subfamília *Lentiviridae* [14], caracteriza-se por produzir infecção crônica no hospedeiro e danos progressivos em seu sistema imune. Dois tipos principais foram caracterizados em humanos [25]: o tipo 1 (HIV-1), predominante em todo o mundo e responsável pela pandemia de AIDS; e o tipo 2 (HIV-2), reportado primeiramente na África Ocidental [15] e menos patogênico que o HIV-1 [26]. Evidências genéticas e epidemiológicas indicam que estes vírus têm infectado humanos por, pelo menos, algumas décadas. Tanto o HIV-1 quanto o HIV-2 são intimamente relacionados a certos vírus de macacos africanos, isto é, os vírus da imunodeficiência símia (SIVs), indicando, portanto, que a origem das infecções pelo HIV esteja relacionada a eventos de transmissão entre espécies diferentes de primatas [27, 28]. Evidências distintas reforçam a transmissão zoonótica dos lentivírus de primatas: similaridades na organização do genoma viral; relações filogenéticas; prevalência no hospedeiro natural; coincidências

geográficas e rotas de transmissão plausíveis [29]. O HIV-2 está mais relacionado, genômica e filogeneticamente, ao vírus da imunodeficiência símia (SIV_{SM}), do que a qualquer vírus derivado de humanos [30]. O vírus SIV_{SM} infecta macacos ”sooty mangabeys” cujo habitat natural coincide com áreas onde o HIV-2 é endêmico, como a África Ocidental. A relação estreita observada entre seqüências de SIV_{SM} e HIV-2 derivada de animais e humanos da mesma área geográfica, ilustram a ocorrência do contato do homem com os animais através da caça ou da criação destes como animais de estimação nas rotas de transmissão [28].

Em contraste, elucidar a origem do HIV-1 tem sido mais difícil. Em 1999 o estudo de Gao e cols. [29] analisou a filogenia das cepas de SIV conhecidas e identificaram duas linhagens principais e altamente divergentes, que infectam duas subespécies de chimpanzés, uma da África Central, *Pan troglodytes troglodytes*, e outra da África Oriental, a *Pan troglodytes schweinfurthii*. Destes, apenas a linhagem de SIV que infecta o chimpanzé *Pan troglodytes troglodytes* mostrou estar relacionada com o HIV-1. Outra evidência é que esta cepa foi encontrada na mesma área geográfica da África onde podem ser encontrados todos os grupos do HIV-1 e seus subtipos. O chimpanzé *Pan troglodytes troglodytes* foi considerado o reservatório primário do HIV-1 [28]. Em 2006, um trabalho mostrou sinais de reatividade para HIV-1 em amostras fecais de gorilas oriundos de florestas de regiões remotas nos Camarões. Das quatro subespécies de gorilas, o Vírus da Imunodeficiência dos Gorilas (SIV_{GOR}) foi encontrado apenas em *Gorilla gorilla gorilla.* Análises filogenéticas mostraram que os gorilas adquiriram o vírus da imunodeficiência símia SIV_{GOR} dos chimpanzés, e que vírus da linhagem SIV_{CPZ}/SIV_{GOR} tem sido transmitida aos seres humanos em pelo menos quatro ocasiões, levando a formação dos grupos M, N, O e P do HIV-1 [31].

A descrição do grupo P foi feita em 2009. Plantier et cols. publicaram um estudo que relata a identificação, em uma mulher camaronesa, de um novo vírus humano de imunodeficiência. Este novo vírus relaciona-se com o SIV_{GOR} e não mostra evidências de recombinação com outras linhagens do HIV-1. Este vírus

representaria uma nova linhagem do HIV-1, que difere do HIV-1 grupo M, N e O; tendo sido proposto a designação de HIV-1 grupo P (figura 2) [32].

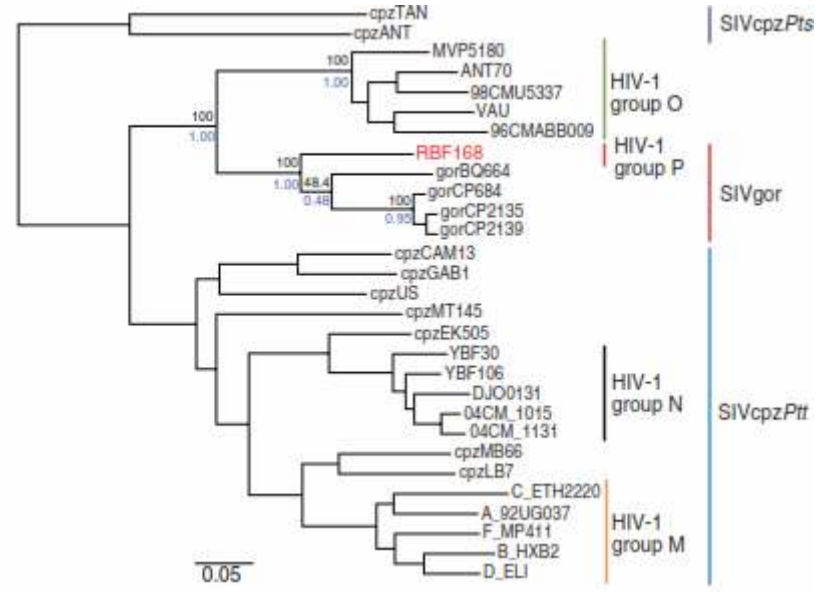

Figura 2: Máxima verosemelhança inferida a partir de alinhamentos de aminoácidos concatenados que correspondem a sequências parciais disponíveis para SIV_{GOR}BQ664. A análise compreende 1.052 posições de aminoácidos. Os valores de suporte em preto acima do ramos são de 1.000 *bootstraps* (mostrado como percentuais), enquanto as probabilidades posteriores da análise Bayesiana dos aminoácidos são mostrados em azul abaixo dos ramos (mostrado como proporções). Fonte: [32]

Outros trabalhos mostram através de análises evolutivas que o ancestral do HIV-1 tem a sua introdução na população humana estimada inicialmente em 1931 (IC95%: 1915–1944) [33]. Em estudos retrospectivos de amostras estocadas em bancos de soros detectou-se a presença do HIV em uma amostra africana, da República Democrática do Congo (RDC), de 1959; primeiramente por testes sorológicos [34] e, posteriormente, através de amplificação por reação em cadeia da polimerase (PCR) e sequenciamento [35]. A presença desta amostra, o alto número de subtipos de HIV-1 co-circulando, a alta diversidade intra-subtipo e o alto número de amostras recombinantes e de amostras não classificadas, sugere ser esta região um dos focos iniciais da epidemia do HIV-1 grupo M [36, 37]. Sequências de HIV-1 que datam de época anterior ao reconhecimento da AIDS são fundamentais para definir o tempo de origem e

escala de tempo da evolução dos vírus. Outras sequências historicamente documentadas servem de calibração importantes para converter a distância evolutiva em tempo. A amostra ZR59 era a única caracterizada antes do ano 1976. Um estudo posterior relatou a amplificação e caracterização de sequências virais da biópsia de um linfonodo fixado em parafina, que foi obtido em 1960 a partir de uma mulher adulta (DRC60), de Leopoldville, no país à época chamado de Congo Belga, hoje Kinshasa, República Democrática do Congo (RDC). Desta forma foi possível realizar o primeiro estudo comparativo de evolução viral com amostras da era pré-pandêmica de AIDS. A distância genética encontrada entre DRC60 e ZR59 foi considerável e demonstrou que a diversificação do HIV-1 no Centro-oeste da África ocorreu bem antes da pandemia de AIDS ser reconhecida, sendo estimada entre 1884 e 1924 [38].

1.4 – Organização do genoma viral:

O genoma dos retrovírus pode ser encontrado sob duas formas: uma fita simples de RNA com polaridade positiva presente em duas cópias na partícula viral, com um tamanho de 9,5 kilobases (kb) [39] e uma dupla fita de DNA, que pode apresentar-se integrada ao genoma da célula hospedeira (provírus) ou livre, na forma circularizada. A infecção pelo vírus pode produzir na célula cerca de 20 cópias do genoma viral sob a forma de DNA circularizado, no entanto, apenas uma cópia será bem sucedida na sua integração ao genoma celular e levará até o final o ciclo infeccioso [40].

Em comum com outros retrovírus, seu genoma contém duas longas repetições terminais (LTRs) que flanqueiam os três genes principais, que produzem as proteínas estruturais do vírus: *gag* (antígeno de grupo específico), *pol* (protease, transcriptase reversa e integrase), e *env* (envelope). Embora idênticos, os LTRs possuem funções diferentes: o localizado na extremidade 5´ (5´LTR) regula a expressão dos genes virais enquanto que o do extremo 3´ (3´LTR) é importante para terminação da transcrição. As extremidades LTRs são geradas durante a transcrição reversa, estando presente somente nos provírus.

Podem ser divididas em 3 sub-regiões, U3, R e U5. A figura 3 esquematiza a organização do genoma do HIV-1.

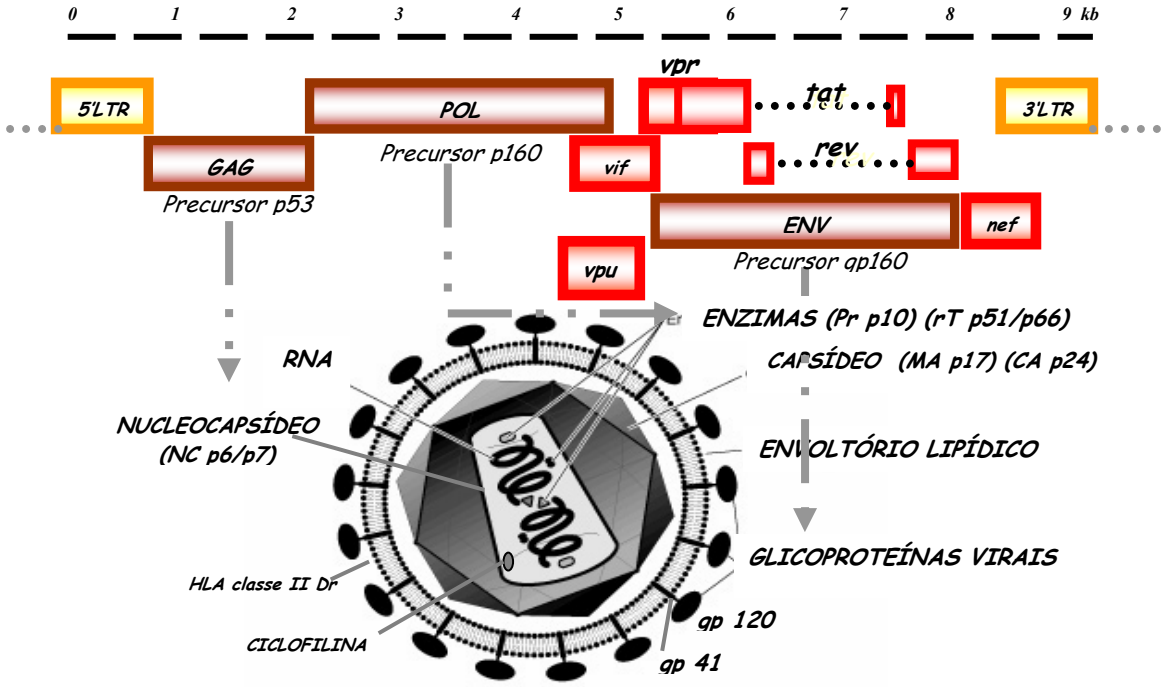

Figura 3: Organização genômica do HIV – 1. Fonte: Ferreira & Ávila 2001, Diagnótico Laboratorial das Doenças Infecciosas e Auto-Imunes

GAG

O gene *gag* produz proteínas necessárias à formação da partícula viral. Este gene é traduzido em uma poliproteína precursora $Pr55^{gag}$, cujo processamento proteolítico origina a proteína de matriz (MA, p17), que se associa à face interna do envelope e ao capsídeo viral; a proteína do capsídeo (CA, p24), constituinte da estrutura capsídea que abriga e protege o RNA genômico viral e enzimas virais; e a proteína do nucleocapsídeo (NC, p7), que se associa ao RNA genômico viral contido no interior do capsídeo [41]. A proteína p6, também é derivada da $Pr55^{gag}$, provavelmente afeta a liberação de partículas virais em brotamento na superfície celular, pois foi verificado que na ausência da p6 as partículas virais não são eficientemente liberadas e,

conseqüentemente, se acumulam na superfície celular [42]. Holguin e cols. [43], sugeriram que a variabilidade nos domínios de $P6^{gag}$ estaria envolvida na liberação das partículas virais.

POL

O gene *pol* codifica três proteínas com atividade enzimática necessárias à replicação viral: protease (PR), transcriptase reversa (RT) e integrase (IN). Os quadros abertos de leitura ("*open reading frames*") desse gene se sobrepõem à porção 3' do gene *gag* em 241 nucleotídeos. A expressão do gene *pol* ocorre quando há um "frameshifting" ribossomal, ou seja, quando o ribossomo desliza uma base para trás durante a tradução do gene *gag*, levando a uma mudança no quadro de leitura, resultando na síntese de uma poliproteína precursora $Pr160^{gag\text{-}pol}$ como resultado do ponto de fusão *gag/pol* [44].

As proteínas de replicação viral são sintetizadas como partes da proteína $Pr160^{gag\text{-}pol}$ e são então proteoliticamente liberadas no vírion maduro [45]. A protease (PR, p10) é responsável por eventos específicos de clivagem que levam à liberação da protease madura, transcriptase reversa e integrase a partir da poliproteína $Pr160^{gag\text{-}pol}$. Além disso, esta enzima também é responsável pelo processamento da poliproteína precursora produzida pelo gene *gag*, a $Pr55^{gag}$ [46], cuja proteólise ocorre de maneira seqüencial e é requerida para a maturação ordenada do *core* viral e geração de partículas virais infecciosas [47]. Demonstrou-se que mutações em um sítio ativo (aminoácido 25) da protease viral resultam na produção de partículas imaturas não-infecciosas devido à não-clivagem das proteínas precursoras [42], indicando que a replicação das partículas virais infecciosas é inteiramente dependente do processamento proteolítico realizado pela protease viral, o que faz desta enzima um alvo para a ação dos medicamentos anti-retrovirais.

A transcriptase reversa (RT) do HIV-1 é composta por duas subunidades, um polipeptídeo de 66kDa (p66) e outro de 51kDa (p51), fazendo da enzima na forma madura um heterodímero p66/p51 [48]. A RT apresenta múltiplas atividades catalíticas, incluindo uma atividade DNA polimerásica, que copia moldes de DNA ou RNA [49], e uma atividade de ribonuclease H (Rnase H), que

degrada especificamente a cadeia de RNA contida em um complexo híbrido RNA-DNA [50]. Essas atividades são responsáveis pela ocorrência de alguns processos exclusivos da replicação dos retrovírus.

A integrase (IN, p31) é essencial para a integração do DNA retroviral no cromossomo da célula hospedeira [51], devido, em parte, à sua propriedade de DNA ligante [52].

ENV

A partir do gene *env* é produzida uma glicoproteína precursora de massa molecular igual a 160kDa, denominada gp160. Seu processamento proteolítico, realizado por uma protease celular, gera uma proteína de superfície de 120kDa (SU, gp120) e uma proteína transmembranar de 41kDa (TM, gp41). Graças à sua localização na superfície do vírion, as glicoproteínas do envelope viral desempenham importantes papéis no reconhecimento e entrada do vírus na célula hospedeira e na subseqüente fusão de membranas, etapas fundamentais no ciclo de biológico do HIV-1.

Assim como outras glicoproteínas destinadas à membrana plasmática, a gp160 é sintetizada no retículo endoplasmático rugoso. Para que isto ocorra é necessária a produção de um precursor de 88kDa que contém uma sequência sinal amino-terminal hidrofóbica de 28 a 30 aminoácidos, que direciona a proteína para a via secretória da célula. Esta seqüência é clivada durante a translocação do precursor no interior do retículo endoplasmático rugoso, onde ocorre a glicosilação e dobramento da molécula, formando uma estrutura terciária apropriada. Logo após a ocorrência destes eventos, os monômeros da gp160 são submetidos à oligomerização, processo requerido para o transporte da glicoproteína do retículo endoplasmático rugoso para o Complexo de Golgi, onde finalmente ocorre a sua clivagem em gp120 e gp41. Após a clivagem, o complexo gp120-gp41, associado de forma não covalente, é transportado para a superfície celular, onde é incorporado aos vírions em brotamento [53].

A proteína de superfície gp120 apresenta em sua estrutura domínios conservados (C1 a C5) e domínios hipervariáveis (V1 a V5). Esta proteína contém os determinantes que interagem com o receptor celular CD4 [54], uma

proteína expressa na superfície das células T e dos macrófagos e descrita como um componente essencial e específico do receptor para o HIV. Além do receptor CD4, co-receptores virais essenciais também estão envolvidos no mecanismo de entrada do vírus na célula alvo, como o CXCR4 e o CCR5. A interação entre a gp120 e o CD4 causa mudanças estruturais que facilitam a ligação ao co-receptor e a subseqüente entrada do vírus na célula [55].

A função primária da gp41 (TM), uma molécula de 345 aminoácidos localizada na membrana viral, é mediar a fusão entre as membranas viral e celular após a ligação ao receptor. Um peptídeo de fusão N-terminal hidrofóbico rico em glicina é presumivelmente o iniciador da fusão, e uma região transmembranar é importante tanto para a fusão quanto para ancorar a gp120 na membrana viral [56].

Em adição, o HIV contém no seu RNA seis genes (*vif, vpu, vpr, tat, rev* e *nef*) que contribuem para a sua complexidade genética. *Nef, vif, vpr* e *vpu* foram classificados no passado como genes acessórios, e não são absolutamente necessários para a replicação *in vitro.* No entanto, a regulação e a função destes genes acessórios e das suas proteínas têm sido estudadas e caracterizadas em maior detalhe nos últimos anos. Os genes acessórios, *nef, tat* e *rev*, são produzidos precocemente no ciclo de replicação viral [57].

Tat e *Rev* são proteínas reguladoras que se acumulam no núcleo e ligam-se a regiões definidas do RNA viral: o TAR (elemento de resposta à trans-activação), encontra-se no LTR; e o RRE (elemento resposta rev), encontra-se no gene *env*, respectivamente. A proteína Tat é um potente ativador pós-transcricional da região promotora LTR e é essencial para a replicação viral na maioria dos sistemas de cultura *in vitro* [58]. A *Tat* e a *Rev* estimulam a transcrição do DNA proviral do HIV–1 em RNA, promovem a elongação do RNA, estimulam o transporte de RNA do HIV–1 do núcleo para o citoplasma e são essenciais para a tradução. A *Rev* é também um fator nuclear de exportação importante na troca da expressão precoce das proteínas reguladoras pelas proteínas estruturais que são sintetizadas mais tardiamente [57].

Nef pode induzir a regulação negativa das moléculas de CD4 e de HLA de classe I e II na superfície das células infectadas com HIV–1, o que pode representar um mecanismo de escape importante do vírus a um ataque imediato por células T $CD8^+$ citotóxicas, de modo a evitar reconhecimento pelas células T $CD4^+$. A proteína Nef pode também interferir com a ativação das células T ao se ligar a várias proteínas que estão envolvidas nas vias intracelulares de transdução de sinal [57]. Em macacos Rhesus infectados com SIV, foi verificado que a presença de um gene *nef* intacto era essencial para garantir uma elevada taxa de produção de vírus e progressão para a doença. Por outro lado, foi identificado numa coorte Australiana de não-progressores a longo-termo, um vírus com deleções no *nef* [59]. Entretanto, artigos indicam que alguns destes doentes desenvolveram posteriormente sinais de progressão para a doença juntamente com um declínio de células T $CD4^+$. Assim, apesar das deleções do gene *nef* terem como conseqüência uma redução da replicação viral, nem sempre evitam a evolução da infecção pelo HIV para AIDS [57].

Vpr parece ser essencial para a replicação viral em células como os macrófagos. A Vpr pode estimular o LTR juntamente com uma variedade de promotores virais e celulares. Verificou-se que a Vpr é importante para o transporte do complexo de pré-integração viral para o núcleo [60] e pode reter as células na fase G2 do ciclo celular.

Vpu é importante para o processo de formação de vesículas, uma vez que mutações no *vpu* estão associadas com a persistência das partículas virais na superfície da célula hospedeira. A proteina *Vpu* é também envolvida quando os complexos CD4-gp160 são degradados no retículo endoplasmático e, por isso, permite a reciclagem da gp160 para a formação de novos vírions [61].

Vif codifica uma proteína importante para os mecanismos intracelulares de transporte dos componentes virais. Foi demonstrada a co-localização da *vif* juntamente com a vimentina, uma proteína pertencente ao citoesqueleto celular. Os vírions deficientes em *vif* podem ser transmitidos célula a célula, mas não a partir de um meio livre de células. *Vif* parece também afetar a morfogênese viral.

Algumas publicações salientaram um importante papel para a vif no apoio à replicação viral [62]. Os isolados HIV–1 defectivos em vif não se replicam em células T $CD4^+$ ou em macrófagos. Os isolados defectivos em vif são capazes de entrar na célula alvo e iniciar a transcrição reversa, mas a síntese de provírus mantém-se incompleta. A fusão *in vitro* de células permissivas com não permissivas origina um fenótipo não permissivo, sugerindo que a replicação do HIV depende da presença ou ausência de um inibidor celular. Este fator inibidor endógeno foi identificado como APOBEC3G [63]. A APOBEC3G ("apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G") pertence a uma família de enzimas intracelulares que desaminam especificamente a citosina em uracil no mRNA ou no DNA, resultando numa acumulação de mutações G-para-A que levam à degradação do DNA viral. Ao formar um complexo com a APOBEC3G, a vif bloqueia a atividade inibitória da APOBEC3G. A atividade antiviral da APOBEC3G é altamente conservada entre várias espécies, enquanto que o bloqueio da APOBEC3G pela vif é altamente específico do HIV. A vif do HIV–1 não forma complexos com a APOBEC3G dos murinos ou dos macacos. Na ausência de vif, a APOBEC3G é incorporada nas novas partículas virais formadas e nas células alvo subseqüentemente infectadas, e a síntese de DNA proviral é bloqueada. Em contraste, na presença de vif, a APOBEC3G é complexada, degradada e não é incorporada nos novos viriões formados. A APOBEC3G é expressa em linfócitos e macrófagos que representam as células alvo primárias da infecção pelo HIV [57]. Durante a produção de partículas virais, vif é encontrado no citoplasma de células infectadas e é encapsulado em pequenas quantidades nos virions. Vif neutraliza A APOBEC3G humana (hA3G) em células produtoras de vírus por mecanismos diferentes [64]. A figura 4 ilustra estes mecanismos.

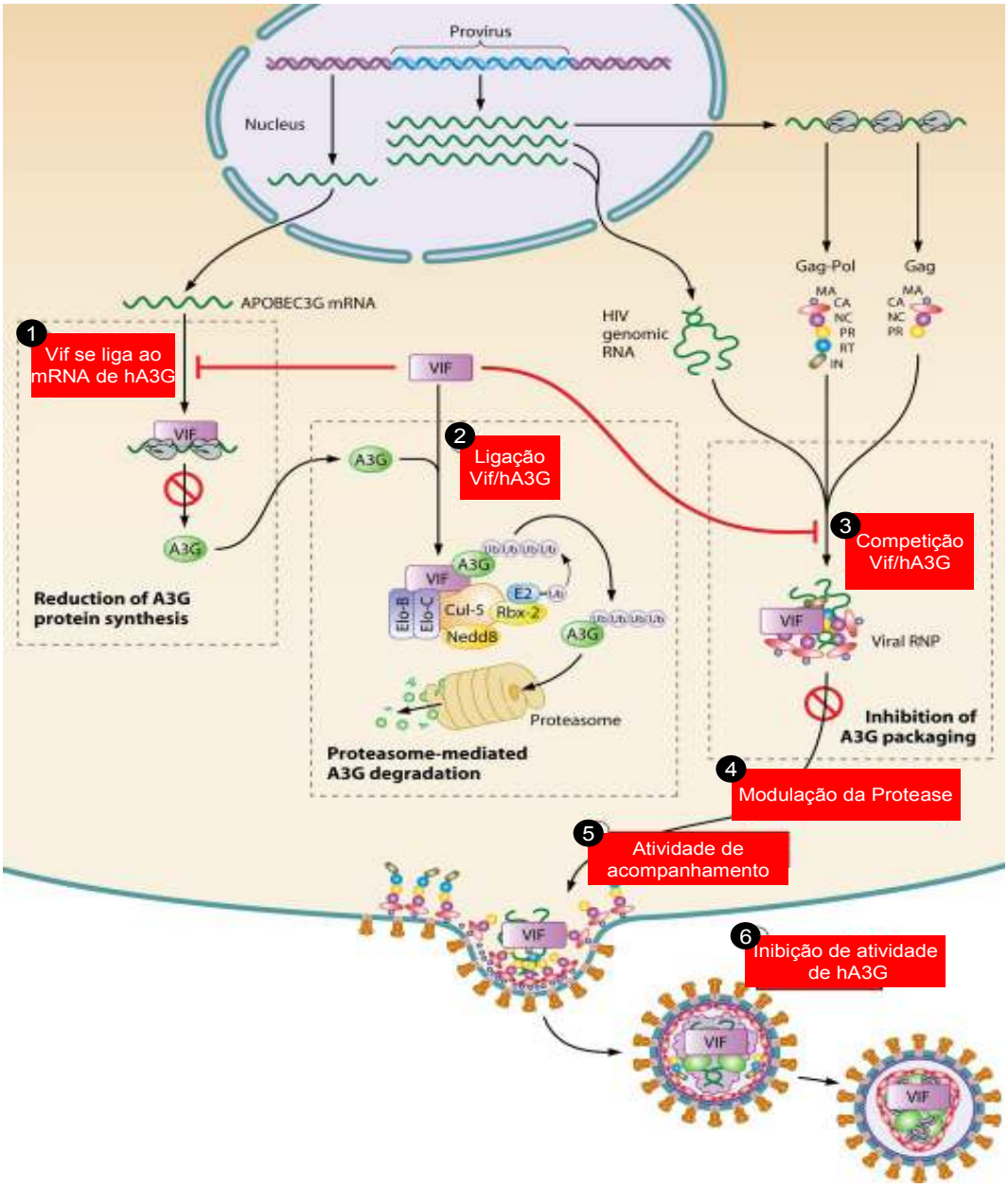

Figura 4: Representação esquemática das funções de Vif e APOBEC 3G humana (hA3G) na montagem e replicação do HIV-1. Adaptado a partir de [64]

(1) Foi mostrado que Vif impede a tradução do mRNA da hA3G, provavelmente através de um mecanismo de ligação ao mRNA. (2) Vif liga-se às proteínas hA3G e recruta um ligase E3 ubiquinina que medeia a poliubiquinação de hA3G e sua degradação. (3) Vif compete com hA3G para a ligação a componentes virais como o domínio do nucleocapsídeo de gag e/ou RNA genômico viral. Juntas, essas três diferentes ações de Vif na tradução, degradação e embalagem não só destroem hA3G de células produtoras de vírus, mas também previnem hA3G de ser incorporada em virions. (4 e 5) Vif intracelular também pode influenciar a montagem viral através da modulação de clivagem mediada pela protease viral dos precursores do Gag (4) e suas atividades de acompanhamento (5), permitindo assim que os eventos tardios, como os precursores de maturação, o início da transcrição reversa, e a maturação do dímero de RNA possam ocorrer após o brotamento viral. (6) Finalmente, Vif pode ser capaz de inibir diretamente a atividade das poucas moléculas hA3G que são empacotados em virions selvagens.

1.5 – Classificação do HIV:

As análises filogenéticas de numerosas cepas de HIV, provenientes de diversas localidades geográficas permitiram a classificação do HIV em tipos, grupos, subtipos, sub-subtipos e formas recombinantes circulantes (CRFs) [65, 66].

Os dois tipos distintos do vírus da AIDS, o HIV-1 e o HIV-2 são diferenciados com base na sua organização genômica e nas relações filogenéticas entre si e entre os outros retrovírus de primatas. Para o HIV-1 foram descritos quatro grupos distintos: M (major), N (new) [67], O (outlier) [68] e P [32], já discutidos acima. Destes, o mais prevalente é o grupo M, responsável pela atual pandemia, que está subdividido em nove subtipos A, B, C, D, F, G, H, J, K, que se acredita ter como origem a África Central [69].

O subtipo F foi inicialmente dividido em três sub-subtipos F1, F2 e F3 [70], mas análises posteriores levaram à reclassificação do sub-subtipo F3 em subtipo K [36]. Um ano após, Gao e cols [71] subdividiram o subtipo A em A1 e A2 e, posteriormente, foi descrito o sub-subtipo A3 [72].

O surgimento de formas recombinantes pode ser considerado uma propriedade fundamental dos retrovírus em razão da natureza diplóide de seu genoma de RNA e da possibilidade da transcriptase reversa atuar ora numa ora noutra fita em células infectadas por mais de uma variante viral durante a síntese do DNA proviral [65, 73].

A identificação de genoma do HIV com recombinação inter-subtipo com representatividade importante em alguma população ou área geográfica, desde que não seja pertencente a indivíduos relacionados entre si que ainda possuem o mesmo padrão de recombinação, denomina-se como forma circulante recombinante – CRF. Estas formas são identificadas por número conforme a ordem de sua descoberta, sendo cada número seguido das letras representativas dos subtipos encontrados na amostra. Até o momento (Dezembro de 2011), 51 CRFs foram descritas (Los Alamos National Laboratory, http://www.hiv.lanl.gov).

O grupo O é composto por um pequeno número de vírus divergentes originários de países do oeste da África [74] e não contempla subtipos. O grupo N contém dois vírus originários de pacientes Camaroneses, que mostram uma composição genética com seqüências intermediária entre os grupos M e O [67].

O grupo P foi o último a ser descrito, e foi isolado e sequenciado a partir de uma mulher nascida nos Camarões [32].

O HIV-2 e o HIV-1, quando comparados, mostram-se relacionados em termos de morfologia, tropismo celular e organização genética global, mas diferem significativamente em termos de seqüências nucleotídicas, com apenas 42% de homologia entre si [15, 75]. A análise da diversidade genética do HIV-2 revelou a existência de sete linhagens evolutivas distintas e aproximadamente eqüidistantes, que, por analogia com o HIV-1, foram denominadas subtipos A,B,C,D,E,F e G, indicando que também no HIV-2 existe uma considerável diversidade genética e biológica [76, 77]. Em 2010, foi descrito a primeira CRF para o HIV-2, contendo os subtipos A e B; sendo denominada HIV-2 CRF01_AB [78].

1.6 –Mecanismos geradores da diversidade genética do HIV:

A variabilidade genética constitui uma das principais características do HIV-1. A enzima transcriptase reversa (RT), que transcreve o RNA viral em cDNA, é altamente propensa a erros e não possui mecanismo de reparação [79], levando à ocorrência de falhas nas incorporações nucleotídicas, com substituições, deleções, inserções, duplicações e recombinações [73]. Como resultado, no HIV-1 e em outros retrovírus, observa-se uma taxa de erro de incorporação de aproximadamente 10^{-4} por base por ciclo replicativo [80], sugerindo que no HIV-1, que possui um genoma de 9,7kb, a taxa de mutação gire em torno de uma substituição de nucleotídeo por genoma por ciclo replicativo [81]. Além disso, as rápidas mudanças do vírus *in vivo*, juntamente com as pressões seletivas do hospedeiro contribuem para a variação genética. Como consequência, a população viral se diversifica ao longo do tempo,

tornando-se mais heterogênea, evoluindo para um conjunto de variantes virais relacionadas entre si, porém distintas [82], denominadas “quasiespécies”, existentes não apenas entre indivíduos diferentes como também em um mesmo indivíduo infectado [83]. Uma forma de recombinação é a infecção por dois vírus geneticamente distintos em uma mesma célula alvo, de forma sequencial ou, menos frequentemente, de forma simultânea. Após a transcrição reversa, caso ambos os vírus sejam integrados, no próximo ciclo podem ser produzidos vírus heterozigotos com genoma recombinante por leitura alternada no momento da transcrição reversa [84]. A figura 5 ilustra esta geração de virus recombinantes.

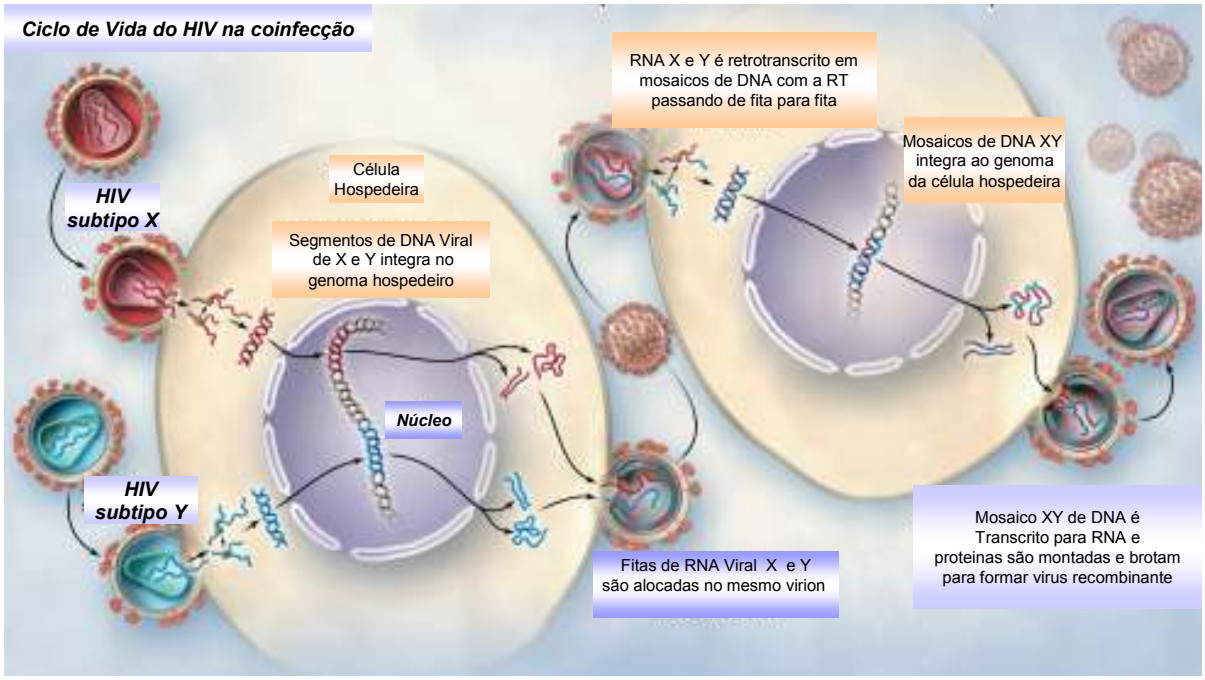

Figura 5: Geração de vírus recombinantes por infecção de vírus geneticamente distintos em uma mesma célula. Fonte: Adaptado a partir de [85].

1.7 – Diversidade e distribuição dos subtipos de HIV no Mundo:

A Epidemiologia Molecular compreende a utilização de ferramentas moleculares em estudos epidemiológicos que, no âmbito da pandemia de HIV–1, se refere basicamente a estudos da propagação e distribuição de formas moleculares em diferentes áreas geográficas e populações com diferentes

características epidemiológicas, incluíndo oscilações transitórias nos padrões de transmissão [84, 86, 87].

Estes estudos têm sido de fundamental importância para o reconhecimento de que a recombinação é o principal mecanismo de evolução do HIV na pandemia. A epidemiologia molecular tem sido também aplicada a estudos de prevalência de isolados resistentes aos antirretrovirais. Vários métodos moleculares têm sido utilizados, entretanto a análise filogenética de sequências dos vírus é o método mais confiável e o que revela mais informações sobre os padrões regionais e global de propagação da infecção por HIV [87]. A aplicação de métodos filogenéticos para estudos epidemiológicos para o HIV e outros vírus RNA é possível em consequência de sua rápida evolução, permitindo a reconstrução acurada de eventos de transmissão, até mesmo em "clusters" intimamente relacionados [88].

Os estudos iniciais de epidemiologia molecular do HIV se baseavam na análise de segmentos dos genes *gag* e *env*; mais recentemente, o gene *pol* tem sido o alvo mais comum em função da utilidade e necessidade de detecção de mutações associadas à resistência aos anti-retrovirais. Em razão do extraordinário potencial de recombinação do HIV–1 e da gravidade da pandemia, a caracterização do genoma completo de milhares de cepas se encontra disponível em bancos públicos de sequências [89], podendo prover informações filogenéticas mais relevantes epidemiologicamente que poderiam ser perdidas na análise de fragmentos menores do genoma. Em todas as opções de estudo, é fundamental dispor de amostras representativas de cada região geográfica e de grupos com diferentes exposições a situação de risco, incluindo indivíduos recentemente infectados [87].

A literatura recente revela um aumento progressivo de variantes genéticas do HIV–1. A relevância do estudo da epidemiologia molecular do HIV–1 é reforçada por recentes publicações que exploram a associação da diversidade genética à: susceptibilidade da resposta imune, incluindo anticorpos neutralizantes [90] e imunidade mediada por células [91, 92] e ao desenvolvimento de mutações relacionadas à resistência às drogas

antirretrovirais [93], o que tem implicações óbvias para o desenvolvimento de vacinas que possam abranger o escopo global da diversidade do HIV.

Estudos têm enfatizado o papel da recombinação na geração de diversidade, diversidade intra-subtipos, frequência de superinfecção, novos surtos epidêmicos e mudanças na dinâmica de epidemias já estabelecidas [87].

O HIV–2 está basicamente restrito à África Ocidental, e a sua detecção em outras regiões (Portugal, França, Alemanha, Estados Unidos e Índia), geralmente reflete suas inter-relações com a África Ocidental [94].

As infecções causadas por vírus do grupo O ocorrem principalmente em Camarões e no Gabão, tendo sido também detectados casos em outros países Africanos. Fora do continente Africano, foram reportados casos na Bélgica, França, Alemanha, Espanha, Noruega e Estados Unidos [95]. Porém, com exceção da infecção que ocorreu na França, todos os demais casos são de indivíduos originários ou vinculados à região Centro-Oeste da África [96]. Os isolados pertencentes ao grupo N foram todos obtidos de pacientes de Camarões [67], assim como o isolado do grupo P [32].

Na pandemia de HIV–1, o grupo com maior dispersão é o M, tendo seus subtipos, CRFs e URFs (formas recombinantes únicas) distribuições específicas nos diferentes continentes.

A primeira infecção documentada por subtipo B foi evidenciada em um americano homossexual do sexo masculino, em 1978 [97]. Nos anos seguintes, o subtipo B propagou-se entre HSH e usuários de drogas injetáveis (UDI) na Europa Ocidental e nas Américas. A epidemia pelo subtipo B na Europa Ocidental [98] e na América Latina parece ser devida a introduções múltiplas oriundas dos Estados Unidos. Entretanto, em alguns países, variantes locais do subtipo B que derivam de introduções pontuais, têm sido reportadas. Um exemplo foi a introdução de uma variante no final dos anos oitenta na Tailândia [99], tendo-se disseminado para outros países naquela região. Outras variantes do subtipo B de origem monofilogenética com disseminação mais limitada têm sido reportadas na Ucrânia e na Rússia [100, 101], Trinidad e Tobago [102], Coréia [103, 104] e Cuba [105].

A variante do sudeste asiático, usualmente denominada B' (ou ainda Thai B) foi introduzida inicialmente por UDI na Tailândia em 1988 [99, 106]. A disseminação foi majoritariamente via UDI para países e localidades de países vizinhos: Myanmar (com transmissão heterosexual concomitante), China, Manipur (nordeste da Índia), Malásia e Singapura [107]. Uma subvariante B' homogênea de origem monofilogenética tem sido identificado em doadores de sangue remunerados na China, infectados a partir de procedimentos não seguros de coleta, majoritariamente nas províncias centrais de Henan e Hubei [108, 109]. Genomas completos de vírus da sublinhagem de Henan, assim como outros vírus B' da China, Tailândia e Myanmar têm sido caracterizados [110-113]. A variante B' está relacionado com CRF07 e CRF08_BC, os quais circulam amplamente na China e com CRF15_01B, um recombinante minoritário encontrado na Tailândia.

A variante Ucraniana do subtipo B (denominada IDU-B), foi introduzida por UDI na cidade portuária de Nikolayev em 1994 [101]. Após uma disseminação limitada na Ucrânia e na Rússia, esta variante recombinou com o subtipo A, gerando o CRF03_AB [100], o qual causou um surto epidêmico entre UDI na cidade de Kaliningrado e tem sido detectado ocassionalmente em alguns países que integravam a Federação das Repúblicas Soviéticas (FRS).

A existência de uma variante específica do subtipo B entre os IDU no noroeste europeu é corroborada pelo grande número de polimorfismos que não são encontrados entre HSH [114].

A epidemia entre UDI em países da FRS é em sua maior parte causada por uma variante sub-subtipo A1 (comumente designada como IDU-A ou FSU-A) [115-117] derivada de introdução única, tendo sido relatada primeiro na cidade portuária de Odessa, sudoeste da Ucrânia em 1995 [101]. Desde 1996, verifica-se a disseminação mais pronunciada desta variante entre UDI, em vasta área geográfica, afetando países da FRS na Europa Oriental e Ásia Central, incluindo Rússia [115-117], Belarus [118], Moldova [119], Estônia [120], Cazaquistão [117] e Uzbequistão [121, 122]. A transmissão heterosexual dessa variante também tem sido reportada na Rússia e em Belarus [117, 118]. Apesar de toda essa

dispersão geográfica e da duração da epidemia, ainda se observa, surpreendentemente, uma baixa diversidade genética [117]. Uma subvariante de IDU-A com características polimórficas na protease é responsável pela eclosão de algumas das recentes epidemias na Rússia, Belarus, Cazaquistão e Uzbequistão [121, 122].

A existência de um novo sub-subtipo A, denominado A3, circulante na África Ocidental foi proposta [72]. Os vírus A3 foram detectados na Costa do Marfim, Nigéria, Guiné-Bissau e Benin. Em Dakar, no Senegal, 9,4% das infecções em prostitutas foram atribuídas ao A3. A designação desta variante como um sub-subtipo está baseada nas distâncias genéticas em relação aos vírus A1 e A2, dentro dos limites para distâncias inter-sub-subtipos. Entretanto, em árvores filogenéticas obtidas a partir de genoma completo, as variantes A3 agrupam-se com 100% de "bootstrap" com as referências A1 e se interdigitam com o *cluster* A1 da Europa Oriental e com todas as demais referências A1 da África Ocidental (Quênia, Uganda e Tanzânia). Estes dados sugerem que os Vírus A3, antes de representarem um novo sub-subtipo, podem representar, na verdade, uma variante do sub-subtipo A1 da África Ocidental [87].

Variantes de origem monofilogenética do subtipo C têm sido encontradas na Índia, Etiópia e no Brasil. A variante chinesa (C_{IN}) [123, 124] tem se difundido através da transmissão heterossexual, com um papel central dos UDI na disseminação no nordeste da Índia no estado de Manipur [125, 126]. A propagação dessa variante teve origem em Myanmar [111, 127, 128], Yunnan - província chinesa [129, 130] e no Nepal [131]. As cepas de Myanmar e as chinesas são estreitamente relacionadas entre si, e podem representar uma subvariante dentro da variante C_{IN}, a qual está relacionada a duas CRFs: CRF07_BC e CRF08_BC [132, 133].

A existência de duas variantes do subtipo C na Etiópia, denominadas C' e C'', tem sido proposta a partir da análise parcial de alguns fragmentos do genoma (gene pol e região V3 do envelope) [134, 135], nos quais 20% dos vírus analisados revelaram recombinação entre ambas as linhagens. Todavia, em análises de genoma completo, um *subcluster* isolado foi encontrado na Etiópia,

mas os dados não foram corroborados pela análise de algumas regiões genômicas [136]. É possível que a recombinação ampla entre as duas variantes tenha obscurecido a distinção entre elas, alterando a estrutura das árvores realizadas a partir do genoma completo e de alguns segmentos adicionais de genoma. Em Cuba, uma variante do subtipo C relacionada com a variante Etíope tem circulado em uma minoria de HSH, tendo sido caracterizados dois genomas completos [87].

As variantes virais circulantes no sul da África parecem se originar de múltiplas introduções [137, 138], embora em outro estudo [135], formem um *cluster* monofilogenético com vírus da Índia e da variante C' etíope em algumas regiões genômicas.

Existem dois *clusters* filogeneticamente distintos no subtipo D. O *cluster* da África Oriental, circulante em Uganda e Quênia, e o *cluster* da África Ocidental que compreende os isolados da RDC, Camarões, Chad e amostras de uma epidemia recente na África do Sul [139-141]. Este *cluster* é subdividido em dois *subclusters*, e um deles inclui a grande maioria dos vírus da RDC e os da África do Sul, enquanto o outro inclui os vírus de Camarões e Chad [141]. As CRF05_DF e CRF10_CD estão relacionados às variantes africanas ocidental e oriental, respectivamente.

Duas variantes distintas do subsubtipo F1 têm sido encontradas no Brasil e na Romênia, formando grupos separados quando analisados por árvores filogenéticas [142]. Uma variante distinta predomina na Romênia em adultos e crianças (infectadas de forma horizontal por equipamentos para injeção contaminado e por transfusão sanguínea), tendo a introdução inicial supostamente ocorrido entre adultos, em razão da maior distância encontrada entre os isolados destes [143].

No subtipo G, uma variante de origem monofilogenética foi identificada em UDI na Galícia – noroeste da Espanha [144, 145], embora mais recentemente tenha sido verificada uma ampla circulação desta variante em Portugal, onde a transmissão ocorre tanto por contato heterossexual como pelo uso compartilhado de seringas e agulhas [146, 147]. Ao subtipo G, com

predominância da variante local, tem-se atribuído cerca de 18% das infecções em Portugal [146]. Entre UDI em Lisboa, a proporção de infectados com o subtipo G foi determinada como 49,5% para *gag* e 24% para *env*, e 29 de 30 vírus G_{env} foram determinados como pertencentes à variante local [147]. A média das distâncias entre os isolados de 11% no *env* sugere que o subtipo G foi introduzido em Portugal há mais de uma década [147]. Análises filogenéticas revelaram uma estreita relação entre a variante portuguesa/galiciana com os vírus oriundos de Camarões e do Gabão [148]. Esta variante está relacionada à CRF14_BG [145] e a diversas formas recombinantes únicas (URFs) isoladas em Portugal e na Galícia [145]. Em Cuba, uma variante local do subtipo G, caracterizada através da análise de genoma completo, é encontrada em 5% dos infectados e majoritariamente transmitida por contato heterossexual [105]. Esta variante está relacionada à CRF20_BG e a duas outras CRFs BG – CRF23_BG e CRF24_BG [149, 150], que se disseminaram entre HSH na cidade de Havana [145, 151]. Uma variante nigeriana do subtipo G também foi caracterizada por seqüenciamento parcial do genoma [152], formando três *clusters* no *env* e dois no *gag*.

A lista de CRFs cresce progressivamente, tanto em consequência do aparecimento de novos recombinantes em áreas onde há a co-circulação de diferentes clades, como pela caracterização atual de antigas formas não detectadas anteriormente. As recombinações geográficas em *hotspots* (regiões do genoma com alta frequência de recombinação) são o berço da maioria das CRFs, algumas das quais têm se propagado para outros locais fora do local de origem. As CRFs representam apenas um pequeno número das milhares de formas recombinantes geradas nessas áreas. Tais formas acabam sendo selecionadas devido às suas propriedades biológicas e à chance de introdução em locais apropriados para sua transmissão.

Recentemente foi publicado um estudo [153] que objetivou apontar tendências globais em relação à epidemiologia molecular do HIV-1, analisando 2 períodos subsequentes – de 2000 a 2003 e de 2004 a 2007. De maneira geral, a distribuição global dos subtipos foi similar entre os dois períodos estudados e

corroborou estimativas prévias [154]. Três tendências epidemiológicas foram notadas. Primeiro um crescimento global tanto absoluto quanto relativo dos subtipos A, F, G, H, CRF01_AE, CRF02_AG e outras CRFs. Em contrapartida, os subtipos D, J, K, CRF03_AB e as formas URFs decresceram em número, e, assim sendo sua proporção global tornou-se menor. Por último, os subtipos B e C cresceram a taxas abaixo da média, resultando em uma participação proporcional menos na epidemia como um todo, embora o subtipo C ainda seja responsável pelo maior aumento absoluto no numero de infecções [153]. A figura 6 faz uma comparação da distribuição global dos subtipos e recombinantes de HIV-1 nos períodos de 2000 a 2003 e de 2004 a 2007.

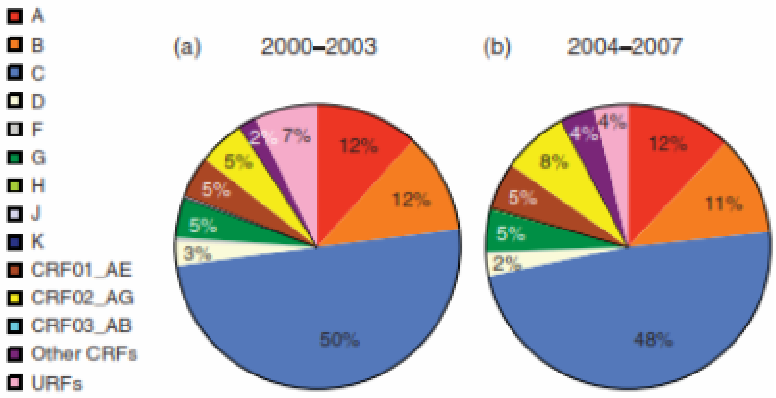

Figura 6: Esquema representativo da proporção de cada subtipo ou forma recombinante na epidemia global de HIV-1. Representação do período que compreende os anos 2000 a 2003 em (a) e do período de 2004 a 2007 em (b). Fonte: [153]

No período de 2004 a 2007, o subtipo C foi responsável por quase metade das infecções ao nível global – 48%. Aos subtipos A e B foram atribuídos 12% e 11%, respectivamente, ficando o CRF02_AG com 8%; CRF01_AE e o subtipo G ficaram com 5% cada e 2% correspondeu ao subtipo D. Os subtipos F, H, J e K juntos não chegaram a 1% e outras CRFs e URFs foram responsáveis por 4% das infecções em todo o mundo. Somando-se todas as CRFs chegou-se a 16% e ao adicionar-se todas as URFs, estimou-se que 1 em cada 5 cepas possui alguma recombinação [153]. Em relação à América Latina, o comparativo revelou decréscimo dos subtipos “puros” e aumento das formas recombinantes no período de 2004 a 2007. Nesta região, o subtipo B

continua sendo o mais prevalente, entretanto sua prevalência diminuiu de cerca de 75% para 68%; verificou-se redução também para as outras duas formas mais encontradas – o subtipo C que correspondeu a 9% no período de 2000 a 2003 e a cerca de 6,5% entre 2004 e 2007 e o subtipo F, onde a redução foi de 4,7% para 3,5%. O total de recombinantes dobrou em prevalência, saindo de 11% para 22%, sendo mais pronunciado entre as CRFs (de 2,3% para 11,3%) do que entre as URFs (9% para 10,7%). A figura 7 faz uma comparação da distribuição na America Latina e de alguns países da região dos subtipos e recombinantes de HIV-1 nos períodos de 2000 a 2003 e de 2004 a 2007.

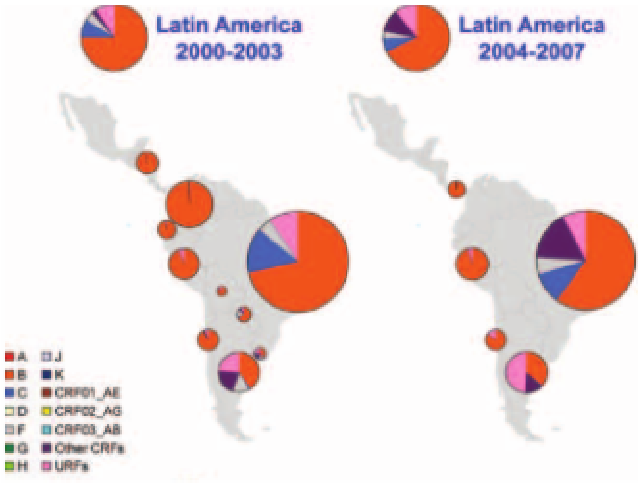

Figura 7: Esquema representativo da proporção de cada subtipo ou forma recombinante na epidemia de HIV-1 na America Latina e de alguns países da região. Representação do período que compreende os anos 2000 a 2003 a esquerda e do período de 2004 a 2007 a direita. Fonte: [153]

1.8 – Diversidade e distribuição dos subtipos de HIV no Brasil:

O Brasil tem a maior diversidade de subtipos e formas recombinantes na América do Sul. O subtipo B é o mais prevalente no Brasil e estima-se que a sua introdução tenha ocorrido na segunda metade da década de 1960 [155]. Estudos têm revelado que a sua frequência varia ao longo do território nacional e ao longo do tempo como por exemplo: 96,0% em Brasília – DF [156]; 92,6% em Miracema – RJ [157]; 87,9% e 81,2% em São Paulo – SP [158, 159]; 81,2% no

Ceará; 51,6% na região Amazônica [160], 32,0% em Porto Alegre [161] e 7,1% em gestantes da cidade de Criciúma [162].

No Brasil, uma variante local (B_{br} ou B'') foi identificada com base na caracterização de um tetrapeptídeo no topo da alça V3 [163-166]. Esta variante foi descrita primariamente em uma mulher japonesa que havia emigrado da América do Sul, sendo posteriormente documentada em território nacional. O tetrâmero GPGR foi descrito como altamente conservado no topo da região V3 do envelope viral de amostras pertencentes ao subtipo B, e tem importância na indução de anticorpos neutralizantes [167], na imunidade mediada por células [168] e no desempenho de diversas funções biológicas. As diferenças observadas na variante B'' podem exercer ainda influência na patogênese e no desenvolvimento de vacinas [165]. Um estudo revelou que este perfil GPGR é evidenciado em apenas 46,5% das amostras brasileiras, enquanto o tetrâmero GWGR tipicamente brasileiro foi encontrado em 40% das amostras [164]. Apesar de ser encontrado em todas as regiões do Brasil, diferentes taxas de prevalência podem ser detectadas nas diferentes regiões, com maior prevalência desta variante na região Sudeste. A prevalência também parece variar ao longo do tempo, mas a proporção de amostras com o tetrâmero GWGR permanece mais de 30 vezes acima da média mundial [169].

No Brasil estima-se que o subtipo F foi introduzido no final da década de 1970 [155], e que sua prevalência, que inicialmente representou cerca de 10 a 15% das infecções por HIV, esteja em declínio. Uma causa seria a extensa recombinação com o subtipo B que tem gerado frequentes URFs e tem feito com que amostras de subtipo F puro sejam encontradas com pouca frequência no Brasil [170]. Um recombinante BF de origem brasileira é o ancestral da CRF12_BF e de recombinantes relacionados [171], tendo estes vírus ampla circulação na Argentina sendo esporadicamente detectados em outros países da América do Sul (exceto Brasil).

Uma variante do subtipo C de origem monofilogenética [172], disseminou-se rapidamente na Região Sul do país nos últimos anos. Proporções de 30 a 45% [173-176] são atribuídas a essa variante entre os casos desta região, sendo

que em indivíduos com diagnóstico recente esta proporção chega a 58% na cidade de Porto Alegre [161] e mais recentemente em gestantes da cidade de Curitiba, chegou a 78,6% [162]. Esta variante não tem sido associada a nenhuma forma particular de transmissão. Casos esporádicos da variante brasileira têm sido detectados na Argentina, Uruguai e Paraguai [177, 178]. Segundo estudo de Salemi e cols [138], a origem desta variante teria se dado nos anos noventa e a sua taxa de expansão seria cerca de duas vezes maior do que o subtipo B e do subtipo C africano. Entretanto, em um estudo da Organização Mundial da Saúde, em 1994, observou-se que uma em cada quatro amostras subtipadas desta região foi atribuída ao subtipo C, o que corrobora estudo posterior que estima a introdução do subtipo C no Brasil no inicio da década de 1980 [179].

No território nacional outros subtipos foram descritos, tais como os subtipos D [180, 181], A [182] e formas recombinantes, compreendendo os subtipos prevalentes em nosso meio: recombinantes B/F [158, 183-185], B/C [186]. Alguns estudos têm relatado a presença da CRF02_AG no Rio de Janeiro e em São Paulo [159, 187, 188].

Em 2006, De Sá Filho e cols publicaram um estudo onde se identificou duas CRFs (CRF28_BF1, CRF29_BF1) [185]. Estas CRFs seriam as primeiras descritas no Brasil e, juntamente com CRF12_BF, seriam as CRFs encontradas no continente sul-americano até então, todas elas compreendendo a recombinação entre os subtipos B e F. Alguns pontos de recombinação presentes nas novas formas propostas também são encontradas em outras formas recombinantes B/F, sugerindo a presença de um ancestral em comum e a existência de áreas mais propensas à recombinação ao longo do genoma do HIV–1.

A presença de duas CRFs e estruturas recombinantes relacionadas indicavam neste momento que a epidemia brasileira poderia ser mais complexa do que o previamente suposto [185]. Estudos publicados nos últimos cinco anos reiteraram esta percepção; ainda em 2006, houve a descrição da CRF31_BC [189], em 2008 de mais duas CRFs relacionadas aos subtipos B e F1

(CRF39_BF1, CRF40_BF1) [190] e mais recentemente em 2010 foi descrita a CRF46_BF1 [191]. A figura 8 ilustra as CRFs descritas em amostras obtidas no Brasil.

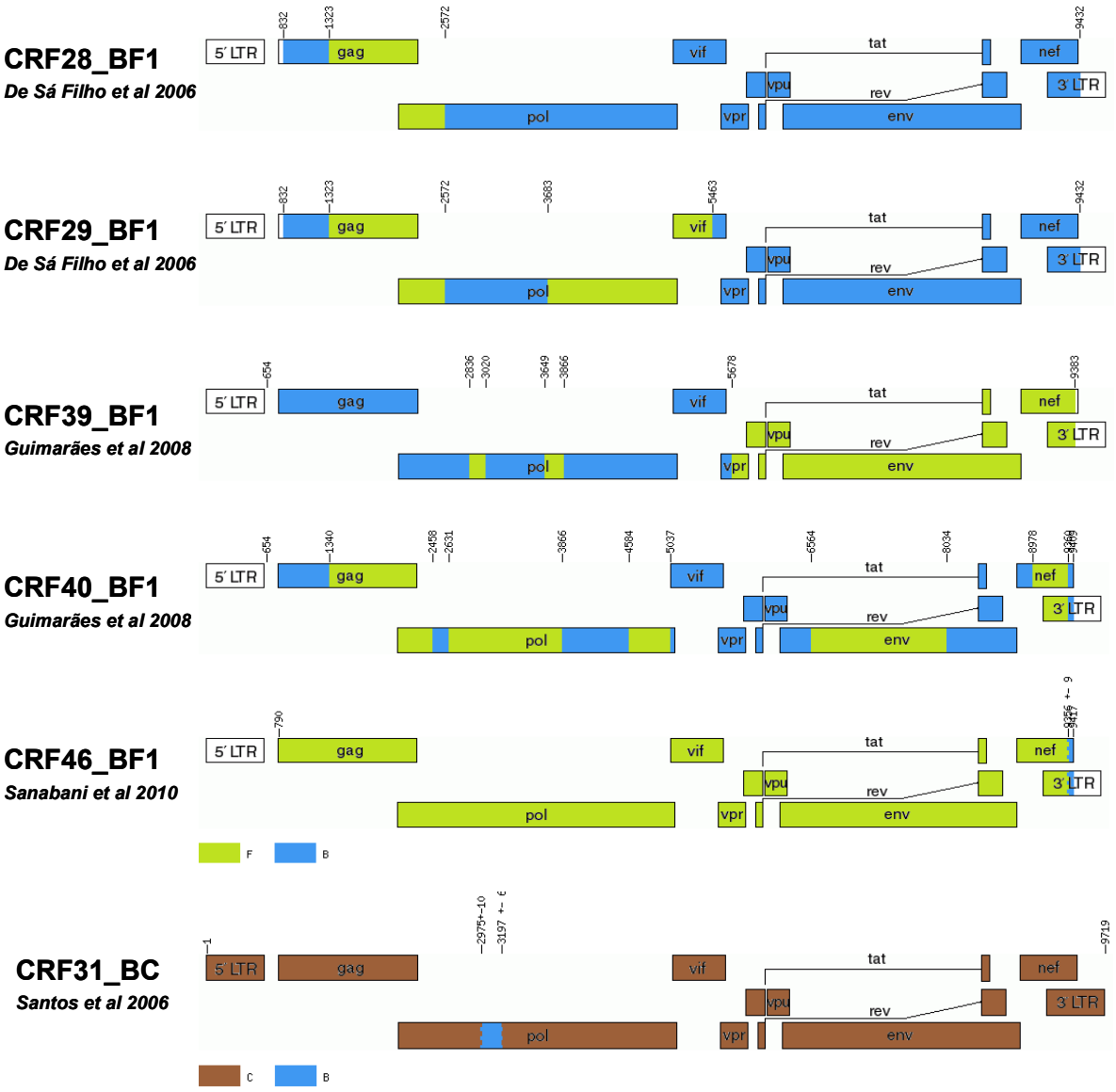

Figura 8: Esquema ilustrativo das CRFs descritas no Brasil. Subtipos envolvidos e pontos de recombinação estão indicados, assim como as referências que descreveram as respectivas CRFs. Elaborado a partir de ilustrações obtidas em http://www.hiv.lanl.gov.

1.9 – A infecção pelo HIV:

A infecção pelo HIV compromete profundamente o sistema imune, vulnerabilizando o organismo a infecções oportunistas. O linfócito T $CD4^+$ constitui uma célula alvo da infecção pelo HIV, devido à afinidade do vírus pelo marcador de superfície CD4, também expresso nos macrófagos, células dendríticas, monócitos e da glia levando à redução quantitativa e qualitativa destes tipos celulares. A queda da população de células T $CD4^+$ ao longo da evolução para a AIDS é uma característica da infecção pelo HIV. A contagem de linfócitos T $CD4^+$ é considerada um importante marcador da progressão para a doença, assim como para a introdução e posterior avaliação da terapia antirretroviral. Em contrapartida, nos últimos anos, alguns pesquisadores têm suspeitado que busca de respostas talvez tenha se concentrado no local errado. Esses autores acreditam que o intestino e outros tecidos de mucosa, e não o sangue periférico, são o principal sítio de infecção pelo HIV e perda de células T $CD4^+$ [192, 193]. Alguns estudos têm enfatizado a velocidade com a qual o HIV deteriora o sistema imune. Estudos demonstraram que o SIV, com rapidez e de forma seletiva, infecta e destrói as células T $CD4^+$ de memória em poucos dias de infecção. Este evento resulta na perda da maioria das células T $CD4^+$ $CCR5^+$ funcionais do corpo. O maior foco de destruição são os tecidos de mucosa, onde a maioria das células T expressa CD4 e CCR5 (com um fenótipo de memória) – receptores para a fusão e entrada do HIV. Embora a literatura tenha relatos prévios da perda acentuada de células T $CD4^+$ $CCR5^+$ de memória em macacos infectados pelo SIV, o mais importante dos estudos mais recentes é a descrição da infecção seletiva dessas células. Assim sendo, estes achados sugerem que essas células são mortas diretamente por destruição mediada pela infecção pelos vírus [192, 193].

Os estudos de Mattapallil e cols. [194] e de Li e cols. [195], evidenciam que a infecção direta das células T $CD4^+$ de memória é o principal mecanismo responsável pela perda dessas células em indivíduos infectados pelo SIV, ao menos na fase de infecção primária. Uma das possíveis consequências é que

aparentemente a infecção por SIV elimina essencialmente a resposta de células T $CD4^+$ de memória ao SIV e a outros potenciais patógenos infecciosos oportunistas em poucos dias, e não em anos de infecção. Estudos em primatas não humanos demonstraram claramente que infecções oportunistas subclínicas ocorrem em hospedeiros recentemente infectados. À luz destas observações, alguns autores defendem que seria importante começar o tratamento anti-retroviral o mais cedo possível, objetivando preservar a arquitetura celular, principalmente no intestino e tecidos de mucosa. A introdução da terapia em fase bastante inicial da infecção de SIV em macacos e do HIV em indivíduos recentemente infectados, resultou em uma acentuada melhora nos resultados obtidos na restauração do "pool" de células T $CD4^+$ intestinais (embora a restauração não tenha sido completa) quando comparada com indivíduos ou macacos nos quais o início da terapia foi postergado [192].

Uma questão que tem sido explorada é a dimensão qualitativa da resposta de células T, em relação ao seu impacto na patogênese da AIDS. Uma mudança na predominância de resposta do tipo 1 (T_H1) para o tipo 2 (T_H2) e a produção das citocinas associadas podem estar relacionadas e até mesmo facilitar a progressão da infecção pelo HIV. Alguns estudos relatam que os clones de linfócitos T_H2 e T_H0 são mais permissivos para a replicação *in vitro* do HIV, do que os clones de T_H1. Outros estudos indicam que a progressão para a doença associa-se com o aumento da secreção de IL-10, citocina característica de resposta T_H2. A controvérsia sobre o papel do tipo de resposta T_H na patogênese do HIV tem, entretanto, uma variável importante que são as co-infecções. A modulação da resposta T_H2 por algumas doenças parasitárias pode facilitar a replicação do HIV em indivíduos co-infectados .

O receptor de quimiocina CCR5 é expresso 8 vezes mais em células T_H1 do que em células T_H2, e o CXCR4 é expresso 4 vezes mais em células T_H2 do que em células T_H1. Embora as células T_H2 suportem preferencialmente vírus X4 e T_H1 vírus R5, eventualmente as cepas R5 podem se replicar melhor em células T_H2 [196].

O curso típico da infecção pelo HIV consiste em três fases ou estágios: infecção primária – fase aguda; latência clínica e AIDS [197].

O período correspondente à fase de infecção primária é caracterizado pelo desenvolvimento de uma síndrome viral aguda, que ocorre três a seis semanas após a infecção e é observada em 50 a 70% dos indivíduos infectados. Este período se caracteriza também por níveis elevados de viremia e ampla disseminação do HIV, relacionada à produção de vírus, particularmente ao nível dos órgãos linfóides. A replicação viral durante a fase primária conduz inicialmente à ativação de resposta imune mediada por células, com o aumento do número de: linfócitos T $CD8^+$ ativados que expressam CD38, CD45RO, e HLA-DR; aumento do número de células NK; elevadas concentrações de marcadores imunes, CD8 solúvel (sCD8), receptor tipo II solúvel de TNF (sTNF – RII), neopterina e CD30 solúvel (sCD30) no sangue, além do aumento da concentração de IFN–γ, TNF–α e IL–1β. De forma marcante o aumento inicial de secreção de IFN–γ tem sido relacionado com a expansão oligoclonal de células T $CD8^+$, sendo o aspecto dominante na resposta imune à infecção primária. Esta resposta imune celular é tardiamente (um intervalo de tempo que varia de uma semana a três meses) acompanhada pelo desenvolvimento da resposta humoral, e a emergência desta imunidade específica está associada a um declínio dramático da viremia e dos níveis elevados dos marcadores imunológicos supra-citados; a infecção entra na fase crônica [198].

A segunda fase caracteriza-se por ser um período de latência clínica que geralmente dura alguns anos. Apesar da ausência de sintomas ou sinais clínicos observada nesta etapa, a doença é ativa, como indicado pela replicação viral persistente nos orgãos linfóides e pela perda progressiva de células T $CD4^+$, que constitui a principal manifestação da deterioração gradual do sistema imune em curso nos indivíduos infectados [197].

Durante a fase crônica, um elevado número de linfócitos $CD8^+$ ativados está presente no sangue periférico, expressando CD38, HLA-DR, CD57 e CD71; desenvolvendo importante papel na resposta antiviral. Os linfócitos T $CD4^+$ embora estejam numericamente diminuídos, se encontram ativados e uma

proporção significativa expressa HLA–DR e CD25. Níveis aumentados de CD71 são detectados nos linfócitos B no sangue periférico e há acréscimo na produção de imunoglobulinas G e M [198].

A concentração de citocinas e de marcadores de ativação imune solúveis no plasma se encontram elevados e estáveis quando mensurados de forma seriada ao longo de meses ou anos, sendo correlacionado com os achados plasmáticos de carga viral (também estáveis) no início da fase assintomática. Entretanto, com a progressão da doença, concentrações aumentadas de sCD8, sCD14, TNF–α e neopterina no soro refletem a ativação dos linfócitos $CD8^+$ e macrófagos, respectivamente. Concentrações elevadas de uma variedade de marcadores imunológicos solúveis no soro estão relacionados à progressão para a doença em indivíduos infectados pelo HIV; entre eles estão a neopterina, β2-microglobulina, sTNF–RII e sCD25. A maior ativação imune geralmente precede o ponto de inflexão dos níveis de linfócitos T $CD4^+$ e aumento da carga viral, dando suporte à hipótese de que a ativação imune é o mecanismo, e não simplesmente uma consequência da patogênese do HIV. De fato, o aumento da ativação imune determinado pelo fenótipo dos linfócitos ou pela quantificação dos marcadores imunológicos solúveis, está associado a uma menor sobrevida. De forma mais marcante, no estágio mais avançado da doença, a sobrevida está antes associada à expressão de CD38 nos linfócitos T do que à carga viral plasmática ou receptor de quimiocina utilizado [198].

A conseqüência inevitável da progressiva deteriorização do sistema imune que ocorre em muitos indivíduos infectados pelo HIV é a doença clinicamente aparente ou AIDS, que inclui sinais e sintomas persistentes e severos de debilitação orgânica, além de infecções oportunistas. A profunda imunossupressão que ocorre durante essa fase da infecção pelo HIV é o estágio final dos eventos imunopatogênicos que tiveram início na infecção primária, quando o vírus se disseminou e se replicou nos orgãos linfóides, como uma continuação do processo infeccioso durante anos através do estágio clinicamente latente, mas biologicamente ativo da infecção [199].

Podem-se obervar pelo menos quatro perfis distintos entre os indivíduos infectados pelo HIV na sua progressão para a AIDS [197]. A maioria deles (70% a 80%), denominados progressores típicos, progride para a AIDS em 8 a dez anos, tendo um longo período de latência (seis a oito anos). Uma pequena porcentagem de indivíduos progride para a AIDS em um período de tempo similar ao dos progressores típicos, mas para estes os parâmetros clínicos e laboratoriais permanencem estáveis por um longo período de tempo, cabendo-lhes a denominação de sobreviventes de longo prazo. Uma porcentagem significativa (10% a 15%) constituída pelos progressores rápidos, apresenta uma progressão para AIDS em um espaço de tempo relativamente curto, que varia de dois a três anos, devido à maior brevidade do período de latência clínica ou até mesmo a ausência deste. Um pequeno subconjunto de indivíduos que se refere como HIV-1 a longo prazo não-progressores (LTNPs), são um grupo de HIV-1 soropositivos que nunca se valeram da terapia antirretroviral e permaneceram infectados com HIV-1 por vários anos - em alguns casos, até 20-25 anos [200] (figura 9).

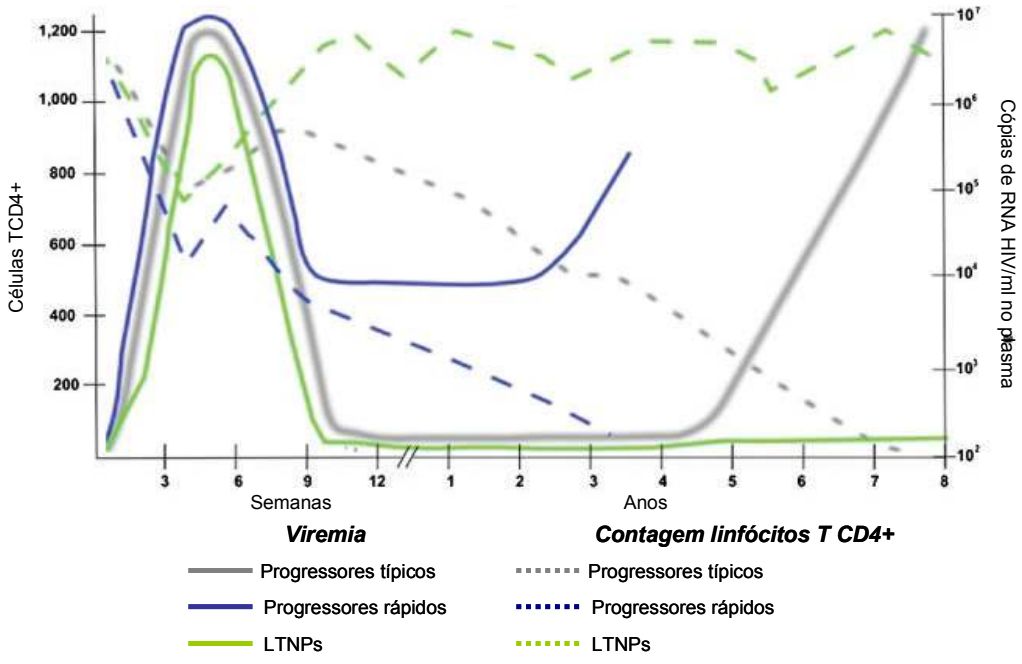

Figura 9: Esquema ilustrativo da viremia e contagem de linfócitos TCD4+ em indivíduos infectados pelo HIV-1 na ausência de terapia antiretroviral – diferenças entre progressores típicos, rápidos e LTNPs. Fonte: [201]

Este grupo corresponde a 2-5% de todos os indivíduos soropositivos para o HIV [202]. Ao longo do curso da infecção, os LTNPs mantêm baixo nível de viremia e níveis de linfócitos TCD4$^+$ elevados, em contraste com os progressores rápidos. Habitualmente, na patogênese do HIV-1, a infecção crônica assintomática pode se estender por 3-20 anos, dependendo da progressão da doença em cada indivíduo [203]. Os LTNPs mantêm entre 5.000 e 15.000 cópias de RNA/ml durante o curso de sua infecção com a maioria registrando menos de 10.000 cópias/ml e muitos tendo 5-20 cópias/ml [200, 204]. Estudos recentes têm mostrado LTNPs com carga viral indetectável, controlando a infecção por 1-2 anos [202, 205]. Um pequeno subconjunto de LTNPs (aproximadamente 1%) são chamados de controladores de elite ou supressores de elite. Nestes indivíduos são observados níveis indetectáveis de RNA viral (<50 cópias/ml) por um período indeterminado de tempo que poderia ser por meses ao invés de anos [206, 207]. Estima-se que cerca de 1 em 300 pessoas infectadas pelo HIV preenchem os critérios para ser um controlador de elite. Uma coorte de 4.586 indivíduos infectados pelo HIV com acompanhamento desde 1986, mostrou que 0,55% são ECs [202, 208]. Em comparação, 3,32% e 2,04% de indivíduos da mesma coorte foram classificados como LTNPs com células T CD4+ maiores que 500 células/ml durante 7 e 10 anos de seguimento, respectivamente [202]. Embora a maioria dos LTNPs tenham contagens de células TCD4$^+$ dentro da normalidade, estes indivíduos podem sofrer várias infecções oportunistas e apresentarem imunodeficiência, como resultado da depleção de CD4 + células T [204]. Alguns estudos têm demonstrado que cepas de vírus de LTNPs são menos evoluídas e, portanto, teriam menor capacidade de escapar da resposta imunológica do hospedeiro, quando comparado com cepas de progressores [209, 210]. No primeiro estudo para sequenciar o genoma completo do HIV-1 de controladores de elite (N=4), Blankson et al. [211] encontraram mutações menores que não afetam a função dos genes do HIV-1 e, portanto, não poderiam explicar a supressão. Além disso, todos os isolados virais permaneciam com seu potencial de replicação e foram capazes de crescer

normalmente em linfoblastos $CD4^+$. Em um experimento subsequente [212], os pesquisadores sequenciaram os isolados virais de um dos controladores de elite, antes e depois da descoberta virológica, a fim de determinar se quaisquer mutações estavam associadas com sua não-progressão a doença. Eles descobriram que foram mantidas as mesmas mutações detectáveis em todos os momentos, o que demonstra que suas mutações não explicam uma mecanismo de controle de infecção e que outros mecanismos devem desempenhar um papel no seu controle a longo prazo de infecção.

1.10 – Detecção de indivíduos recentemente infectados pelo HIV:

A prevalência de infecção pelo HIV pode ser mensurada pelos ensaios sorológicos clássicos, entretanto a estimativa de incidência tem sido de difícil obtenção. As variações na prevalência em um contexto de acesso não refletem a dinâmica mais recente da disseminação, o que enfatiza a importância de se estimar a incidência [213, 214].

No desenho clássico, a incidência de infecção pelo HIV é mensurada prospectivamente pelo seguimento de um grupo de indivíduos soronegativos, realizando retestagem periódica ao longo do tempo. Estudos de coortes prospectivas são de difícil condução, podendo apresentar tendenciosidade no recrutamento e seguimento e têm custo elevado. A adesão à coorte, o processo envolvendo o consentimento e o aconselhamento visando redução de riscos, resultam em estimativas de incidência menores, do que a incidência efetiva, ou seja, fornecem uma incidência subestimada [215]. Os estudos envolvendo coortes não permitem uma amostragem representativa da população em geral, o que é essencial para estimativas regionais e/ou nacionais [216].

Wiliams e cols. descreveram um método estatístico baseado em dados de prevalência para estimar a incidência a partir de certos pressupostos sobre as taxas de crescimento da epidemia e de mortalidade por AIDS [217]. Esta metodologia pode ser útil na ausência de outras alternativas, entretanto, não é o

mais adequado em locais onde existam intervenções relevante de prevenção, incluindo a expansão na oferta de terapia anti-retroviral [216].

Nos últimos anos algumas metodologias laboratoriais têm sido desenvolvidas visando detectar, de forma específica, infecções recentes, tanto no período pré-soroconversão como no período pós-soroconversão. Amostras oriundas de estudos seccionais como, por exemplo, as coletadas visando à vigilância da infecção e determinação de prevalência, podem ser utilizadas com esse propósito [216].

O período entre a infecção e o aparecimento de anticorpos específicos anti–HIV é conhecido como fase de infecção aguda. A duração desta fase (comumente denominada de janela imunológica) é breve (cerca de 10 a 20 dias), o que exige habitualmente grandes casuísticas para sua eventual detecção. Indica-se esta estratégia para indivíduos soronegativos com exposição recente a situações de risco. Em anos recentes, os bancos de sangue têm introduzido testes que visam cobrir esta fase da infecção, objetivando um controle mais apurado na triagem de sangue e seus produtos. Metodologias moleculares e de detecção de antígenos específicos são empregadas nesta fase (RNA HIV – 1 ou Ag p24) [218-221]. Estes testes são tecnicamente mais complexos e de custo mais elevado, o que limita sua implementação em larga escala [216] e, eventualmente, podem não ter sensibilidade suficiente [222].

Alguns testes foram desenvolvidos ou avaliados para a testagem em indivíduos soropositivos visando à detecção de infecção recente. O reduzido número de amostras testadas (somente as soropositivas), menor necessidade tecnológica e *expertise* técnica, fazem destes métodos opções mais adequadas para a utilização em países com recursos limitados e/ou de amplo território. Em 1998, Janssen e cols. [223] descreveram uma modificação em um ensaio sorológico comercial de primeira geração para diagnóstico (3A11, Abbott Laboratories) visando à detecção de soroconvertidos recentes. O ensaio foi alterado na diluição da amostra e no tempo de incubação, com o objetivo de diminuir sua sensibilidade (3A11-LS). Soroconvertidos recentes possuem títulos mais baixos de anticorpos quando comparados aos que têm infecção de longo

termo e sua infecção não é evidenciada pelo 3A11-LS, enquanto os soroconvertidos de longo termo possuem reatividade para ambos os ensaios. Para a realização do 3A11-LS é necessária a utilização de calibradores e controles adicionais para determinação do ponto de corte e monitoramento da performance do ensaio, uma vez que se trata de um teste originalmente qualitativo. Estudos com amostras sucessivas de soroconvertores sugerem que a "janela" assim obtida é de cerca de 129 dias (109-149 dias, 95% IC) para indivíduos infectados com o subtipo B do HIV-1. Os CDC desenvolveram e distribuiram estes reagentes para alguns laboratórios. O fabricante interrompeu a fabricação deste teste, e um segundo teste comercial (Vironostika HIV – 1 EIA, Organon Teknika) foi modificado de modo similar (Vironostika LS–EIA) [224] e tem sido utilizado para a detecção de infecção recente [225-228].

Ao longo do tempo, cresceu o interesse de implementar estes dois testes menos sensíveis em outras regiões do mundo. Entretanto, dois estudos demonstraram que tanto o 3A11-LS como o Vironostika LS–EIA tinham diferentes "janelas" para os subtipos B e E, em populações testadas na Tailândia [226, 229]. O maior período de janela obtido para os indivíduos infectados pelo subtipo E, foi atribuído à utilização de derivados antigênicos do subtipo B nestes ensaios. A presença de dois ou mais subtipos em diversas partes do mundo e a crescente diversidade viral, a utilização destes testes não se mostra adequada para estimar incidência. Alguns estudos foram conduzidos na África [228] e na Ásia [230], todavia a acurácia destas estimativas têm sido questionadas. Apesar da importância de estimar a incidência de infecção por HIV, estes ensaios menos sensíveis não foram implementados amplamente em outras partes do mundo em função dos múltiplos subtipos circulantes e das dificuldades de interpretação dos dados obtidos [216].

A pouca disponibilidade e as limitações dos ensaios menos sensíveis motivaram muitos laboratórios a investir em uma abordagem alternativa para a detecção de infecções recentes [226, 231, 232]. Baseado no desenvolvimento da resposta imune humoral, alguns ensaios utilizando diferentes estratégias foram implementados para: determinados epítopos ou respostas antígeno-

específicas; avidez/afinidade de anticorpos e quantidade de anticorpos no soro [216].

Entre outros testes desenvolvidos para avaliar a infecção pelo HIV recentes, o teste de avidez tem uma abordagem qualitativa em que: o tratamento anti-retroviral, a baixa contagem de células $CD4^+$, a baixa carga viral bem como seu uso em locais onde subtipos não B do HIV-1 têm sido observadas não tiveram efeito aparente sobre o índice de avidez (AI) [232, 233].

Parekh e cols. [234] desenvolveram um ensaio imunoenzimático de captura competitivo (BED-CEIA, Calypte), o qual mede, de forma indireta, o incremento na proporção de anticorpos anti-HIV da classe IgG na amostra estudada. A inclusão de um peptídeo sintético quimérico ramificado de região imunodominante da gp41 de alguns subtipos (B, E e D) visa minimizar os problemas vivenciados com os testes menos sensíveis. Após o relato de preocupações emergentes relacionadas com cenários de alta prevalência do HIV e da diversidade viral [235], duas estratégias de correção/fatores foram propostos para minimizar a superestimação das taxas de incidência usando o teste BED-CEIA [236, 237]. A aplicação destes fatores de correcção tem sido discutida [238-243] e mais recentemente o grupo de trabalho técnico sobre ensaios de incidência em HIV da Organização Mundial de Saúde publicou um documento com as recomendações e os pontos críticos para a avaliação de incidencia do HIV-1 ao nível populacional [244].

1.11 – Resistência aos antiretrovirais:

Resistência às drogas antirretrovirais (HIVDR) se refere à capacidade do vírus de suportar os efeitos de determinada droga antirretroviral (ART), que tem como objetivo evitar a sua replicação. Os vírus resistentes à droga continuam a replicar-se na presença da droga para a qual eles se tornaram resistente. Como consequência da expansão da terapia, o surgimento de HIVDR é inevitável. Durante a última década, o acesso aos ART que visam inibir a replicação do HIV tem aumentado dramaticamente em países de baixa e média renda. A partir de dados preliminares, estima-se que mais de 6 milhões de pessoas estavam

recebendo ART nesses países no final de 2010, em comparação com apenas 400.000 no final de 2003. O conhecimento insuficiente entre pacientes e profissionais de saúde, a adesão sub-ótima aos regimes de tratamento, e mecanismos de acompanhamento do paciente inadequados estão entre os muitos fatores que levam ao fracasso do tratamento e, eventualmente, a resistência às drogas. Se o paciente desenvolve resistência em seu regime de primeira linha, a resposta terapêutica deixa de ser eficaz. A fim de manter o controle virológico, estes indivíduos precisam receber um tratamento de segunda linha. Em 2010, em países de baixa e média renda, os regimes de tratamento de segunda linha eram, em média, pelo menos, seis vezes mais caros do que o tratamento de primeira linha. Dessa forma a sustentabilidade econômica dos programas de tratamento dependem da manutenção de níveis de resistência os mais baixos possíveis [245].

A resistência aos medicamentos pode ser adquirida ou transmitida. É adquirida em pacientes nos quais a replicação do vírus em curso ocorre quando da terapia antiviral abaixo do ideal. Embora a terapia antiviral subótima seja uma conseqüência da insuficiência de drogas ativas, em geral é resultado de interrupções no tratamento ou de adesão incompleta. Estima-se que a resistência às drogas transmitida, seja responsável por cerca de 15% das novas infecções nos EUA [246], 10% na Europa [247], 5% na América do Sul e Central, e menos de 5% na maior parte da África Subsaariana e no Sul e Sudeste da Ásia [248, 249].

Combinações de drogas ART são eficazes no tratamento do HIV, independentemente do subtipo viral. No entanto, o nível de resistência aos anti-retrovirais difere entre as variantes do HIV. Até o momento, temos um conhecimento limitado sobre as mutações de resistência em subtipos não-B do HIV-1 e sua relevância clínica, apesar de que mais de 90% dos pacientes com HIV-1 em todo o mundo estão infectados por variantes de subtipos (ou formas recombinantes) não B do HIV-1. A maioria dos dados sobre resistência são de infecções pelo subtipo B nos países desenvolvidos. Dados enzimáticos e virológicos indicam que polimorfismos que ocorrem naturalmente entre

diferentes subtipos do HIV podem influenciar a suscetibilidade dos ART ao HIV-1 e a propensão para adquirir certas mutações que conferem resistência. Além disso, vias de resistência às drogas em diferentes subtipos podem determinar resistência cruzada e o uso potencial de regimes de segunda linha específicos. O potencial genético para produzir diferenças entre os subtipos nos padrões de mutações que conferem resistência é suportado por uma variação natural entre o conteúdo genético dos subtipos de HIV– variação que chega a 40% em *env* e 8 a 10% em *pol* e *gag*. Esta questão adquire especial relevância tendo em vista o fato de que o *pol* codifica as enzimas transcriptase reversa, protease e integrase, que são os principais alvos da terapia antirretroviral [250].

Dados bioquímicos e virológicos indicam que a variação natural nos aminoácidos pode afetar a magnitude da resistência conferida por certas mutações [251]. Como exemplo, os virus relacionados ao tipo 2 (HIV-2) e vírus do grupo O do HIV-1 mostram alto nível de resistência inata a alguns inibidores da transcriptase reversa não nucleosídeos (ITRNNs) como resultado de mutações na transcriptase reversa que estão presentes como polimorfismos naturais [252, 253]. Além disso, as diferenças de nucleotídeos entre os subtipos definem o número de transições ou transversões necessárias para desenvolver a resistência a diferentes classes de ART, podendo afetar a barreira genética para resistência [254, 255].

Atualmente as classes de drogas utilizadas na prática clínica são seis: Inibidores da Transcriptase Reversa Análogos de Nucleosídeos (ITRNs), os Inibidores da Transcriptase Reversa Não Análogos de Nucleosídeos (ITRNNs), os Inibidores da Protease (IPs), os Inibidores da Integrase (INIs), os Inibidores da Fusão (IFs) e por fim os Inibidores de CCR5.

Os ITRNs são pró-fármacos que devem ser trifosforilados ou, no caso do Tenofovir (TDF), difosforilado na sua forma ativa. Esta dependência de fosforilação intracelular complica a avaliação *in vitro* da atividade dos ITRNs porque a fosforilação ocorre em taxas diferentes em diferentes tipos de células e leva a discrepâncias entre a potência *in vitro* e *in vivo* dos ITRNs.

Há dois mecanismos bioquímicos de resistência aos ITRNs que são causados principalmente por mutações na região N-terminal que codifica a transcriptase reversa da polimerase do HIV-1. Um mecanismo é mediado por mutações que reduzem a afinidade da RT para um ITRN, impedindo a sua adição à cadeia de DNA [256]. Outro mecanismo é mediado por mutações que favorecem a remoção hidrolítica de um ITRN que foi incorporado à cadeia primária do HIV-1 [257, 258].

Os ITRNNs inibem a RT do HIV-1 alostericamente pela ligação a uma porção hidrofóbica próxima ao sítio ativo da enzima. Esta porção de ligação é bem menos conservada do que o sítio ativo da enzima de ligação de dNTP. Como resultado, entre os isolados do grupo M há uma maior variabilidade em sua suscetibilidade a ITRNNs do que aos ITRNs [259]. Os ITRNs e os ITRNNs são usualmente sinérgicos. Várias mutações que conferem resistência aos ITRNNs aumentam a susceptibilidade a determinados ITRNs [260] e várias mutações de resistência aos ITRNs aumentam a susceptibilidade aos ITRNNs [261, 262].

Mais de 80 mutações não-polimórficas aos IPs têm sido relatadas [263]. A maioria destas contribui para uma menor susceptibilidade *in vitro* a um ou mais IPs [264, 265]. As figuras 10, 11 e 12 descrevem a relação das mutações relacionadas a resistencia aos ART para ITRNs, ITRNNs e IPs, respectivamente.

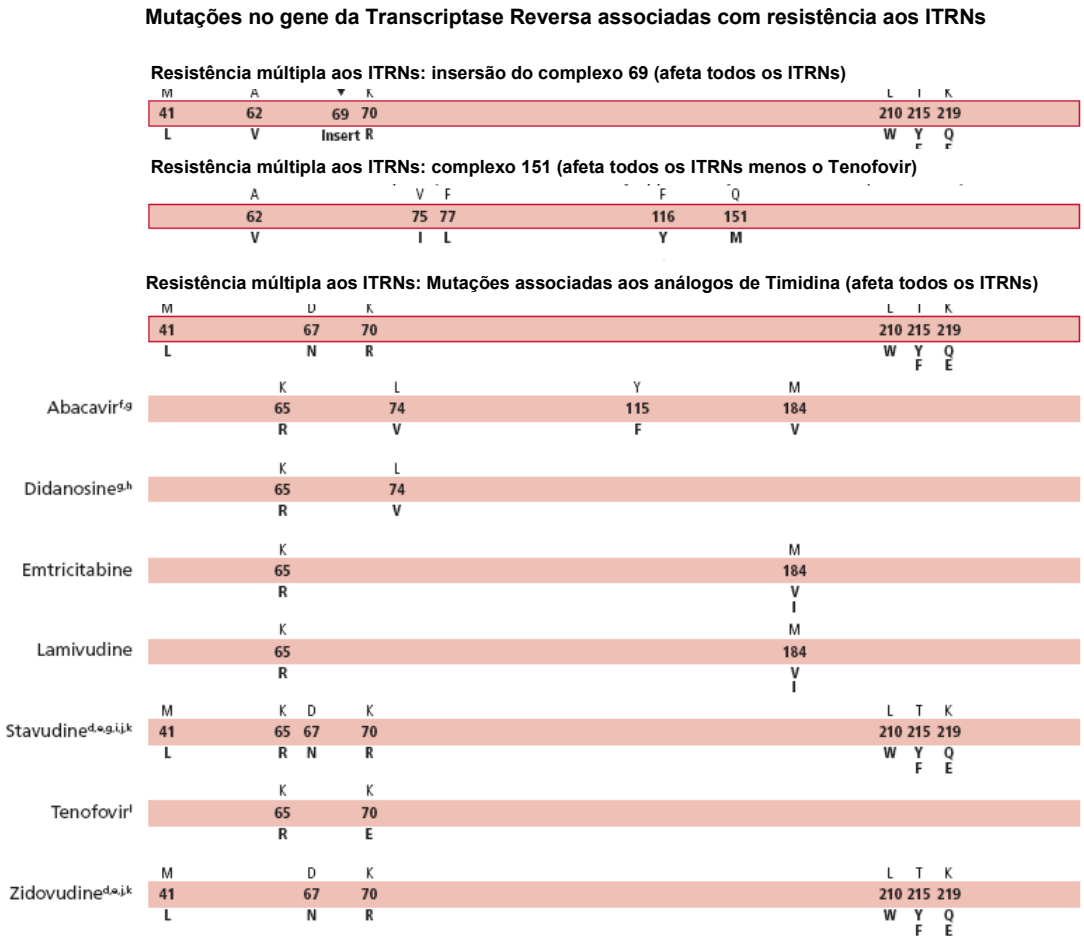

Figura 10: Esquema ilustrativo das mutações do gene da Transcriptase Reversa associadas com resistência aos ITRNs. Adaptado a partir de: [266].

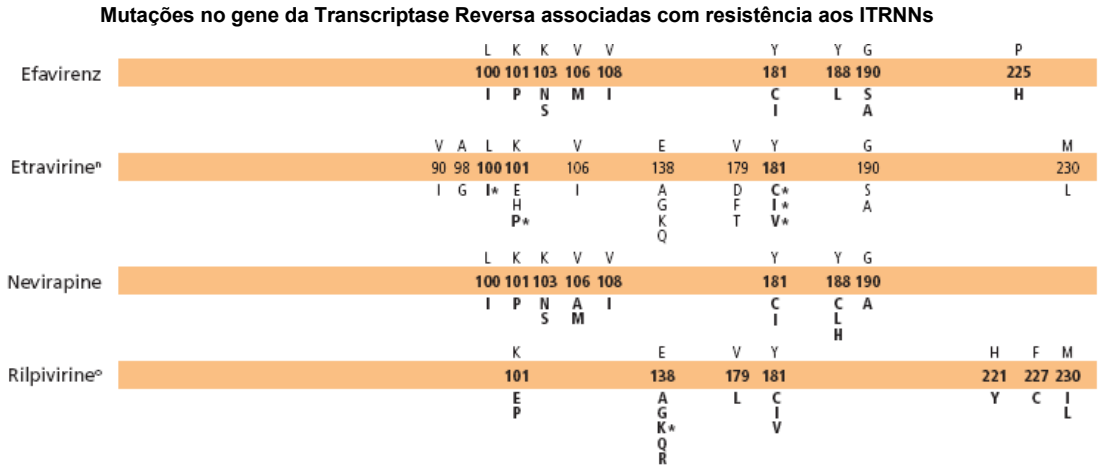

Figura 11: Esquema ilustrativo das mutações do gene da Transcriptase Reversa associadas com resistência aos ITRNNs. Adaptado a partir de: [266].

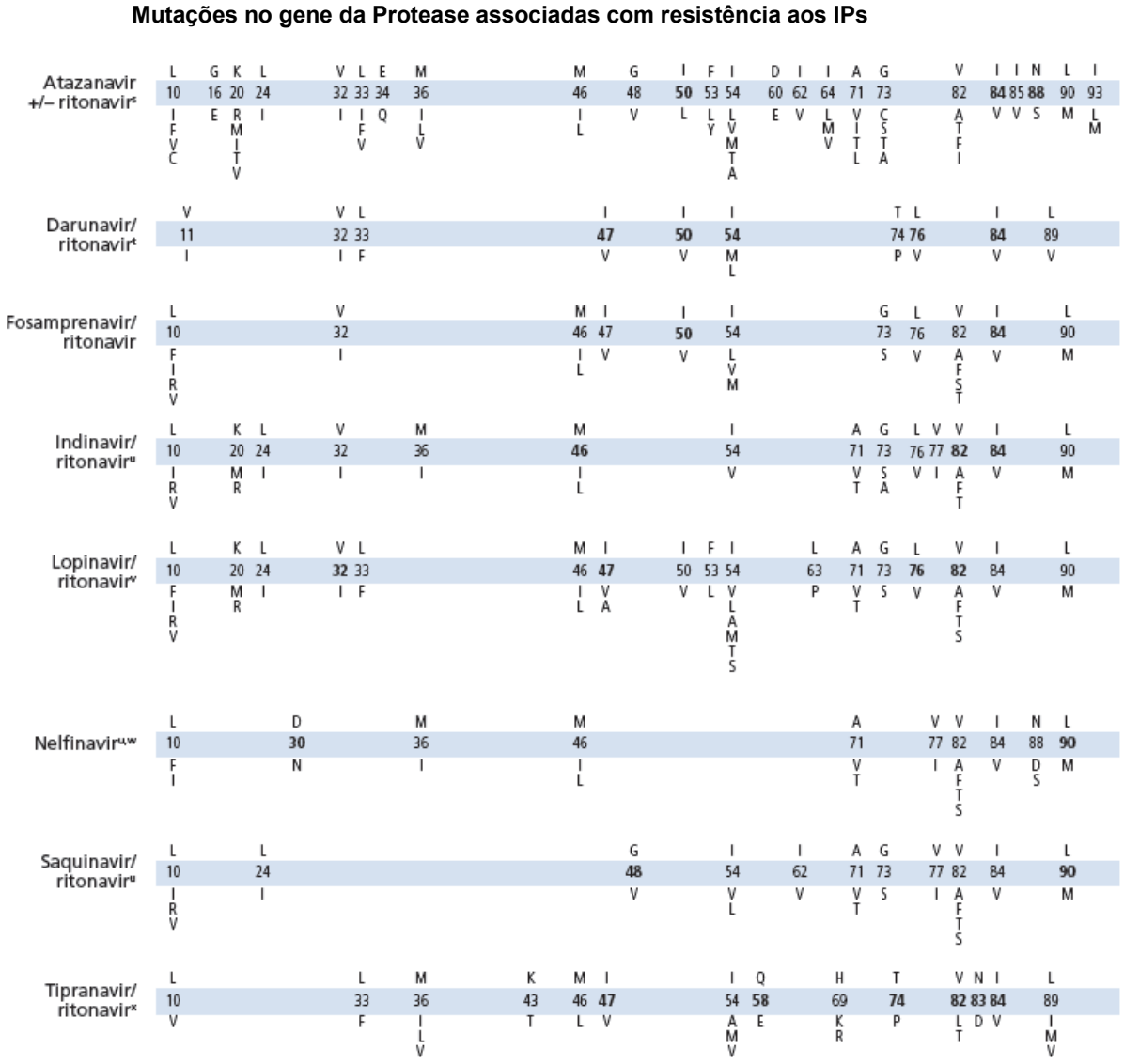

Figura 12: Esquema ilustrativo das mutações do gene da Protease associadas com resistência aos IPs. Adaptado a partir de: [266].

1.12 – Resistência Primária (transmitida) aos Antirretrovirais:

Apesar dos avanços na terapia antirretroviral que revolucionaram o manejo do HIV e o controle de epidemias regionais [267-269], a resistência aos antirretrovirais surgiu em todas as localidades em que tais drogas foram usadas. A resistência primária representa um desafio para o controle do HIV-1 por reduzir a eficácia da terapia anti-retroviral de primeira linha e afetar os resultados

clínicos. Dados de todo o mundo que estimam a prevalência de resistência antirretroviral evidenciam a resistência antirretroviral ao longo dos anos [270-273]. O Brasil é um país que desde 1996 há uma política de acesso universal à terapia antiretroviral altamente ativa (HAART), com mais de 200.000 indivíduos infectados pelo HIV-1 em tratamento hoje em dia [274, 275] e a primeira pesquisa brasileira sobre a taxa de resistência primária às drogas foi publicada em 1999 [276]. Depois de uma década, foram realizados estudos regionais [187, 277-282] e nacionais [175, 275, 283, 284], sendo possível detectar de forma geral um incremento na prevalência da resistência primária às drogas antirretrovirais; o que faz emergir a necessidade de um maior debate sobre a necessidade da realização do teste de genotipagem antes do início da terapia antiretroviral [285].

2 – Justificativa

A diferenciação de indivíduos recém infectados vírus HIV daqueles infectados de longa data é difícil, porém extremamente importante para a estimativa da incidência, aprimorando as estratégias de acompanhamento clínico e preventivo. Estudos de pacientes com infecção recente são ferramentas úteis para a verificação da eficácia de políticas públicas de controle da pandemia e funcionam como sinalizadores de controle epidemiológico, podendo ser um indicador precoce na detecção de uma eventual variação nos padrões de disseminação nas populações afetadas [286]. Além de ser uma ferramenta da epidemiologia, a facilidade de execução técnica, o relativo baixo custo e o fato de não exigir nenhum outro recurso tecnológico além do necessário para a realização dos testes de triagem credencia a sorologia para a identificação de amostras de indivíduos recentemente infectados pelo HIV-1 como procedimento eletivo para a utilização em larga escala, podendo ser utilizado por vários centros em nosso país, contribuindo assim para um melhor entendimento e vigilância desta pandemia em nosso meio.

A vigilância em relação à diversidade viral e à resistência primária em nosso meio pode ser favoravelmente impactada com a identificação de amostras de indivíduos recentemente infectados, pois possibilita avaliar com maior acurácia as tendências mais recentes da epidemia. Até o momento, a maioria dos estudos em nosso meio avaliou a totalidade das amostras sem levar em consideração o período de transmissão destas cepas e/ou analisou populações específicas em estudos seccionais; o que dificulta a comparação ao longo do tempo. Diante desta carência de dados que possam ser melhor comparados em diferentes populações, nossa proposta é identificar indivíduos com infecção recente pelo HIV-1 e estudar as taxas de prevalência e incidência estimadas, levando em conta as variáveis sócio-demográficas, assim como a análise da diversidade e resistência primária aos antiretrovirais ao longo do tempo nas populações que buscam testagem em quatro CTAs de diferentes cidades da região metropolitana do Rio de Janeiro por um período contínuo de três anos. No decorrer deste trabalho a literatura apontou para a importância da associação de metodologias visando o aumento da especificidade na detecção dos casos recentes de infecção pelo HIV-1, o que nos levou à realização de um estudo comparativo entre dois métodos com vistas à proposição de um algoritmo de análise.

3 – Objetivos

3.1 – Objetivos Gerais

1) Identificar em centros de testagem e aconselhamento indivíduos recentemente infectados pelo HIV.

2) Comparar os aspectos biológicos e de resistência do HIV-1 entre os indivíduos recentemente infectados e os cronicamente infectados ao longo do estudo

3.2 – Objetivos Específicos

1) Determinar a prevalência e a incidência da infecção por HIV na população estudada.

2) Comparar a prevalência e a incidência da infecção por HIV ao longo do estudo.

3) Caracterizar o perfil sócio-demográfico na população estudada (casos incidentes, prevalentes).

4) Comparar diferentes métodos sorológicos para a caracterização de infecção recente por HIV-1.

5) Propor um algoritmo visando uma melhora da especificidade na identificação de indivíduos com infecção recente pelo HIV-1 a partir de métodos sorológicos e marcadores imunológicos específicos e não específicos.

6) Determinar a freqüência dos subtipos de HIV-1 e de genomas recombinantes nos casos de infecção recente frente a uma amostragem dos indivíduos soropositivos de longo termo.

7) Determinar a freqüência de resistência primária aos antiretrovirais nos casos de infecção recente frente a uma amostragem dos indivíduos soropositivos de longo termo.

8) Avaliar a reatividade e a avidez de anticorpos através do uso de métodos de identificação de infecção recente pelo HIV-1, em indivíduos com perfis distintos de progressão para a AIDS, incluindo indivíduos não progressores de longo termo.

4 – Artigo 1

"Prevalence, estimated HIV-1 incidence and viral diversity among people seeking voluntary counseling and testing services in Rio de Janeiro, Brazil."

Este artigo se relaciona com os objetivos específicos 1 e 6 e foi publicado no periódico *BMC Infection Disease.* 2010 Jul 28;10:224.

1) Determinar a prevalência e a incidência da infecção por HIV na população estudada (primeiro ano do estudo);

6) Determinar a freqüência dos subtipos de HIV-1 e de genomas recombinantes nos casos de infecção recente frente a uma amostragem dos indivíduos soropositivos de longo termo (primeiro ano do estudo).

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RESEARCH ARTICLE Open Access

Prevalence, estimated HIV-1 incidence and viral diversity among people seeking voluntary counseling and testing services in Rio de Janeiro, Brazil

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Abstract

Background: BED-EIA HIV-1 Incidence Test (BED-CEIA) has been described as a tool to discriminate recent (RS) from long-term (LTS) seroconversion of HIV-1 infection, contributing to a better understanding of the dynamics of the HIV/AIDS epidemic over time. This study determined the prevalence, estimated incidence and HIV-1 subtype infection among individuals seeking testing in Voluntary Counseling and Testing centers (VCTs) from Rio de Janeiro, Brazil.

Methods: Demographics and behavioral data were obtained from 434 individuals, diagnosed as HIV-positive among 9,008 volunteers screened from November 2004 to October 2005 in three VCTs located in the Rio de Janeiro Metropolitan area, Brazil. BED-CEIA protocol was performed to identify RS. DNA samples from RS and a subset of LTS (under a proportion of 1:2) were selected for gp120 C2-V3 and *pol* (protease and reverse transcriptase) regions genomic sequencing.

Results: Overall HIV-1 prevalence was 4.8%. Sixty-one of 434 seropositive individuals were classified as RS, corresponding to an incidence rate of 1.68%/year (95%CI 1.26% -2.10%). Estimated incidence between Men Who Have Sex with Men (MSM) was 11 times higher than among heterosexual men and 55% of the new cases were identified in volunteers aged 25-40 years. A similar distribution of different HIV-1 subtypes was found among RS and LTS.

Conclusions: Our data suggest that prevention for MSM remains a challenge and efforts focusing on prevention targeting this population should be prioritized. No significant changes in HIV-1 subtypes were observed among the RS and LTS subgroups. One case of HIV-1 AUK (pol)/A (env) recombinant genome was detected for the first time in Brazil.

Background

The HIV pandemic continues to spread unabated, with about 33.4 million people living with HIV worldwide. Globally, approximately 2.7 million new infections are believed to occur in 2008 [1]. Hosting the largest HIV-1 infected population in Latin America, Brazil has a complex HIV-1 epidemic characterized by an increase of women participation over time, a pronounced decrease among injecting drug users and persisting high-risk behaviors and a higher proportion of new infections among gay and bisexual men [2]. Approximately 620,000 individuals aged 15 to 49 years old are estimated to be infected with HIV in Brazil. According to the former National AIDS Program (recently renamed as the Department of STD, AIDS and Viral hepatitis) 544,846 AIDS cases were reported from 1980 to June 2009. Rio de Janeiro is the second Brazilian state in terms of number of reported AIDS cases, corresponding to 14% of the overall national AIDS cases [3]. Sentinel

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studies in pregnant women estimate an overall prevalence of HIV-1 infection of 0.61% [4]. Serology for HIV infection is available at no cost in the Voluntary Counseling and Testing centers VCTs) all over the country, where the volunteers have full access to prevention activities and individual counseling.

In Brazil, twenty years ago, facilities offering voluntary counseling and testing (VCT) at not cost at the point of delivery were established as a key strategy to promote the access of the Brazilian population to early diagnosis and prevention of HIV and others sexually transmitted diseases (STDs) and to stimulate agile referral to treatment. Such VCT centers became a central piece of Brazil's initiative to curb the epidemic over the years and nowadays comprise a comprehensive network of over 380 public health facilities spread all over the country. In cities with VCT centers, the number of HIV tests per 1,000 people is 2.4 times higher than in the localities where there is no VCT center. At present, in Rio de Janeiro state, 13 VCT centers are fully operative - eight of them located in the metropolitan area [5].

In order to better understand recent epidemiological trends in HIV transmission it is key to estimate incidence and to assess viral diversity among recently infected individuals. Serological differentiation between recent (RS) and long-term seroconverters (LTS) has been described as a powerful tool to track the trends of the HIV/AIDS epidemic [6]. A variety of approaches have been described over time [7,8]. One of those assays is based on the capture and detection of increasing proportions of HIV-1 IgG in the serum over the time (BED-CEIA) that uses a branched peptide based on gp41 of B, D and E HIV-1 subtypes [9,10]. Due to some concerns that have emerged in scenarios with high HIV prevalence and/or viral diversity [11], two correction strategies/factors were proposed to minimize the putative overestimation of incidence rates using BED-CEIA. One of them proposes the exclusion of certain specimens on clinical grounds, by relying on trend differences rather than absolute incidence estimates, using secondary confirmatory testing or adjustments for misclassification [12]. The second correction factor adjusts the findings in order to avoid misclassification of subtype C infected individuals [13].

Brazil is a huge country presenting heterogeneous HIV/AIDS epidemiological trends [3] and virological scenarios, with distinct HIV-1 subtypes and recombinant infections [14] in different regions and/or population strata. The aim of this study was to assess the prevalence and to estimate incidence of HIV-1 infection among individuals seeking HIV testing in VCT centers located in the Rio de Janeiro Metropolitan area, as well as to track HIV diversity among RS and LTS over time.

Methods

Study Population

Frozen sera collected from a consecutive sample of individuals from November 2004 to October 2005 (n = 9,008), recruited from three VCT centers located in municipalities of Rio de Janeiro Metropolitan area were analyzed in this study. The VCT centers were selected based on their role as reference centers for people seeking HIV testing at no cost in the Rio de Janeiro Metropolitan area. These sites are the places where any Brazilian citizen can be tested for HIV infection for free, keeping anonymity if desired, with easy access, and in the context of a welcoming environment. They are localized mainly in the outskirts of Rio de Janeiro, which corresponds to a catchments area of about 5 million people, the vast majority of them from poor social strata.

Demographic information was obtained from baseline data collection forms, administered in private by trained interviewers. Among the population studied, the records provided information on gender, age and sexual orientation for all individuals. In 96.5% of HIV-1 seroreactive individuals (419 of 434) it was possible to obtain additional information on testing such as: reasons for being tested, previous testing, and presence of symptoms associated to HIV/AIDS, as well as a history of risky injection behaviors. We should observe here that the protocol of the present study did not interfere in any sense with the routine procedures adopted by each one of the VCT centers and in this sense allows for individuals the right to refuse to answer one or more questions they may consider as embarrassing or annoying. Individuals were entitled to be counseled and tested, as defined by the guidelines of the Brazilian Ministry of Health (BMoH), irrespectively of their answers (or the absence of answers) to the short standard form used as a routine procedure in the VCT centers.

The present research protocol conformed to the Helsinki Declaration and to the local legislation. Informed consent was obtained from the participants and the refusal to provide information perceived as sensitive explicitly guaranteed by the study protocol. The study was approved by the Oswaldo Cruz Foundation (FIOCRUZ) - Evandro Chagas Clinical Research Institute Ethical Board, registration CAAE-0032.0.009.000-04.

Serological testing algorithm

With the goal of enabling this research protocol without disturbing the routine procedures of the VCT centers, two parallel blood tubes (BD Vacuntainer® ref. 367958) were collected from each individual. One of them remained in the VCT centers, in order to conduct the standard serological testing and delivery of results with

the corresponding post-test counseling, in accordance with the Brazilian Ministry of Health (Ordinance N°59 of January 28, 2003). The second tube was sent to the Laboratory of AIDS & Molecular Immunology, IOC (FIOCRUZ), and then stored at -20°C until the delivery of the results of the HIV testing. This strategy assured the non-interference in the VCT center routine, as well as the possibility to have 100% of the HIV seroreactive samples viable to perform BED-CEIA methodology. These samples were then thawed, aliquoted into sterile microtubes and stored at -70°C for subsequent studies. Two aliquots of serum and one aliquot containing the blood clot were available for each HIV seroreactive sample.

In order to identify recent HIV-1 infections, serum samples reactive for HIV antibodies were subjected to testing in vitro by a quantitative competitive capture enzyme immunoassay (BED-CEIA), to determine the proportion of anti-HIV-1 specific IgG in relation to the total IgG, according to the manufacturer's instructions [9]. Briefly, serum (or plasma) samples were added to the wells of a microplate coated with anti-human IgG. HIV specific and not specific IgG molecules were captured on the solid phase, representing populations of antibodies found in the sample. After the incubation and washing protocol, a synthetic chimeric polypeptide containing the HIV-1 immunodominant epitope of gp41 subtype B, E and D was added and incubated for one hour at 37°C. At this stage the peptide bound to their respective antibodies (anti-HIV-1 specific). After further steps the reactions is revealed in the presence of TMB (tetramethylbenzidine) and read using a spectrophotometer (wavelength 450 nm). The color intensity obtained in each sample is directly proportional to the amount of specific anti-HIV-1 antibodies. The controls (negative, low positive and high positive) (Calypte HIV-1 BED Incidence EIA) and calibrators are analyzed along with samples. The criteria for validation of the test, definition of samples to confirm and set the time of infection were used in accordance with the instructions and worksheet provided by the manufacturer [10]. Annual HIV seroincidence and 95% confidence intervals (CIs) were calculated on the basis of the BED-CEIA results, using the 153-day window period and the seroincidence formula described elsewhere [9,10]. Two correction strategies, here named CF1 [12] and CF2 [13] were further applied.

DNA Extraction and PCR amplification of HIV-1 env and pol regions

Proviral DNA was extracted from the blood clot using the phenol/chloroform method [14]. The samples were amplified for the gp120 C2-V3 region using a nested PCR protocol with ED5/ED12 and ED31/ED33 oligonucleotide sets respectively as outer and inner primers [15], generating a fragment of approximately 564 bp. Similarly, for the *pol* gene we amplified a 1 Kb fragment covering the protease (PR) and part of the reverse transcriptase (RT) using the outer DP10/LR54 and inner DP16/LR49 primer sets as previously described [16,17]. The samples were amplified in the thermocycler GeneAmp® PCR System 9700 (Perkin Elmer - Norwalk, USA) using the following conditions: 3 cycles at 97°C for 1 minute, 55°C for 1 min, 72°C for 2 minutes. Thirty two cycles at 94°C for 45 seg, 55°C for 1 minute, 72°C for 2 minutes. Final cycle was performed at 72°C for 10 minutes. PCR products were visualized after electrophoresis in 1% agarose gels (Gibco/BRL, Life Technologies - USA) and purified (GFX™ PCR DNA and Gel Band Purification Kit) following the manufacturer's protocol (Amersham Biosciences, GE Healthcare - UK).

DNA sequencing and Phylogenetic analyses

Purified PCR products corresponding to *env* and *pol* regions were respectively submitted to automated sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction - ABI PRISM (Perkin Elmer, Foster City, USA), according to manufacturer's instructions. Fifty to 100 ng of amplified DNA were used for sequencing reaction in both senses using the *env* inner primers ED31 and ED33. For the *pol* region, sequence reactions were performed using DP16 and LR49 in addition to the RT12 and LR51 in order to cover the whole fragment in both directions Sequencing reactions were performed in the thermocycler GeneAmp® PCR System 9700 (Perkin Elmer - Norwalk, USA) using the following conditions: 25 cycles of 96°C for 30 seconds, 50°C for 20 seconds and 60°C for 4 minutes. Each reaction product with a final volume of 20 μl was incubated for 15 min with 80 μl of 75% isopropanol, followed by centrifugation, discarding of the supernatant and drying the precipitate in the thermalcycler at 70°C for about 10 minutes. The pellet was suspended with 10 ul of Hi-Di Formamide, followed by a shock of 5 minutes at 95°C and 1 minute on ice for denaturation and placed on the ABI model 3100 DNA Sequencer (Applied Biosystems, Foster City, CA).

Generated DNA sequences (sense and anti-sense) of each sample were assembled using SeqMan, included in the DNASTAR package [18]. Sequence electropherograms obtained for the *env* C2-V3 and *pol* regions were respectively aligned against a set of reference strains from HIV-1 group M subtypes using CLUSTAL X [19]. SIVcpz *env* C2V3 or *pol* sequences were used as outgroup in both analyses. Gap stripping and minor adjustments were manually performed. Phylogenetic inferences were performed by the neighbor-joining algorithm using the program Mega 3.0 (Molecular

Evolutionary Genetics Analysis, version 3.0), and the reliability of the branches determined by analysis of bootstrap calculated based on 100 re-samplings [20]. TREEVIEW [21] was used for visualization of the phylogenetic trees. Screening of *pol* recombinant forms were assessed through the Rega HIV-1 Subtyping Tool website [22] and further confirmed by bootscanning analysis (sliding window of 400 bp, incremental steps of 10 bases, and the Kimura two-parameter model) as implemented in Simplot version 2 (Ray S. Simplot v2.5.0 http://sray.med.som.jhmi.edu/SCRoftware/simplot/).

Data Analysis

Data were entered into contingency tables and analyzed using chi-square or Fisher's exact test.

Results

Overall 9,008 persons were tested for HIV infection in the three VCT centers. Men comprised 41.4% of the tested population, of whom 10.7% (n = 399) referred to have had sex with other men. Twenty-eight percent of clients were younger than 25 years, 40.8% were 25-40 years old, and 31.2% were older than 40 years.

The overall prevalence of HIV infection was 4.8%, higher for men compared to women (6.5% *vs*. 3.6%). Prevalence was 6 times higher for MSM (24.8%) vis-à-vis heterosexual men (4.3%). The age group more affected was young adults aged 25 to 40 years old, with a prevalence of 6.3% (Table 1). The major motivations for testing could be obtained for the HIV seropositive individuals. In general, about thirty-five percent of the individuals with valid answers admitted to be recently exposed to risk as the main reason to seek and HIV test, over one quarter said testing had been taken after the suggestion made by professionals from a health service. Forty individuals (roughly corresponding to 10% of the individuals with valid answers) reported that symptoms related to HIV/AIDS were their main motivation for testing, whereas 15% reported they were mainly motivated by the desire to prevent themselves against HIV. In this subgroup, only five people were returning for a new test (all of them with prior negative results), and 404 individuals with valid answers (96.42%) reported unprotected sexual intercourse as their main risk factor. Eight individuals (less than 2%) reported to have shared syringes in the context of illicit substance self-administration.

Table 1 Prevalence of HIV-1 infection in 9,008 individuals screened in 3 VCTs of Rio de Janeiro state from November 2004 to October 2005.

Variables (n)	Seropositives		P value
	%Prevalence (n)	95%Confidence Interval	
Overall– 9,008	4.8 (434)	4.4 - 5.3	-
Gender			
Female (5,282)	3.6 (191)	3.1 - 4.1	< 0,0001[a]
Male (3,726)	6.5 (243)	5.7 - 7.3	
Sexual practice (men)			
Heterosexual (3,327)	4.3 (144)	3.6 - 5.0	< 0,0001[a]
MSM (399)	24.8 (99)	19.9 - 29.7	
Age - years			
< 25 (2,525)	2.6 (65)	1.9 - 3.2	< 0,0001[b]
25 a 40 (3,671)	6.4 (233)	5.5 - 7.1	
> 40 (2,812)	4.8 (136)	4.0 - 5.6	

a = X^2

b = Fisher's exact test

Based on the BED serology results, sixty-one out of the 434 seropositive individuals (14.0%) were classified as RS, with an overall incidence rate of 1.68%/year (95% CI 1.26% -2.10%). Data including the estimated incidence re-calculated according to CF1 (1.09%/year) and CF2 (1.17%/year) are also presented. Incidence rates were also calculated according to the different exposure categories and age strata (Table 2). Overall, higher incidence rates were observed for MSM when compared to heterosexual men (8.55-11.96% *vs* 0.56-1.12%), and young adults aged 25-40 years old.

Molecular analyses of the C2-V3 *env*, RT and Protease genomic regions were performed for 52 out of the 61 RS (85%) and 114 out of the 122 (93%) randomly selected LTS samples. Overall, 80.1% (n = 133) individuals were infected by subtype B viruses, 8.4% by subtypes F (n = 13) and C (n = 1), and recombinant forms were found in 11.4% (n = 19) of the isolates. No significant differences were found between RS and LTS for subtype distribution; however, a higher proportion of non-B subtypes were found in the RS group (11.5% *vs* 7.0%). Roughly 75% (14 out of 19) of recombinant samples were between B and F subtypes and none of them showed the same breakpoint of recombination in the *pol* region, or subtype determination between *pol* and *env* regions, confirming the high frequency of URFs BF, as usually described in our region [23].

An HIV-1 $A_{PR}UK_{RT}A_{ENV}$ mosaic genome was detected among the LTS. Based on the bootscanning analysis (data not shown), this viral genome was composed by a fragment of subtype A in the protease portion, one fragment of around 400 bp classified as unknown (U), located at the 5' side of the reverse transcriptase, followed by a subtype K fragment of 301 bp from position 2952 to 3253 (relative to HXB2 isolate). The gp120 C2-V3 region was assigned as subtype A.

Table 2 Estimated HIV-1 Incidence of HIV infection among 9,008 individuals (434 seropositive samples) screened in 3 VCTs of Rio de Janeiro Metropolitan area from November 2004 to October 2005 - conventional estimation and estimation with 2 corrective factors (CF)

	Recent Infections	Estimate Incidence[a] %/year (95% CI)	Estimate Incidence[b] %/year (95% CI)	Estimate Incidence[c] %/year (95% CI)
Overall = 434	61	1.68 (1.26 - 2.10)	1.09 (1.32 - 1.87)	1.17 (0.88 - 1.47)
Gender				
Female (191)	30	1.40 (0.90 - 1.90)	0.97 (0.62 - 1.31)	1.04 (0.67 - 1.41)
Male (243)	31	2.10 (1.36 - 2.84)	1.27 (0.82 - 1.72)	1.37 (0.89 - 1.85)
Exposure categories (among men)				
Heterosexual (144)	15	1.12 (0.55 - 1.68)	0.56 (0.28 - 0.84)	0.60 (0.30 - 0.91)
MSM (99)	16	11.96 (6.10 - 17.82)	8.55 (4.36 - 12.74)	9.17 (4.68 - 13.67)
Age (years)				
<25 (65)	9	0.87 (0.30 - 1.44)	0.56 (0.19 - 0.92)	0.60 (0.21 - 0.99)
25 to 40 (233)	34	2.33 (1.55 - 3.12)	1.55 (1.03 - 2.07)	1.67 (1.11 - 2.23)
>40 (136)	18	1.59 (0.86 - 2.33)	0.99 (0.53 - 1.45)	1.07 (0.57 - 1.56)

[a] Incidence estimated with conventional estimation [9]
[b] CF1 = Incidence estimated with Corrective Factor 1 [12]
[c] CF2 = Incidence estimated with Corrective Factor 2 [13]

Discussion

Data generated from multiples studies conducted under the scope of the HIV/AIDS National Surveillance System, clearly indicate that the epidemic in Brazil persists as a concentrated one [3,4]. Like other countries in the region, our epidemic is driven mostly by Men who have sex with men [3,4]. The 4.8% overall prevalence found in our study conducted on three VCTs located in the outskirts of Rio de Janeiro State, are in accordance with data from studies conducted among clients seeking HIV testing in VCT services. These studies found a higher prevalence among these clients than in the general population in which rates are around 0.6%. [24-27]. Our data showing a prevalence of HIV infection 6 time higher among MSM when compared to heterosexuals participating in the same study, corroborates with a variety of studies which results showed high rated of HIV infection among this population (9.2% -32.2%). In a report of the Population Council, published recently, one study using the respondent-driven sampling (RDS) method points to a high prevalence (7%; 95%CI 5-11%) among MSM in the city of Campinas, state of São Paulo. Of special concern are the high prevalence found between very young MSM (aged 14-19) among whom a prevalence of 4% (95%CI 1-9%) was found, suggesting the epidemic in this population is far to be curbed [28]. Finally, all these data are in accordance with the AIDS national report system that register a proportion 11 times higher of MSM AIDS cases reported when compared to cases reported among heterosexual men [29], reinforcing the conclusion that MSM remain disproportionately affected by the epidemic in Brazil.

The approach adopted by our study has been found to be highly sensitive (82% [95%CI 77-86%]) and specific (89% [95%CI 85-92%]) [30], and has been used to estimate HIV national incidence in the USA [31], where viral diversity is similar to the scenario made evident for the regions assessed by the present study, where most of the infections are attributed to subtype B.

Different factors have been described as associated with BED-CEIA misclassification, which can lead to overestimated HIV incidence, including: HIV-infected individuals under antiretroviral therapy, patients with advanced immunodeficiency, drug naïve individuals with persistent low to undetectable viral loads, and different HIV subtypes [11,32,33]. In order to overcome these putative caveats we applied 2 correction factors (CF1 and CF2) to improve the incidence estimate rates in our study. Indeed, rates obtained after correction with CF1 and CF2 were close to each other and lower than the one obtained with the conventional method. Although lower estimates were obtained after the use of the correction algorithms, the proportions relative to the different categories remained approximately the same. This speaks in favor of the internal consistency of our findings, irrespectively of the inclusion or not of the correction factors. Recently, a series of publications discuss the pertinence and adequacy of such corrections [34-38] and, as to the best of our knowledge, this subject is still far from a consensus.

In our study, women found to be HIV-infected were almost invariably infected in consequence of unprotected sex with HIV infected male partners. This is a common pattern in contexts where blood transfusions are safe and HIV drug use is uncommon, like in Rio de

Janeiro. This city metropolitan area has been relatively spared by the injecting drug users-driven boom of new HIV infections secondary to the shared use of injection paraphernalia, even in its national peak (in the late 1980s/early 1990s), and has declined since then. The number of female IV drug users has been particularly low [39,40]. Since early 90 s the standard of quality of the Brazilian blood banks is comparable to the best international ones [41]. As a result, new AIDS cases secondary to blood transfusions has been negligible in the last decades, specially in urban areas such as the one where our study was conducted (data available from Monitoraids and SINAN-AIDS at: http://sistemas.aids.gov.br/monitoraids/?lang=en-US&pagina=&st_lPanel=&indexMenu= -1).

No significant changes in the HIV-1 subtype distribution could be discerned among the RS compared to LTS. Subtype B was predominant, followed by subtype F and BF recombinant viruses, as previously described for the Brazilian Southeast region [42-44]. A different scenario may be observed for heterosexual men *vs.* women, when we compare HIV-1 infections with recombinant virus (almost 1:7 *vs.* 1:15, p = 0.19). One case of AUK recombinant infection was detected for the first time in Brazil.

Conclusions

To the best of our knowledge, this is the most comprehensive assessment of HIV-1 prevalence, estimated incidence and subtype profile in the population seeking testing in the Rio de Janeiro metropolitan area and may contribute to better discern recent trends of the local epidemic. Moreover, our data suggests that prevention for MSM remains a challenge and efforts aiming to curb the epidemic in this population should be prioritized. The approach used for this study also permitted the identification of new HIV-1 isolate, as the AUK recombinant genome found for the first time in Rio de Janeiro, Brazil. Unfortunately, no further contact could be made with this individual in order to track the origin and the potential dissemination of this recombinant virus in the country.

Due to the fact our data were obtained from a population who actively sought HIV testing, findings cannot be generalized to the general population or to the sampling frame defined by men who have sex with men living in the metropolitan area of Rio de Janeiro and should be viewed with the necessary caution. Notwithstanding, the present study is the most comprehensive assessment of the population seeking testing in the Rio de Janeiro metropolitan area carried so far and may inform renewed policies aiming to prevent and treat people living with HIV/AIDS in Rio de Janeiro.

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Authors' contributions

CAVC, BG, VGV, FIB, JHP and MGM participated in the conception and design of the study; analysis and interpretation of data, drafting the paper and/or substantially revising it. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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5 – Artigo 2

“Temporal trends in HIV-1 subtype distribution and antiretroviral primary resistance mutations among people seeking HIV diagnosis in voluntary counseling and testing sites in Rio de Janeiro metropolitan area, Brazil.”

Este artigo, encontra-se em fase final de revisão para a submissão e se relaciona com os objetivos específicos 6 e 7.

6) Determinar a freqüência dos subtipos de HIV-1 e de genomas recombinantes nos casos de infecção recente frente a uma amostragem dos indivíduos soropositivos de longo termo.

7) Determinar a freqüência de resistência primária aos antiretrovirais nos casos de infecção recente frente a uma amostragem dos indivíduos soropositivos de longo termo.

Temporal trends in HIV-1 subtype distribution and antiretroviral primary resistance mutations among people seeking HIV diagnosis in voluntary counseling and testing sites in Rio de Janeiro metropolitan area, Brazil.

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RS : Recent seroconverted
LTS : Long term seroconverted

Key words: HIV-1, subtypes, drug resistance mutations, VCT, temporal trends, Brazil

Running title: HIV-1 subtype and transmitted drug resistance among recent infections in Brazil

Background: Thirty years after the first report, HIV-1 remains a global health problem. Remarkable viral diversity and adaptation to immune activity is one of the most challenging aspects to the development of a HIV vaccine. After fifteen years of HAART therapy, primary resistance begins to increase and its surveillance is needed to assure optimal choice of therapy.

Objectives: The aim of this study was to assess the temporal trends of HIV-1 subtype distribution and primary resistance mutation profile in different VCTs sites located in the metropolitan region of Rio de Janeiro state, Brazil.

Study Design: Blood samples were collected from HIV-1 positive individuals seeking HIV-1 diagnosis in four VCTs sites located in the metropolitan region of Rio de Janeiro state, Brazil from 2005 to 2007. Serological differentiation between recent (RS) and long term (LT) HIV-1 infections were determined by BED. Pol viral sequences were obtained for 106 LT individuals identified in 2005 and 151 recent infection from 2005-2007. HIV-1 subtype and p*ol* recombinant genomes were assessed through the Rega HIV–1 Subtyping Tool. Surveillance of HIV-1 primary resistance to protease and reverse transcriptase inhibitors were performed according to Calibrated Population Resistance (CPR) Tool Version 6.0.

Results: In general, **s**ubtype B remains the most prevalent in Rio de Janeiro in both LT and RS HIV-1 infected individuals. An increased proportion of recombinant samples were detected especially in heterosexual men as a result of the emergence of CRF02_AG and URF samples bearing a subtype K fragment among RS. Overall, the frequency of transmitted drug resistance mutations did not change when comparing time of infection (LTS 16.0% *vs* RS 15.9%) or age (<25yo 17.9% vs >25yo 16.6%) during the study period. However the high levels detected in both cases are of concern for future therapeutical options. Of note, different scenarios of drug resistance were found when HIV-1 subtype diversity was considered.

Conclusions: Our study shows that HIV diversity and primary/transmitted resistance have become increasingly complex, in Brazil, reaching levels that genotyping test prior treatment may be strongly considered as police to guarantee the best therapeutic options. Continuous surveillance of the emergence of non prevalent HIV-1 subtypes and CRF/URF are also of major priority.

Background:

HIV remains a global health problem with an estimated 33.3 million people living with the virus in 2009. Although the global prevalence rate has remained stable from 2001 to 2009 (0.8%), regional variations are observed, varying from <0.5% to >40% [1]. In Brazil, the prevalence of HIV-1 infection in individuals from15 to 49 years old is estimated on average 0.6%, varying from 0.8% to 0.4% between men and women [1].

In the first half of the last century, HIV-1 group M diversified into genetic subtypes but is still confined to western-central Africa [2]. In the second half, the global spread took place resulting in the differential global distribution of HIV-1 subtypes and recombinant genomes [3]. The high mutation and viral replication rates associated to the recombination role of reverse transcriptase result in a large genetic variability of HIV strains worldwide.

Although the global and regional distributions of individual subtypes and recombinants are broadly stable, the CRFs play an increasing role in the HIV pandemic – 51 CRFs have been described so far (Los Alamos National Laboratory, http://www.hiv.lanl.gov) [3]. Data from Brazil have been available since the nineties showing a predominance of subtype B in distinct country region, followed by subtype F and innumerous URF_BF and exception for the Southern states, where subtype C and CRF31_BC predominates Indeed, the diversity of HIV-1 is one of the most challenging aspect to the development of a HIV vaccine [3].

Despite advances in antiretroviral therapy that have revolutionized HIV management and the control of the spread of regional epidemics [4-6], resistance to antiretroviral drugs has emerged in all localities in which such drugs are used. Primary or transmitted resistance

represents a challenge for the control of HIV-1 due to the impact for the efficacy of antiretroviral therapy and clinical outcomes. Data from reports around the world have shown high levels of primary antiretroviral resistance through the years in developed countries [7-10]. Brazil is a middle-income country that since 1996 has a policy of universal access to highly active antiretroviral therapy (HAART). Nowadays more than 200,000 HIV-1 infected individuals under treatment and the national cross-sectional surveys already performed pointed out to an intermediate level of HIV-1 primary drug resistance (5-15%) [11, 12]. In this paper, we aimed to assess HIV subtype diversity and primary resistance mutations overtime in populations tested and newly diagnosed from 2005 to 2007 in VCTs located in Rio de Janeiro metropolitan area.

Study Design:

Patient Population

From 2005 to 2007 an overall of 27,807 individuals seeking HIV diagnosis in four VCTs located in the metropolitan region of Rio de Janeiro state, one in capital and three in the periphery, were enrolled in the present study. Blood samples were obtained and submitted to routine serological assays to assess HIV infection. Seropositive samples from each year were further submitted to the differentiation between Recent Seroconvertion (RS) and Long Term Seroconvertion (LTS) infections as performed by the BED-CEIA protocol [13] according to the manufacturer's instructions. From these 151 samples from RS (2005-2007) and a random subset of 106 LTS samples diagnosed in 2005 (1RS:2LTS) were included in the present analysis. This study was approved by the

Evandro Chagas Clinical Research Institute Research Board, registration CAAE–0032.0.009.000-04.

HIV-1 subtyping

DNA were sequenced for a fragment of the polymerase region covering the protease and part of the reverse transcriptase as previously described [14]. DNASTAR package was used for sequence edition [15]. Phylogenetic inferences were based in neighbor-joining distances [16] using Mega 3.1 software packages [17]. *Pol* recombinant forms were assessed through the Rega HIV–1 Subtyping Tool website [18].

HIV-1 primary resistance

HIV-1 primary resistance was evaluated according to calibrated population resistance (CPR) Tool Version 6.0. The CPR tool is a program for analyzing pools of human immunodeficiency type 1 (HIV-1) sequences, providing a standard approach for determining the proportion of submitted sequences containing a mutation suggestive of transmitted HIV-1 drug resistance giving a list of standard surveillance drug resistance mutations (SDRMs) [19].This approach was endorsed by the World Health Organization (WHO) for epidemiological surveillance of transmitted HIVDR [20, 21]. The analysis was done by CPR website (http://cpr.stanford.edu/cpr.cgi) accessed in October of 2011.

Data Analysis

Four major groups (LTS of 2005, RS of 2005, 2006 and 2007) were evaluated for VCT location, gender, sexual practice for men, pregnancy in women and age. Data were

analyzed using chi-square or Fisher's exact test. An alpha below or equal to 0.05 was considering significant.

Results:

The genetic characterization of HIV-1 subtypes among the LTS (2005) and RS (2005-2007) showed a predominance of subtype B (80.2%). Among the non-B samples mostly were from subtype F1 (22 in 23 samples), and one was assigned to subtype C. In general, during the studied period approximately one in every ten tested samples presented a recombinant profile between subtypes. These data are detailed in table 1. The comparison between the samples from the LTS 2005 and RS 2006 groups showed a higher proportion of recombinants ($P < 0.01$). We evaluated the following two groups of recombinant forms, URFs - that had no relation with CRFs, and URFs that presented subtypes and points of recombination that were compatible (in the genomic region studied) with some of the previously described CRFs. This last group was designated CRF- related, because only complete sequencing of the samples could establish this characterization. Although no statistically significant difference was obtained, the ratio of URFs: CRFs related decreased from 3:1 in both groups in 2005 to 1:1 in the 2006 and 2007 samples. Differences were not observed between men and women, but men in the RS 2006 group showed a higher frequency of recombinants than in the f LTS 2005 group ($P < 0.01$); similarly, there was a greater recombinant frequency among men in the samples from recently infected individuals (RS, 2005, 2006 and 2007) than in the samples from individuals chronically infected - LTS 2005 ($P = 0.04$). Regarding the analysis of sexual practices among men, we observed more recombinants among MSM in the RS 2006

group than in the LTS 2005 group (P = 0.03). Table 1 summarizes the absolute and relative values. The variable with significant difference is marked in bold.

The analysis of the frequency of surveillance drug resistance mutations (SDRM) revealed that 16% of all samples analyzed had at least one of these mutations. Protease inhibitors (PI) SDMRs were found in 5.2% of the sequences. For the reverse transcriptase inhibitors 9.9% of the mutations conferred resistance against nucleoside analogues (NRTIs) and 8.3% were related to non-nucleoside (NNRTIs). The frequency of resistance mutations found in the same sample for the NRTIs and NNRTIs was 3.7% and two samples (0.9%) had resistance mutations against all three drug groups. The comparative analysis of time of infection in each of these groups – (RS vs LTS) over the study period, gender, sexual practices for men, pregnancy, VCTs location and categorized age did not reveal any significant differences. When the analysis was performed according to the subtype found, several scenarios emerged. Values were above average for subtypes B and F, whereas no SDMR was observed for the samples containing a recombinant fragment of subtype B (12 BF, 1 CB, 1 DB) and the recombinant AG - 7 samples. Table 2 depicts the frequency of mutations against the three drug classes according to in each of the variables studied. Regarding SDRMs, more than half were associated with NRTIs and one in every four mutations in this group were the M184V/I. The second most prevalent mutation in this group was K219R, and half of these mutations were found in the more recently-infected group - RS 2007, even though the number of samples for this time period was only 20% of the total. Approximately one in four mutations found was associated with NNRTIs; K103N was the most prevalent, followed by Y181C and Y188C/L. The PI SDRMs accounted for one in five mutations found and the M46L/I and L90M mutations

accounted for class of drugs over half of the mutations found in this class. Table 3 shows the frequency of each mutation found for each class of drug as a function of the groups studied over time.

Discussion:

In this study we chose to sample over a period of time (between 2005 and 2007) from individuals with recent infection (around 6 months) because this approach could provides the most timely information regarding HIV-1 viral diversity and primary resistance to HIV-1. Previous studies evaluating samples without discriminating the timing of infection information mostly reflect those strains that were transmitted in the past, once that, even in areas where incidence rates are high, the proportion of new infections do not reach 20% of the total number of infections, according to previous reports from the same sites currently included in this analysis [22]. To enhance the comparison analysis we included samples from individuals infected within 6 months (RS), and another group of samples collected in 2005 that were classified as infections having occurred more than 6 months from the date of collection/diagnosis (LTS). Subtype B remains the most prevalent subtype in Brazil, followed by recombinants mostly BF1 and subtype F1 as previously described [23-26]. The increased proportion of recombinants found in the samples of this study (7.5% in LTS vs. 13.2% in RS) and a higher proportion of CRF-related samples compared to URFs were observed over time and this finding correlates with recent data published by WHO-UNAIDS [3]. Interestingly, this trend was due to the increase of CRF over time among men, and was more pronounced among heterosexuals (2.6% in LTS vs. 12.5% in RS) than among MSM

(5.0% vs. 15.6%), which is in accordance with the highest proportion found primarily among women (13.8% in LTS). These data suggest that among the new infections a greater transmission of recombinants may have occurred from women to men than from men to women. A second hypothesis is that men could constitute a population with more imported infections, which may have a new effect on the diversity of the local epidemic. This hypothesis was supported when we found that 35% (7/20) of new infections with recombinants were=classified as related to the recombinant CRF_02AG, with a prevalence of over 70% among men (5/7) and representing an increasing proportion of recombinant RS along the years included in the study (25% in 2005, 30% in 2006 and 50% in 2007). CRF_02AG is the fourth most prevalent recombinant around the world with a known epidemic in West Africa and to a lesser extent in Central Africa, North Africa and the Middle East [3]. So far there have been few reports in our setting [27-29], which is consistent with our findings because all CRF_02AG samples were classified as RS. Another finding that supports the recent inclusion of new recombinant forms in our setting was the occurrence of four recombinants that had a fragment of subtype K, and three of them were characterized as RS. The geographic distribution of subtype K is similar to CRF_02AG – and mostly includes Western, Central and Southern Africa. To date only two reports of subtype K have been published in our country [22, 30].

Regarding the detection of SMDRs our data indicate that the overall levels of resistance were virtually unchanged during the study period. However, the consistently high levels of resistance in at least one of the classes of drugs in the four groups (ranging from 12.7% to 19.3%) were of concern. In the beginning of the HAART era, in 1996 and 1998, two studies were conducted in Brazil, one nationwide [31] and the other in Rio de Janeiro

[32]. The overall prevalence rate ranged from 0.0% to 0.9%, and resistance was found only to NRTIs in the nationwide study. In 2001 a study was conducted in thirteen VCTs located in the metropolitan regions of s eight states in Brazil (including Rio de Janeiro with 83 samples). This study evaluated 45% of the patients taking HAART and found an overall prevalence of 6.6% with a uniform distribution between the drug classes – (2.4% for NRTI, 2.0% for NNRTIs and 2.2% for PIs) [11]. A study conducted in Rio de Janeiro from 2000-2002 which included 56 individuals from the Army Health Service depicted a diverse resistance landscape with a 14% prevalence and only NRTI resistance [27]. In 2002 a similar profile was found in the city of Recife in Northeastern Brazil, which had a much lower prevalence - NRTI resistance at 3,6% also in the year 2002 [24]. Up until now, the concern with primary resistance in Brazil has been restricted to surveys that can detect trends, but routine genotypic testing in drug-naive populations prior to treatment has not been required. Some surveys have reported intermediate and high levels of primary resistance to at least one class of antiretroviral drugs, including 11.4% an AIDS clinic in Bahia [26], 8.1% in a study that included 6 capitals, including Rio de Janeiro where the prevalence was 14.89% [12] and 7.0% in another study of 400 patients nationwide [33]. In 2011, two studies published data on recently infected individuals, reporting a prevalence ranging from 9.5% in Ceará [34] to 13.9% in Sao Paulo [35]. Our results are in agreement with these recent reports that suggest a growing primary resistance level in Brazil and taken together may contribute to the discussion on the need for implementation of resistance testing before starting ARV treatment, at least in cities that have the highest rates of resistance. In early studies that found low levels of resistance, participants were mostly male (70% to 82%) and /or a limited number of

samples were analyzed for a limited period of time. Therefore, the comparison between sex, age, regions of the same metropolitan area and over time in the same location were not possible. Our decision to study RS individuals in VCTs allowed us to make these comparisons because more women participated in the study (1:1.04 females: males), and the current ratio of new infections among men and women has been described as being close to 1:1 [36]. There was no difference in relation to sex, but among men the prevalence among MSM was twice higher than in heterosexual men. This high level of primary resistance corroborates the findings of a recent study conducted in a respondent-driven sample with MSM in nine Brazilian cities where the rate found was 21.4% [37] . This finding can be justified because MSM were the group most affected by HIV infection at the beginning of the epidemic and consequently, a higher proportion of MSM were among the first patients in need of ARV therapy they therefore constitute a proportionally larger group with a longer exposure to therapy, which would increase the chances of new infections with a resistant virus in this group.

A similar scenario has been well described in other countries with a similar epidemiological profile [38, 39]. In our study, considering the limited number of pregnant women analyzed (n = 11), no resistance was identified; this is a positive finding with regards to the prevention of vertical transmission.

In relation to age, the prevalence was higher in individuals with more than 49 years and almost reach the level found for MSM, but has the limitation of the number of samples in this category; 25 which correspond for only 1 in each ten samples. In contrast, the category of individuals from 25 to 49 years correspond to the large majority of the

samples, 180 (73.8%) and as consequence the rate is close to the overall rate – 15.6% vs 16.0%.

The greatest difference was found when we analyzed resistance according to subtype. Surprisingly recombinants of subtype B did not show any level of resistance, which may be due to the reduced number of samples with that profile. For the group of samples containing CRF_02AG the absence of resistance could be explained by the origin of these samples. They could be from the African continent, from where we receive many immigrants in Brazil and where ARV treatment is not widespread, among samples with subtypes B and F the overall prevalence of resistance was similar, but differences in prevalence were found regarding resistance to specific drug classes. The findings described in this study reinforce the idea that HIV-1 viral diversity and primary resistance have become increasingly complex and it may be essential, at least in some regions of the country, to obtain primary resistance information to inform treatment decisions. A potential limitation of this study is related to the occurrence of false-recent classification in BED-CEIA that could occur in scenarios with high viral diversity.

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Table 1. Diversity in *pol* sequences obtained in four VCTs located in metropolitan area of Rio de Janeiro, Brazil.

	2005 Prevalent N (%)	**2005-2007 Incident N (%)**	**Overall N (%)**
OVERALL	106 (41,2)	151 (58,8)	257 (100,0)
B	89 (84,0)	117 (77,5)	206 (80,2)
F1	9 (8,5)	13 (8,6)	22 (8,6)
BF1	5 (4,8)	7 (4,6)	12 (4,7)
CRF_02AG	0 (0,0)	7 (4,6)	7 (2,7)
K recombinants	1 (0,9)	3 (2,0)	4 (1,5)
Other forms	2 (1,8)	4 (2,7)	6 (2,3)
Subtype			
Pure	98 (92,5)	131 (86,8)	229 (89,1)
Recombinant	8 (7,5)	20 (13,2)	28 (10,9)
Pure Subtype			
B	89 (90,8)	117 (89,3)	206 (90,0)
other than B	9 (9,2)	14 (10,7)	23 (10,0)
Recombinant			
URF	6 (75,0)	11 (55,0)	17 (60,7)
CRF (related)	2 (25,0)	9 (45,0)	11 (39,3)
MALE - FEMALE	59 (58,2) - 47 (41,8)	77 (51,0) - 74 (49,0)	136 (52,9) - 121 (47,1)
Subtype			
Pure	**57 (96,6)** - 41 (87,2)	**66 (85,7)** - 65 (87,8)	123 (90,4) - 106 (87,6)
Recombinant	**2 (3,4)** - 6 (13,8)	**11 (14,3)** - 9 (12,2)	13 (9,6) - 15 (12,4)
Pure Subtype			
B	51 (89,5) - 38 (92,7)	59 (89,4) - 58 (89,2)	110 (89,4) - 96 (90,6)
other than B	6 (10,5) - 3 (7,3)	7 (10,6) - 7 (10,8)	13 (10,6) - 10 (9,4)
Recombinant			
URF	2 (100,0) - 4 (66,7)	4 (36,4) - 7 (77,8)	6 (46,1) - 11 (73,3)
CRF (related)	0 (0,0) - 2 (33,3)	7 (63,6) - 2 (22,2)	7 (53,9) - 4 (26,7)
MSM - HETERO [#]	20 (33,9) - 39 (66,1)	32 (50,0) - 32 (50,0)	52 (41,8) - 71 (58,2)
Subtype			
Pure	19 (95,0) - 38 (97,4)	27 (84,4) - 28 (87,5)	46 (88,5) - 66 (93,0)
Recombinant	1 (5,0) - 1 (2,6)	5 (15,6) - 4 (12,5)	6 (11,5) - 5 (7,0)
Pure Subtype			
B	18 (94,7) - 33 (86,8)	24 (88,9) - 24 (85,7)	42 (91,3) - 57 (86,3)
other than B	1 (5,3) - 5 (13,2)	3 (11,1) - 4 (14,3)	4 (8,7) - 9 (13,7)
Recombinant			
URF	1 (100,0) - 1 (100,0)	2 (40,0) - 1 (25,0)	3 (50,0) - 2 (40,0)
CRF (related)	0 (0,0) - 0 (0,0)	3 (60,0) - 3 (75,0)	3 (50,0) - 3 (60,0)

Table 2. Distribution of relative frequency (%) of SDRM by class in sequences obtained in four VCTs located in metropolitan area of Rio de Janeiro, Brazil.

	N analysed (%)	any	PI	NRTI	NNRTI	NRTI + NNRTI	NRTI + NNRTI + PI
OVERALL	257 (100)	16,0	5,2	9,9	8,3	3,7	0,9
Time of Infection							
Recent (RS)	151 (58,8)	15,9	4,2	12,3	7,2	3,6	0
Long Term (LTS)	106 (41,2)	16,0	6,7	6,7	9,6	3,8	1,9
Over Time							
LTS in 2005	106 (41,2)	16,0	6,7	6,7	9,6	3,8	1,9
RS in 2005	57 (22,2)	19,3	5,4	16,7	7,4	3,7	0
RS in 2006	39 (15,2)	15,4	8,1	8,3	8,3	5,6	0
RS in 2007	55 (21,4)	12,7	0,0	10,4	6,2	2,1	0
Gender							
Male	136 (52,9)	16,2	4,5	10,2	9,4	4,7	0,8
Female	121 (47,1)	15,7	6,0	9,6	7,0	2,6	0,9
Sexual practice (men)							
MSM	52 (42,3)	25,0	5,9	12,2	14,3	4,1	0
Heterosexual	71 (57,7)	12,7	4,3	10,4	7,5	6,0	1,5
Pregnancy (women)							
Yes	11 (8,3)	0	0	0	0	0	0
No	110 (91,7)	17,3	6,7	10,6	7,7	2,9	1,0
VCT							
Madureira	54 (21,0)	16,7	9,4	13,2	9,4	7,5	1,9
Nova Iguaçu	116 (45,1)	13,8	5,3	8,3	7,4	3,7	1,0
Caxias	57 (22,2)	21,1	3,6	7,4	13,0	1,9	0
São Gonçalo	30 (11,7)	13,3	0	14,8	0	0	0
Age							
15 to 24 ys.	39 (16,0)	17,9	5,3	7,9	5,3	0	0
25 to 49 ys.	180 (73,8)	15,6	4,6	9,4	8,8	4,1	0,6
More than 49 ys.	25 (10,2)	24,0	12,0	22,7	13,6	9,1	4,5
Subtype							
B	206	17,5	6,1	11,3	8,7	4,1	1,1
F	22	18,2	0	9,5	14,3	4,8	0
Recombinants with B	14	0	0	0	0	0	0
AG	7	0	0	0	0	0	0

Table 3. Distribution of frequency (n) of each SDRM found in sequences obtained in four VCTs located in metropolitan area of Rio de Janeiro, Brazil.

	2005 LTS	**2005 RS**	**2006 RS**	**2007 RS**	**2005-2007 RS**	**Overall**
PI SDMR	10	4	3	0	7	17
L24I	1	0	0	0	0	1
D30N	1	1	0	0	1	2
M46L/I	2	0	3	0	3	5
L76V	1	0	0	0	0	1
V82L	1	0	0	0	0	1
I85V	1	0	0	0	0	1
N88D	1	1	0	0	1	2
L90M	2	2	0	0	2	4
NRTI SDMR	13	20	4	7	31	44
M41L	2	2	1	1	4	6
D67N/G	1	3	0	1	4	5
T69D	0	2	0	0	2	2
K70R	3	2	0	0	2	5
M184V/I	4	5	1	1	7	11
L210W	1	0	0	0	0	1
T215C/D/F/S/Y	2	3	2	0	5	7
K219R/Q	1	3	0	4	7	8
NNRTI SDMR	13	4	3	3	10	23
K103N	5	1	2	2	5	10
Y181C	3	1	0	1	2	5
Y188C/L	2	1	1	0	2	4
G190A	2	1	0	0	1	3
P225H	1	0	0	0	0	1
All SDRM	36	28	10	10	48	84

6 – Anexo I: Referente a proposta de Artigo 3

"Serological markers for improved detection of recent HIV-1 infections: Simple algorithms to reduce the occurrence of false-recent sample results in cross-sectional studies."

O artigo se relaciona com os objetivos específicos 4 e 5 e se encontra em fase de revisão pelo grupo de pesquisa visando futura submissão.

4) Comparar diferentes métodos sorológicos para a caracterização de infecção recente por HIV-1.

5) Propor um algoritmo para melhora da especificidade na identificação de indivíduos com infecção recente pelo HIV-1 a partir de métodos sorológicos e marcadores imunológicos específicos e não específicos.

Serological markers for improved detection of recent HIV-1 infection: Simple algorithms to reduce the occurrence of false-recent results in cross-sectional studies.

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Abstract:

Objective: This manuscript proposes a simple algorithm for HIV-1 incidence estimation that aims to minimize the impact of samples with discordant results and identify samples with false-recent results that are associated with disagreements between serological testing methods.

Methods: The strategy was based on two serological methods [i.e., the BED-CEIA and AxSYM HIV-1/2 group O Avidity Index (AI) tests] used to detect recently acquired human immunodeficiency virus type 1 (HIV-1) infection. This strategy included adopting a gray zone (G-Z) around previously proposed cut-offs and evaluating specific (T cell lymphocytes) and nonspecific (ß2-microglobulin) markers of immune status. Two scenarios were examined. The first was an actual scenario consisting of serum samples that were reactive to HIV antibodies collected consecutively from individuals visiting four voluntary counseling and testing (VCT) centers located in the metropolitan area of Rio de Janeiro, Brazil. The second scenario was created with a bias to evaluate the possible impact of the proposed algorithm in situations with different incidence rates.

Results: In the first scenario, the overall discordance determined from simple comparison of the two methods was 12.0%. This disagreement was reduced significantly ($P<0.0001$) to 4.7% by applying the G-Z criteria. Following evaluation of the immune status of the subjects, the proportion of indeterminate samples was reduced to 1.9%, which was a significant reduction ($P=0.027$) compared to the G-Z approach. In the second scenario, the disagreement values were 17.6%, 6.9% and 3.1%. As for the first scenario, this disagreement was significantly reduced following each new step of the algorithm ($P<0.0001$ and $P=0.0075$). The majority of the false-recent samples were detected using the BED HIV-1 Incidence Test (BED-CEIA method), specifically, 12 of 13 and 20 of 21 samples were misclassified in scenarios one and two, respectively.

Conclusions: Application of the G-Z criteria contributed to a significant reduction in the number of indeterminate samples when the BED-CEIA and AI tests were used to evaluate recent HIV-1 infections. The G-Z approach also proved useful when evaluating immune system status in samples with false-recent results. The data obtained suggest that the AI test is more specific than the BED-CEIA test. The algorithm proposed here exhibited no difference between the scenarios tested, for which different sample proportions were initially characterized as recent infections. The ability of this algorithm to reduce the proportion of indeterminate samples is likely associated with the proportion of individuals with late diagnoses in the given sample population, and the approach appears to be an option for scenarios with a high proportion of patients with late diagnoses.

Background

It is difficult to accurately differentiate individuals recently infected with human immunodeficiency virus (HIV) from those who have been infected for an extended period of time. Nonetheless, it is extremely important to make this differentiation when estimating HIV incidence, key information used to improve strategies for clinical treatment and prevention. Studies of recent infection rates are useful tools for determining the effectiveness of public policies aimed at controlling the HIV pandemic and for the prevention of disease spread. These studies may also identify early indicators of changes in the dynamics of the epidemic among affected populations[1]. A better understanding of HIV infection dynamics allows researchers and policy makers to infer recent trends in the epidemic, to monitor the viral population dynamics, and to analyze immune responses among seroconverted individuals to generate information relevant to vaccine development.

Under the classic design, the incidence of HIV infection is prospectively measured by monitoring seronegative individuals and conducting periodic retesting over time. Prospective cohort studies are difficult and costly to conduct and may be biased with respect to data analysis. Cohort membership, which is the process of obtaining patient consent and providing risk-reduction counseling, results in incidence estimates below the observed effective rates [2]. Studies involving cohorts are not adequade for the analysis of a representative sample of the general population, which is essential for regional and national estimates [3]. Several laboratory methods have recently been developed for the detection of recent infections in both pre- and post-seroconversion periods. Samples obtained during cross-sectional studies, including those collected to monitor infections and to determine prevalence, may be used for this purpose [3].

The period between infection and the onset of detectable specific anti-HIV antibodies is known as the acute phase of the infection. The duration of this phase, which is commonly referred to as the window period, can be short, i.e., approximately 10 to 20 days. Studies that aim to detect HIV infection during this period generally require large samples and/or the inclusion of individuals who are at an increased risk for HIV infection. In recent years, blood banks have introduced tests to cover this infection phase with the aim of screening blood and blood products more accurately. Molecular methodologies and antigen-specific detection (e.g., HIV-1 RNA or p24 Ag) are employed at this early stage [4-7]. These tests are more technically complex and expensive, limiting their large-scale implementation [3]. Moreover, these assays have less than optimal sensitivity [8]. Serological approaches have been developed and evaluated for testing HIV-positive individuals for recent infection. The small number of samples that need to be tested (only HIV + individuals) and the reduced technical expertise required to perform these methods make serological techniques more suitable for countries with limited resources or a large geographical area.

Some laboratories have invested in alternative approaches to detect recent infections [9, 10]. Based on the development of a humoral immune response (HIR), some tests use strategies that involve identifying certain epitopes or antigen-specific responses or determining the avidity/affinity of anti-HIV antibodies and the levels of serum antibodies [3, 9].

Parekh et al. [11] developed a competitive capture BED-EIA HIV-1 Incidence Test (BED-CEIA) to indirectly measure increases in the proportion of anti-HIV immunoglobulin G (IgG) antibodies. The inclusion of a chimerically branched synthetic peptide from the immunodominant region of the gp41 subunit of some HIV-1 subtypes (B, E and D) seeks to minimize the problems that plague less sensitive tests. Due to emerging concerns related to scenarios of high HIV prevalence and viral diversity [12], two correction strategies/factors

have been proposed to minimize the putative overestimation of incidence rates using BED-CEIA [13, 14].

The application of these correction factors has been discussed [15-20] and recently was presented by the WHO Technical Working Group on HIV Incidence Assays consensus, the need to establish false recent rates (FRRs) in representative groups of the broader population for which incidence rates are desired [21]. Among other tests developed to assess recent HIV infection, the avidity assay consists of a qualitative approach, in which antiretroviral treatment, low CD4-cell count and low viral load have no apparent effect on the AI [10]; furthermore, the avidity assay has been used in locations where non-B subtype HIV-1 infections have been observed [22].

Previous studies have compared two or more serologic methods, examining their sensitivities and specificities [23-28]. However, no study has addressed the problem of discordant sample results. The current study proposes a simple algorithm to minimize the occurrence of samples with discordant results by identifying samples with false-recent results that are associated with serological tests measuring the incidence of infection.

Methods

Study design

This study was designed to evaluate a simple low-cost algorithm to be applied to widely used methodologies to reduce discordance in serological tests and the occurrence of false-recent results among samples from recent cross-sectional studies that target new HIV-1 infections. The strategy is based on two serological methods for detecting recent HIV-1 infections. Specifically, the study adopted a gray zone (G-Z) based on previously proposed cut-offs. The study additionally evaluated specific and nonspecific markers of immune system status. The choice of serological methods used to detect recent HIV-1 infection was

intended to reflect a diversity of approaches, including those encompassing different sensitivities and targets. The methods were also chosen to include approaches that have been proposed and applied in previous studies. Based on these criteria, we chose to focus on the quantitative BED-CEIA method (Calypte Biomedical Corporation) and the qualitative avidity index (AI), which was calculated using a method based on an automated AxSYM HIV 1/2gO assay (Abbott Laboratories).

The application of the G-Z criteria is justified because these serological tests do not have the same ranges of results by which classifications are made, and variations in individual humoral immunity are known to occur. Thus, minor technical inconsistencies in mutually opposing directions may result in false disagreement between the proposed tests. For samples that remained discordant following application of the G-Z criteria, an analysis of immunological markers was conducted to exclude samples corresponding to long-term infections. Figure 1 summarizes the study design.

Study population

Two scenarios were evaluated in this study. The first scenario was composed of consecutive HIV-positive samples collected between November 2004 and October 2005 from individuals recruited from four voluntary counseling and testing (VCT) centers located in the metropolitan area of Rio de Janeiro, Brazil. This first scenario tested 10,011 individuals and identified 468 reactive samples. In the second scenario, 83 samples were identified (i.e., in addition to the initial 468 reactive samples) after being characterized as recent infections by the BED-CEIA method. Thus, a total of 551 samples were available for analysis. These additional samples were obtained at the same VCTs during subsequent years. This latter scenario was created with a bias to evaluate the impact of the algorithm on scenarios with

different incidence rates. The serological tests used to detect recent infection would have a negative correlation dependent on the actual incidence rate [15].

This study was approved by the Oswaldo Cruz Foundation (FIOCRUZ) ─ Evandro Chagas Clinical Research Institute Ethical Board (registration number CAAE-0032.0.009.000-04).

Serological testing algorithm

The serological assays for HIV-1 diagnosis followed the Brazilian algorithm. Further differentiation between recent and long-term infections was performed using the BED-CEIA protocol and the AI automated AxSYM HIV 1/2gO assay.

Calypte HIV-1 BED Incidence EIA (BED-CEIA)

Briefly, this assay is a quantitative *in vitro* EIA used to determine the relative proportion of HIV-1-specific IgG to total IgG levels. This assay can be used to estimate the elapsed time since an HIV-1 infection. The BED-CEIA assay uses a branched gp41 peptide with sequences derived from multiple HIV-1 subtypes (B, E and D) and achieves similar performance levels with different subtypes. Confirmed samples with normalized optical density (ODn) values less than or equal to 0.8 were classified as recent seroconverters (RS), and samples with ODn values above 0.8 were classified as long-term seroconverters (LTS).

Antibody avidity index (AI)

Antibody avidity index (AI) values for the recombinant HIV-1 Env and Gag proteins were calculated using the approach first described by Suligoi et al. [10]. Briefly, two aliquots of each sample were subjected to a pretest 1:10 dilution. One aliquot was diluted in phosphate-buffered saline (PBS), and the other aliquot was diluted in 1 M guanidine (G). Following incubation at room temperature for 10 min, both aliquots were subjected to an AxSYM

HIV1/2 group O immunoassay following the manufacturer's instructions. The sample/cutoff (S/CO) ratios of both aliquots were calculated, and the AI of the HIV antibodies was obtained using the following formula: AI=(S/CO G aliquot/S/CO PBS aliquot). Samples with AI values less than or equal to 0.85 were classified as low AI (LA), reflecting recent infection (<6 months since infection). Samples with AI values greater than 0.85 were considered high AI (HA).

Immunological status evaluation

Beta-2-Microglobulin (ß2-M)

The serum ß2-M concentration (mg/ml) was measured in plasma samples stored at -20°C using an enzyme-linked fluorescent assay (Vidas ß2-Microglobulin, BioMerieux, MarcyL'Etoile, France) according to the manufacturer's instructions. A panel containing RS and LTS samples was used to establish a cut-off value for distinguishing false-recent classifications.

$CD4^+$ T Lymphocytes

Immunophenotyping was performed using freshly collected ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood. A single platform method was used to measuring $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T-cell counts. In this method, 20 µl of single-color antibodies (CD4-FITC, CD8-PE, and CD3-PerCP) and 50 µl of whole blood were added to bead-containing TruCount tubes (BD Biosciences). The tubes were incubated for 15 min at room temperature, after which 450 µl of FACS lysing solution was added. The samples were analyzed after 1 hr using a FACSCalibur flow cytometer (BD Biosciences).

Determination and application of gray zone (G-Z) criteria

To determine the values of the G-Z criteria, the results of replicate samples with initial values near the cut-off values were considered for the BED-CEIA method. The replicates were conducted with samples fitting in the category of ODn values less than 1.2, which were confirmed in a subsequent test according to the manufacturer's instructions. The vast majority of samples exhibited coefficients of variation <5%, although there were rare outliers. As the objective of the study was to achieve the maximum specificity, borderline initial ODn values were considered to be those for which the samples' ratings remained unchanged when comparing the initial results and the respective replicates. We therefore defined the n-OD value G-Z criteria to be between 0.6 and 1.0. The AI analysis followed the same criteria, with the requirement for replicate measurements empirically determined as 10% of the cut-off AI (0.77 – 0.93 AI). Based on the replicate analysis, we defined the G-Z criteria as AI values between 0.80 and 0.90. The G-Z criteria were applied to samples with discordant results between the BED-CEIA and AI test methods when this disagreement did not exceed the maximum or minimum G-Z values.

Data analyses

Ratings were set for samples with concordant results using the evaluated test methods. The samples with RS and LA profiles were defined as being from individuals with a recent infection; samples with LTS and HA profiles were classified as coming from volunteers with a long-term infection. Discordant samples subjected to the G-Z criteria were classified as probable recent infection (i.e., samples with RS/G-Z or G-Z/LA profiles) or probable long-term infection (i.e., samples with LT/G-Z or G-Z/HA profiles). Discordant samples that did not fit within the G-Z criteria and those with a G-Z/G-Z profile were analyzed for their immune status by assessing $CD4^+$ T cell counts and/or ß2-M concentrations. A sample was

classified as false-recent if it exhibited an absolute CD4 + T cell count of fewer than 50 cells/mm3 or a ß2-M concentration greater than 4.00 mg/ml (defined using control sets with RS/LA and LT/HA profiles; data not shown). These strict criteria were used with the aim of excluding samples from individuals with AIDS and those who were incorrectly classified using one of the described methods. The remaining samples were classified as indeterminate. Figure 2 illustrates these analyses.

Results

In the first scenario, the concordance between the BED-CEIA and AI test methods was 88.0%, and this value was significantly higher (P=0.014) than findings from the second scenario, for which there was 82.4% concordance. Under the actual scenario (scenario one), which reflected one year of volunteers who sought testing at VCTs in the Rio de Janeiro metropolitan area, approximately 80% of all samples (equivalent to 90% agreement) were determined to be long-term infections. It was not possible to define the profile of 56 of the samples (or 12.0% of the total) due to disagreement between the methods. Among these 56 initially indeterminate samples, 34 fit within the G-Z criteria (60.7%). This proportion was similar (P=0.95) to the percentage of initially indeterminate samples that fit within the G-Z criteria for the second scenario (60 of 97 discordant samples, 61.9%). Among the samples subjected to the G-Z criteria, those classified as probable recent (i.e., RS/G-Z or GZ/LA) accounted for 38.2% and 40% of the total in scenarios one and two, respectively (P = 1.00). Of the samples that still had indeterminate classifications following this analysis, more than half were characterized as false recent results following quantitative analyses of $CD4^+$ T cell lymphocyte counts (<50 cells/mm3) and/or ß2-M concentration (> 4.00 mg/ml).

For this analysis, no significant differences (P=0.80) were observed between scenarios one (59.1%) and two (55.3%) following application of the G-Z criteria. Most of the samples that

were characterized as false-recent used the BED-CEIA test, specifically 12 of 13 samples (92.3%) for the first scenario and 20 of 21 samples (95.3%) for the second scenario.

Discussion

In a recent document published by UNAIDS [21], a novel term was coined to describe a laboratory assay or a combination of one or more assays and clinical information designed to classify HIV-1 infections as recent or long-term. This term, recent infection testing algorithm (RITA), was formulated to control for the primary issues responsible for the inconsistencies observed in previous studies using laboratory assays to estimate HIV-1 incidence. Some of the recommendations of this document were designed to enforce some aspects as the limiting conditions for sample collection, transportation and storage. Indeed, the presence of an AIDS-defining illness and/or a previous HIV diagnosis more than one year ago and/or having received HAART were considered supplementary clinical information that would "correct" the classification of these samples to non-recent [21]. In the study and proposal presented herein, we exclude these samples to avoid misclassification and unnecessary costs. The most significant achievement was addressing the need to determine false recent rates (FRRs) [21]. This need emerged following the presentation of substantial evidence that a proportion of long-standing infections are misclassified as recent infections, which can occur for various reasons. The primary factors underlying the ultimate accuracy of incidence rates are determination of the correct FRR for each population and the use of a minimum sample size. Locations certainly exist where ideal conditions and economic support allow for the evaluation of the FRR for each population. In these regions, more accurate incidence rates will be obtained. However, the majority of individuals affected by HIV live in locations where the economic reality is far from ideal, and application of the RITA as proposed by the UNAIDS report depends on external sources.

Our proposal aims to provide an option for minimizing the FRR without establishing its value. Thus, the proposed approach does not calculate incidence rates but instead provides information for comparing groups over time or in the same period. Some practical examples of this method include determining the ratio of new HIV infections between genders and evaluating the impact of HIV prevention measures in a specific group; to make these comparisons, this method uses the proportion of new infections in tested subjects. The RITA based on a combination of two assays is described as a method for lowering the FRR [21]. Our choice to use two methods in parallel with a G-Z was intended to prevent the exclusion of real recent infection samples.

Our data indicate that the initial concordance between the BED-CEIA and AI tests was relatively high (greater than 80%) in both scenarios. However, in at least one of the tests, this correspondence dropped to 63% among samples from individuals classified as recently infected.

The observation that 60% of the discordant samples were initially eligible for application of the G-Z criteria suggests that most of the identified inconsistencies may be explained by individual variations in humoral immunity, inconsistencies between the techniques or differences between the tests in terms of their respective ranges by which the results are classified. These factors may individually or collectively contribute to any observed disagreements.

Immunological markers, such as $CD4^+$ T cell lymphocyte counts and the $CD4^+/CD8^+$ ratio, offer little specificity in detecting recent infections when examined separately from serological test results. However, the use of immunological markers within defined criteria can be useful for detecting false recent results among individuals with late diagnoses and weakened immune systems. The use of a non-specific marker such as ß2-M has advantages over $CD4^+$ T cell counts, with the primary advantage being sample matrix quality. ß2-M

measurements can be performed with the same serum or plasma sample for both screening and incidence tests. It is therefore possible to perform this test for all of the samples in a cross-sectional study where the study population is not planned. Another advantage of the ß2-M test arising from the nature of the sample is that more accurate information regarding the status is immediately available. This advantage prevents the different sampling dates from having different windows (even if it is possible to determine $CD4^+$ T cell counts), which would make it difficult to compare the samples. In addition, sample collection may last a significant period of time, during which a given sample may develop a different profile (e.g., the initial sample may exhibit a recent infection profile, and the second sample may exhibit the profile of a long-term infection), resulting in an erroneous interpretation. The values proposed here regarding the use of immunological parameters to exclude false recent results were designed to have the highest specificity possible; however, the goal of 100% sensitivity was not attained. Therefore, it is likely that additional false incidents were not identified using the proposed algorithm. Because ß2-M is a non-specific marker, the results for this assay should be interpreted using defined clinical criteria. In studies where it is not possible to know whether the individual has chronic kidney disease or hepatitis, we assume that knowledge regarding specific biochemical and serological markers will not greatly impact the algorithm given that these tests are required for individuals with newly diagnosed HIV.

Extreme disagreements between methodologies are likely to arise from situations that are initially planned for but are uncommon. These disagreements should be observed as exceptions. To a lesser extent, such disagreements may not be accounted for in the descriptions of the tests because they are rare or difficult to observe. With respect to the BED-CEIA test, there are predictable, although infrequent, situations that will have varying degrees of prevalence according to the examined population. These situations include

possible variations in the total IgG titer and/or, more rarely, in the kinetics of specific anti-HIV-1 antibodies. Such a scenario would justify samples with a low ODn value and a high AI. In the case series studied here (i.e., scenario 2, the larger of two), and taking into account the G-Z criteria, these samples [AI> 0.90 (AI) test and an n-OD <0.6 (BED-CEIA test)] comprised 5.9% of the positive samples and likely came from individuals co-infected with another pathogen such as tuberculosis. Another possible difficulty is the eventual lack of reactivity in BED due to the different subtypes that are covered by the peptide used in the BED assay; this effect does not appear to occur with the AI test [22]. Furthermore, samples with the opposite discordant result [i.e., AI <0.80 (AI) test and an n-OD> 1.0 (BED-CEIA test)] may also be explained by rare events. For example, these results may arise from individuals who were super-infected, where one infection was more recent. In this situation, the proportion of specific antibodies (measured using the BED-CEIA assay) is high, and the avidity index would be smaller due to the observed heterogeneity (i.e., high avidity for the clone of the first infection and low avidity for the recent infection). In this study, the proportion of samples that fit this profile was rare. Only one of nearly 138 positive samples, approximately (0.7%), fit this profile. The values of the four samples with this profile were near the limit established by the G-Z (between 0.74 and 0.78 AI), which would be expected if super-infection had occurred. Given that we aimed to use a simple, rapid and inexpensive algorithm, these samples were classified as indeterminate.

Although the AI test has been standardized for recent infection samples, i.e., for individuals infected less than 6 months before the test, no study has estimated the window for the results of this test. We therefore cannot quantitatively estimate the impact of this calculation on the estimated incidence. Regardless, following the application of the algorithm proposed here, only one sample was classified as a false incident using the AI method. In fact, the picture changes when we simulate the same situation using the BED-CEIA test. When considering

the samples exhibiting initial concordance between the tests (RS and LA) and those referred to as probable recent (following the G-Z analysis) as recent infections, a significant difference was observed in the second scenario when comparing the conventional estimated incidence and the corrected incidence using the algorithm [3.98%/year (95% CI 3.35–4.60) *vs.* 2.71%/year (95% CI 2.19–3.23)].

Conclusions

The application of a gray zone contributed to a significant reduction in the number of indeterminate samples when the BED-CEIA and AI tests were applied for the evaluation of recent infection with HIV-1. The inclusion of other immunological parameters, such as $CD4^+$ cell counts and ß2-M in the remaining samples with discordant results (following the G-Z step) proved to be useful for identifying samples with false recent results. The data suggest that the AI test is more specific than the BED-CEIA test. The proposed algorithm showed no difference in scenarios with different proportions of samples initially characterized as recent infections. The ability of this algorithm to reduce the proportion of indeterminate samples is likely associated with the proportion of individuals with late diagnoses in the given sample population.

Competing Interests

None of the authors has any potential financial conflict of interest related to this manuscript.

Authors' contributions

CAVC, BG, VGV, FIB, JHP and MGM participated in the conception and design of the study; analysis and interpretation of data, drafting the paper and/or substantially revising it. All authors read and approved the final manuscript.

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Figure 1. Description of the study design with both scenarios analyzed and criteria adopted

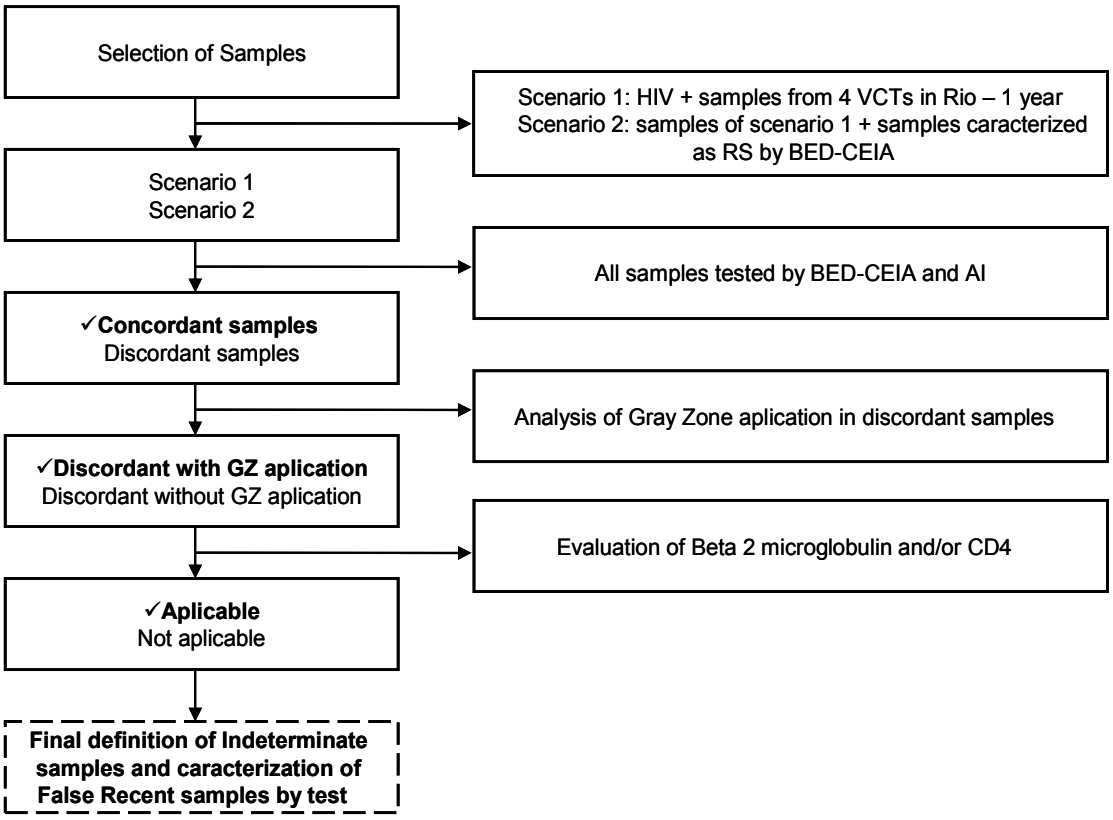


Figure 2. Chart of the algorithmic proposed with possible results and subsequent analysis and/or classification

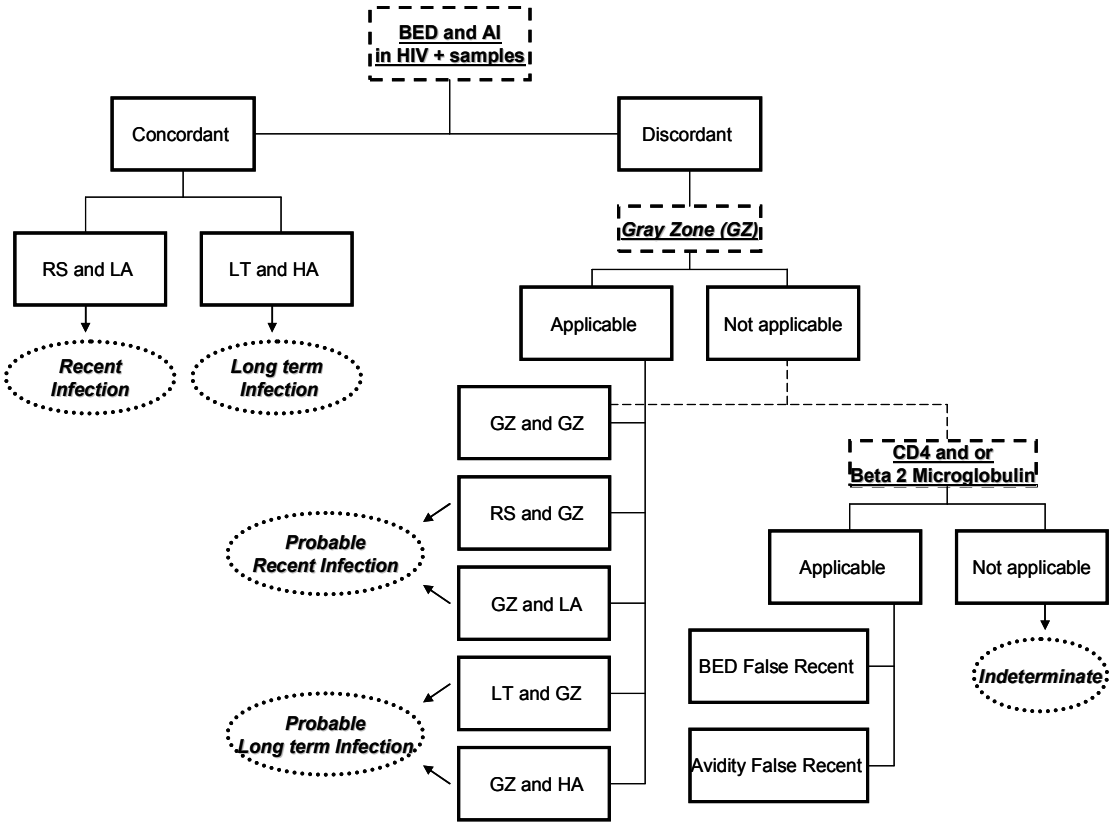
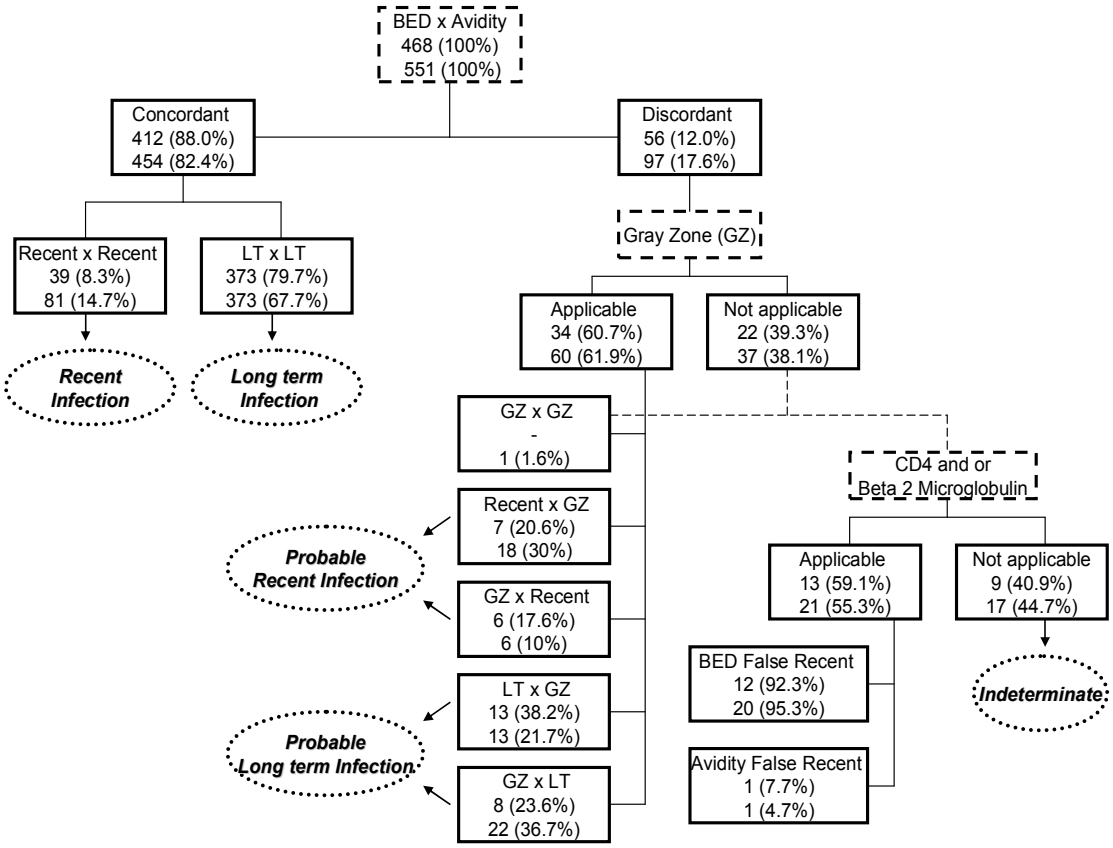


Figure 3. Description of the results in first (first data showed in each square) and second (second data showed in each square) scenario.



7 – Anexo II: Referente a proposta de Artigo 4

"Who attend voluntary counseling and testing sites in Rio de Janeiro metropolitan area? Insights from two major VCT centers, 2005-8."

O artigo se relaciona com os objetivos específicos 1, 2 e 3 e se encontra em fase preleminar de elaboração visando futura submissão.

1) Determinar a prevalência e a incidência da infecção por HIV na população estudada;

2) Comparar a prevalência e a incidência da infecção por HIV ao longo do estudo;

3) Caracterizar o perfil sócio-demográfico na população estudada (casos incidentes, prevalentes e soronegativos).

Who attend voluntary counseling and testing sites in Rio de Janeiro metropolitan area? Insights from two major VCT centers, 2005-8.

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ABSTRACT:

Aims: In this study, we propose to study over time (2005-2008) the population profile and risk factors for HIV infection in two VCTs located in the metropolitan region of Rio de Janeiro state. We also evaluate the occurrence of new HIV infections targeting detect eventual trends in populations tested in these cities.

Methods: Serological assays for HIV-1 diagnoses followed the Brazilian algorithm. Further differentiation between recent and long-term infections was performed using the BED-CEIA protocol. To evaluate association, data were assessed by a decision tree algorithm through the Chi-squared Automatic Interaction Detector (CHAID).

Results: During the study period, the return rate of customers regardless of VCTs request was 1.68% and five seroconversions could be observed, four of them among MSM. The VCT (A) – in the capital received more male customers and a higher proportion of MSM that the VCT (B) – in the outskirts, where there was a larger number of pregnant women being tested. In general, the VCT (B) received a higher proportion of customers younger, unmarried, nonwhite, with lower risk perception, most individuals who reported having had STD in the year prior the colection, fewer proportion with multiple partners and a lower rate of condom use. Motivating for testing, age, STDs, sexual practice for men and time of formal education were the factors more frequent for higher prevalence of HIV infection and the rates were always higher in VCT (A).

Conclusions: In VCTs the prevalence of HIV infection was related to the composition of the population served and in our study was very different even though they were in the same metropolitan region. Although in the capital was found consistently higher prevalence rates, in the periphery are also high and the low consistently use of condoms compose a scenario conducive to more infections. Prevention campaigns should focus on MSM, prevention of STDs in general and seek to intensify efforts in the periphery, in individuals with less education and the importance of retesting as a tool for early diagnosis.

Background

The World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS) have established that early diagnosis of HIV infection is a key component of any initiative aiming to guarantee the right of individuals to access treatment and curb the epidemic, and may reinforce different behavioral interventions promoting safer behaviors in affected communities worldwide [1].

Among these initiatives, the prompt diagnosis of HIV infection, counseling and prophylaxis of vertical transmission of HIV remains a pivotal initiative to be fully implemented yet in many low and middle-income countries [2, 3].

However, according to evaluation issued in 2008 by WHO and UNAIDS, the quality of sero-surveillance in low and middle-income countries still have poorly functioning surveillance systems. The inclusion of HIV testing in national population-based surveys in recent years has resulted in some countries with generalized epidemics receiving higher coverage scores, but many countries with concentrated or low-level epidemics continue to lack data on high-risk populations [4].The comprehensive implementation of voluntary counseling and testing centers (VCTs) has been an important tool adopted in Brazil and many other countries to foster testing for those who want to know their serostatus and receive group and individual in a private and user-friendly environment, for patients referred by community services and other health professionals, and, in some countries such as Brazil, to provide testing and counseling for pregnant women, ideally in a concerted effort with units and/or health professionals providing prenatal care [5, 6]. In a recent review, Guy e cols conclude that data routinely collected from VCT sites can be used to evaluate the impact of public health interventions, but attention to methodological issues will maximize their value for evaluation purposes [7].

In Brazil, the survey of knowledge, attitudes and practices in people from 15 to 64 years of age (PCAP) evaluates the sexual behavior of the Brazilian's citizens. The survey is conducted nationwide and the main objective is to monitor the performance indicators of the Department of STD, AIDS and Viral Hepatitis, especially with regard to the prevention of situations of vulnerability to HIV infection and other sexually transmitted diseases. The PCAP, released in June 2009, was conducted during 2008 in all regions of Brazil and interviewed 8,000 people 15-64 years of age. The survey found that young people demonstrate that they have safer sexual behavior. Moreover, it revealed that the main behavioral differences are between men and women. Among men, 13.2% had more casual partners in the five years preceding the survey, among women, this rate is three times lower (4.1%). One in ten men had at least one partner of the same sex in life, while only 5.2% of women have had sex with other women. For men the introduction in sex life also starts earlier - 36.9% had sex before aged 15, for women the index falls by more than half, 17%. The research also features for education and by region. In both cases, there were no statistically significant [8]. In relation to testing, one study describes changes between 1998 and 2005; when 20.2% and 33.6% of interviewees had been tested, respectively. Six in each ten women aged 25-34 years were tested. In general there was no significant increase in testing among men over time. Testing rates did not increase in those who self-reported as being at high risk for acquiring HIV. Among women, prenatal testing rate increased while work-related testing decreased among men. In 2005, half of those who were tested did not receive any counseling before or after testing [9].

An evaluation of strategies to foster the descentralized response to AIDS suggests that the municipalization policy contributed to improve local response to AIDS [10]. Rates of HIV prevalence are significantly higher in the clientele of VCT than those observed in the general population [11-14]. In general, these studies were conducted in different cities (most of them

in the capitals of Brazil major states) and were cross sectional studies. As a result, data from trends in populations tested in VCTs are not available until now, nor comparison between VCTs located in major cities like capitals and others cities in same metropolitan area.

In this study, we propose to study over time (2005-2008) the population profile, risk factors for HIV infection in two VCTs located in metropolitan region of Rio de Janeiro state (one in capital – Rio de Janeiro city and other in a peripheral city – Nova Iguaçu). We also evaluate the occurrence of new HIV infections (less than six months prior sample collection) targeting detects eventual trends in populations tested in these cities.

Methods

Study design and population

In 2004, we began to conduct a prospective study in three VCTs located in Municipalities of metropolitan region of Rio de Janeiro state (one in the capital [Rio de Janeiro city] and two in the outskirts of the state [located in Nova Iguaçu and Duque de Caxias cities]) [14] . Analysis of these results for each VCT suggested a similar profile between the two VCTs located in the outskirts, but different from the VCT located in the capital. For this study, targeting to compare the profile of a VCT in the capital and another in the periphery, the periphery center was selected based on three criteria: coverage area, number of collections and best proportion of samples with results available and satisfactory data collection. In 2004, Nova Iguaçu VCT was better than Duque de Caxias VCT in all criteria. The VCT of the capital was followed by three consecutive years (2005-2007) while the VCT Nova Iguaçu for four years (2005-2008), the VCTs was here named VCT (A) and VCT (B) respectively. Samples without result of HIV testing and/or no data available were excluded. A search was conducted to find volunteers who returned to VCTs during the study period (based on a mach with VCT general number, gender and date of birth - with day, month and

year of birth). The multiple entries following the first visit were excluded from the database for analysis and the characteristics of these individuals will be described separately. Figure 1 summarizes the study design. To be enrolled, individuals were counseled and tested, those defined by the guidelines of the Brazilian Ministry of Health (BMoH), irrespectively of their answers (or the absence of answers) to the standard short form used as a routine procedure in the VCT centers. The study was approved by the Oswaldo Cruz Foundation (FIOCRUZ) ─ Evandro Chagas Clinical Research Institute Ethical Board, registration CAAE-0032.0.009.000-04.

Serological testing algorithm

Serological assays for HIV-1 diagnoses followed the Brazilian algorithm. Further differentiation between recent and long term infections was performed using the BED-CEIA protocol (Calypte Biom Corp, USA) according to the manufacturer's instructions. Briefly, this assay is an *in vitro* quantitative enzyme immunoassay for the determination of the proportion HIV-1 specific IgG with respect to total IgG as a tool to estimate the elapsed time since HIV-1 infection. The BED-CEIA uses a branched gp41 peptide with sequences derived from multiple subtypes (B, E and D) and achieves similar performances with different subtypes [15].

Data Organization

Data obtained by standard short form were filled in a SPSS file for further CHAID analysis. Socio-demographic variables were: sex, presence of pregnancy for women, age, current marital status, race and education. Information related with behavior were: sexual practice for men, motivation for testing, origin of clientele, use of drugs in last year – considering the date of the visit in VCT center, sharing needles – lifetime and number of sexual partners in

last year. As clinical variable, the occurrence of sexual transmitted diseases (STDs) was asked. Aiming a better understanding and distinguishing feature of any differences between populations tested, the variable motivation for testing had the answer choices grouped according to the characteristics of these responses: spontaneous with risk perception (exposure to risk, window period), spontaneuos relative to prevention (knowledge of HIV status, prevention), suggested by health service or health professional (blood bank, clinical recovery addicts, tuberculosis, STD clinics, symptoms related to HIV/AIDS, other health services), utilization of public health service (admission to employment/military, check previous result, pre-natal, pre-nuptial, pre-operative).

Data Analysis

Chi-squared Automatic Interaction Detection

Chi-squared Automatic Interaction Detection (CHAID) is a method of tree classification originally proposed by Kass in 1980 [16]. CHAID is a non-parametric analysis based on a relatively simple algorithm that is particularly well suited for the analysis of larger datasets. The splitting method use chi-square and F tests. The branch limitations are the number of values of the input and the pruning uses p-values. A CHAID tree is a classification tree that is constructed by repeatedly splitting subsets of the space into two or more child nodes, beginning with the entire data set. To determine the best split at any node, any available pair of categories of the predictor variables is merged until there is no statistically significant difference within the pair with respect to the target variable. To identify the nodes with a relatively high probability, a gain chart was constructed showing the nodes sorted by the number of cases in the target category for each node. For the current analyses, the endpoint variable was the HIV infection. The resulting groups were split until the following criteria were reached – tree depth

was limited to ten levels, no group smaller than 100 was split, no group smaller than 50 was formed, and an alpha level for all statistical tests was 0.05, all statistical analyses were performed using SPSS Answer Tree 16.0 software (SPSS Inc. Chicago, Il, USA). A CHAID tree for each VCT was performed, as well as tables showing statistic significant results.

Trends in HIV-1 infection

After CHAID analysis, the related nodes were compared based in the number of overall individuals tested – of each node – and the number of recent infection characterized by BED-CEIA. Chi-square or Fisher tests (according on sample size) were done to evaluate recent trends. Alpha level equal or below 0.05 was considered significant.

Results

Return Clients

During the study period, the return rate of customers regardless of VCTs request was 1.68% - 377clientes, the vast majority of these being over one (90.5%) or two (7.1%) more samples. According to gender, women had lower prevalence of return to VCT for new testing (0.66% vs. 1.65% for pregnant and non pregnant women). Of the 59 pregnant women who returned, 23 underwent further testing during the same pregnancy while 36 (61%) return further testing in subsequent pregnancy. Among men, the return was equivalent between heterosexuals and MSM – 2.46% and 2.54% respectively. Most customers place the new testing within 1 year after the first collection - about 10% within three months between collections, 15% between 3 and 6 months and 30% between six months and one year. Approximately 30% returned between one and two years, and 14% between 2 and 3 years. About 14% (20 of 145) of clients who reported exposure to risk situation in past or recent

(window period) at the first visit to the VCT, reported that the new collection was motivated by prevention. Twelve clients (8.3%) stated the need to confirm the previous result, a customer returned by symptoms associated with HIV/AIDS, another on suspicion of STD and the vast majority of these (73.1%) reported a new exposure to risk situation. Furthermore, 30% (23 of 77) of those who reported being motivated by prevention in the first time had motivated his return from exposure to a risk situation. Five seroconversions could be observed, with four of them among MSM (one with less than three months between collections, one between 3 and 6 months, one from 6 months to 1 year and one between 1 and 2 years between the two collections) and one in a non pregnant woman (1 year between samplings). All individuals who seroconvert reported exposure to risk situation in both samples.

Comparison between profile from VCT (A) – capital and VCT (B) – periphery

A total of 22,727 customers were included in this study. In the VCT (A), 2,624 customers were seen in 3 years of follow up (mean of 874 and median of 828 per year). During four years in the VCT (B), 20.103 customers (mean of 5,025 and a median of 4.859 per year) were tested and counseling. The socio-demographic profile and behavior of customers were different when compared the VCT (A) with VCT (B). Among the variables analyzed, the only one where there was no statistically significant difference was related to drug use in the last year from the date of collection, p=0.95. The VCT (A) received more male customers and a higher proportion of MSM that the VCT (B), where there was a larger number of pregnant women being tested. In general, the VCT (B) received a higher proportion of customers younger, unmarried, nonwhite, with lower risk perception, most individuals who reported having had STD in the past year, fewer proportion with multiple partners and a lower rate of condom use (table 1).

Risk Factors for HIV infection

VCT (A) - Capital

For the VCT in the capital, the overall prevalence of HIV infection was 11.3%. The CHAID tree obtained was composed by 16 nodes with nine terminal nodes and a depth of four levels (figure 2). At the first level, the variable obtained was the motivation for testing and the four possibilities were divided into three groups. The group tested as suggested by health service or health professional has the bigger prevalence for HIV infection - 21.8%, while those who tested for prevention or use of the structure had 5.6%. The third group - volunteers at risk (11.1%) was the one where we obtained the most depth in the CHAID tree. The variables in order were: number of partners in last year, gender and sexual behavior and finally the education. Thus, the highest prevalence found among the volunteers with risk perception was among MSM with education less than 7 years and with more than one partner in the year preceding the date of the survey - 27.8%. In contrast, those who sought testing after a suggestion by a health professional had a depth of just one level - gender and sexual behavior – Between those, 40% of prevalence of HIV infection was observed for MSM *vs*. 18.55% against the set formed by straight men and women in general – pregnant or not. Among the individuals that search testing for prevention or use the structure the prevalence for HIV infection obtained was higher among individuals older than 24 years - 7.2%, while those under 25 was 1.8% - being 4.5% among men and no infection was observed among the 97 women in this group. Table 2 summarizes the terminal nodes obtained.

VCT (B) - Outskirts

For the VCT in the periphery, the overall prevalence was 3.8%. The CHAID tree obtained was composed by 48 nodes with 26 terminal nodes and a depth of six levels. At the first

level, the variable obtained was the motivation for testing and the four possibilities resulted each one in a new group in the first level. Due to the size (difficulty in visualization) and the complexity of this tree, we chose to separate the original tree in four other considering each possibility obtained in the first level (motivation for testing). The group with the highest prevalence of HIV infection was formed by users who sought testing after suggestion of a medical doctor or health services - 12.3%, this group was also where there was a greater depth - five levels. The first level was the occurrence of STDs in the last twelve months prior to collection. Those who reported STD in the previous year, the frequency of HIV infection was 28.9% - the highest rate was found among MSM with 61.5% and the lowest among heterosexual men aging 15 to 24 years with 2.9%. Among those who reported not having had an STD in the last year, the prevalence of HIV infection was 9.2%. In this group the lowest rate found was 7.2% among non-pregnant women and was higher between those that were unmarried aged from 25 to 49 years old with less education - 19.4%. The highest rate was found among MSM - 26.9% being 10.9% among MSM who reported having one partner in the last year against 43.4% among those who had more than one partner (figure 3). In the periphery VCT the second most affected group was that formed by users that search test spontaneously with risk perception - 7.3%. Among older individuals (above 24 years) the prevalence was about 3 times higher than among younger (15 to 24 years), 9.0% *vs* 2.9%. Among the younger who were married, the prevalence was approximately 6 times higher than in the group formed by single, separated or widowed - 8.5% *vs* 1.4%. Among the olders, the prevalence was higher among those with less education (figure 4). The third group, formed by users who sought testing for prevention the prevalence was 3.6%, with the highest rate among MSM and the lowest (0.9%) between the group formed by straight men, pregnant women and non-pregnant women who were between 15 and 24 years. Among the olders (over 24 years) in this group, the frequency of HIV infection was higher among those

who reported STD episode in the past year (Figure 5). The largest group in this VCT (54.1%) was formed by users who made as utilization of public health services and had the lowest prevalence rate – 0.8%. Between these volunteers, the group with lower education had more prevalence of HIV infection than the users with more than 7 years of study – 1.1% *vs.* 0.5% respectively. In the group with more education was observed more infections in people with a episode of STD (1.9%) in the year prior the sample collection that the subjects that didn't have any STD in the year before the test (figure 6).

Trends in HIV-1 infection

For samples included in CHAID analysis, in the VCT (A) 17.07% of the samples tested were clasified as RS *vs* 15.73% of VCT (B) [P=0.63]. For both VCTs significant difference was found in motivation testing (P <0.01), with a greater frequency of newly infected individuals among those who sought testing by the suggestion of a health professional. In the VCT (A) was identified one recent infection with HIV-1 among approximately 31 people tested in this category while in the VCT (B) were 64 individuals, while those who seek tested using the public infrastructure the ratio was nearly 842 tested for each recent infection found. In the VCT located in the capital, difference was found among those volunteers who had perception of risk and had more than one sexual partner. For this group, between MSMs tested, the proportion was found about 23 people for each infection recently found, while in the group formed by heterosexual men and women in general - pregnant or not pregnant - the ratio found was close to 72 volunteers tested.

In the VCT located in the periphery, five other subgroups had a statistically significant difference detected. In individuals who sought testing after the suggestion of a health professional was detected difference between having or not had at least one episode of STD in the previous year of the VCT visit - 18 in 510 for those who had STD *vs* 32 in 2.707

without DST. For this last subgroup, difference was found among MSM, non-pregnant women and the group formed with pregnant women and heterosexual men. In the group of those who reported seeking testing for prevention, MSM had a recent infection frequency about ten times greater than in the group of heterosexual men and women in general - pregnant or not - about one recent infection for every 22 *vs*. 221 respectively.

In this last group, among individuals with more than 24 years, the occurrence of STDs was determinant for the frequency of recent infections is four times higher than among those who reported no STDs in this group. Overall individuals tested within each subpopulation, as well as their recent infections detected and P values are described in Tables 4 and 5 for VCTs (A) and (B) respectively.

Discussion

Although this kind of data is not available and a comparison cannot be done, the evaluation of the return of customers to VCTs can be generally considered low (less than one in 50 customers). In general, concerns with health indicate a major zeal among women, however; it was among men who observed a higher rate of return for further testing. This finding might possibly be a result of a higher exposure to risk compared with women after counseling done at the first visit to the VCT since four of the five cases of seroconversion were detected among men (approximately one in 36 men retested while among women was found one case in 192 who returned for another test). Among men, although the frequency of return has been equivalent between MSM and heterosexual men, the four cases of seroconversion were found among MSM in a sample of 25 returns. This difference would be explained by the HIV status of partner (all reported that the new risk exposure was sexual intercourse) and/or type of sexual contact [17].

Our results indicate that the use of VCT as a place of testing for pregnant women in the periphery results more testing in young and married women than in the capital and the rate among those who use the public infrastructure for the testing was ten times higher and the majority are coming from another health service. In addition, a higher proportion of nonwhite and less formal education individuals were found in the periphery, where these features are usually found more frequently and are associated with poorer populations. This profile can be reinforced when we observed that only eight percent of those tested in the suburbs seek testing spontaneously and as a result of risk perception. So, indeed, the periphery of the VCT is characterized more as an ancillary service structure of public health - called basic VCT - than as a center for testing of spontaneous [18].

As a feature common to both studied VCTs would be highlight the low frequency of drug users in these services, which could eventually mean that the drug user does not recognize the VCT as a suitable place for their reception and testing, although this population has been classified as priority for VCTs [18]. Thus, the results obtained here should be considered in the context of essentially sexual transmission. Another common sign is the low consistent of condom use; about half of the individuals tested reported that never used condoms with steady partner and about one in three never made it with an eventual partnership. These data suggest that although it was detected an increase in condom use among young people [8], this issue is far from being resolved and should always be targeted by prevention campaigns for individuals of all ages.

Our results concerning the analysis of risk factors for HIV infection, revealed that in both VCTs, 15% of the individuals sought to test after the suggestion of a health professional and that within this group the prevalence was twice that the one found among those who tested on a voluntary basis with a perception of risk, in others words, the detection of HIV infection is still late and dependent more from health professionals than risk perception by the

individual. Our data proved to be consonant with the literature where a remarkable number of studies have undertaken link STDs and HIV [19, 20]. In VCT on the periphery, we observed that the group that had both factors and have sought testing after medical suggestion, prevalence was above 60%. For this group the chance to have tested positive serology for HIV was greater than to be negative, which illustrates the relationship of these factors with higher rates of HIV infection and the potential of the combination of both. The age range where there was a higher frequency of HIV infection was always the one made by users from 25 to 49 years; however the younger – between 15 and 24 years – were those with the lowest prevalence rate, following the logic of historical cohort where sexually active older individuals were probably more at risk.

For those tested with 50 years or more, a well-defined pattern was not observed, in some cases they were grouped with the younger and therefore with lower rates and sometimes aligned with those of the range of 25 to 49 years formed the group with the highest rates. This inconsistency can be explained by a change in profile in this age group, where there was an increase in rates in recent years [21]. In the general scope, less schooling was related to higher rate of HIV infection, which is in agreement with the more contemporary profile described in our country. Nevertheless, an exception was observed. In the VCT of the periphery, among MSM which reported no recent episode of STD and tested after the advice of health professionals, which would be more compatible with the initial characteristic of the epidemic in our midst. Although no significant difference ($P = 0.35$) in relation to recent infections when education was assessing in this group, the results suggest that this difference may be due to late diagnosis, once only one recent infection was detected in 55 individuals with more than seven years of formal education, while three recent infections were found in 53 people with education less than 8 years.

In this study we propose an alternative way to analyze the recent infections, observing if new infections indicate a possible change of scenario in the context of the subpopulations studied with difference detected by CHAID. No significant differences contrary to what was observed in the initial analysis done by the CHAID was found, suggesting that with the population studied was not possible to detect any major change seems to happen in the near future. On the other hand, some differences in the same direction as that found by CHAID were observed, suggesting that this difference was recently determined or - more likely - means that this difference has been maintained over time and that to date no indication of change in the future will occur. This scenario was observed in both VCTs about the motivation for testing - which reinforces the needs of a description of the population served in these VCTs for proper analysis of results. Other situations where this profile was found were always related as factors either recent reporting of STDs or the group of MSM, further reinforcing that these factors have always been and continue to be risk factors for HIV infection. For groups where no statistically significant difference was found we impose restrictions on sample size of some subgroups generated after analysis by CHAID. Notwithstanding, some potential trends would be observed, as a statement that the consolidation between the group with more formal education, the proportion of new infections has been consistently lower than those with less formal education and that younger individuals have proportionally fewer recent infections in the total number of individuals tested than those who are between 25 and 49 years. Although this data is in line with the results of prevalence rates, reveals that even after the onset of sexual activity, some of the new infections will occur few years later - which suggests that prevention efforts related to these specific individuals could in theory avoid part of future infections.

Conclusions

In VCTs the prevalence of HIV infection was related to the composition of the population served and in our study was very different even though they were in the same metropolitan region. Although in the capital was found consistently higher prevalence rates, in the periphery are also high and the low consistently use of condoms compose a scenario conducive to more infections. Prevention campaigns should continue to focus on MSM, prevention of STDs in general and seek to intensify efforts in the periphery, in individuals with less education and the importance of retesting as a tool for early diagnosis.

Competing Interests

None of the authors has any potential financial conflict of interest related to this manuscript.

Authors' contributions

CAVC, BG, VGV, NB, JHP, MGM and FIB participated in the conception and design of the study; analysis and interpretation of data, drafting the paper and/or substantially revising it. All authors read and approved the final manuscript.

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Figure 1. Description of the study design. Absolute and relevant relative proportion of samples tested and/or analyzed in each step is shown as well as missing samples.

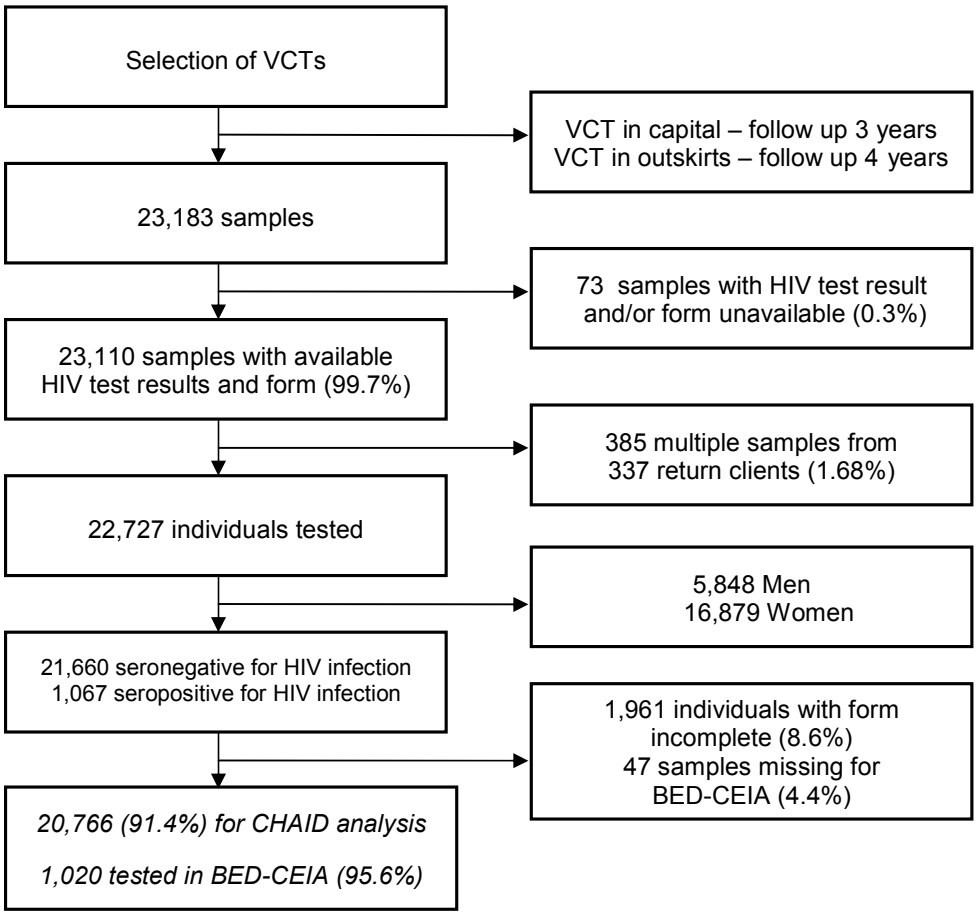


Table 1. Comparison of socio-demographic and behavior profile from populations seeking for HIV testing in two VCTs located in Rio de Janeiro metropolitan area from 2005 to 2008

	VCT capital (A) N (%)	VCT outskirt (B) N (%)	P value
Socio-demografic			
Sex			
Women	1,153 (43.9)	15,726 (78.2)	< 0.0001
Men	1,471 (56.1)	4,377 (21.8)	
Pregnant (women)			
Yes	116 (10.1)	8,727 (55.5)	< 0.0001
No	1,037 (89.9)	6,999 (44.5)	
Age, y			
15-24	719 (27.4)	7,006 (34.9)	< 0.0001
25-49	1,604 (61.1)	11,066 (55.0)	
+ 49	301 (11.5)	2,031 (10.1)	
Current marital status			
Married	911 (38.5)	12,937 (64.5)	< 0.0001
Divorced	186 (7.9)	1,310 (6.5)	
Never married	1,187 (50.1)	5,215 (26.0)	
Widowed	84 (3.5)	602 (3.0)	
Race			
White	663 (64.5)	4,486 (38.5)	< 0.0001
Browns	196 (19.1)	4,700 (40.3)	
Black	169 (16.4)	2,479 (21.2)	
Education, y			
0-7	976 (41.3)	9,086 (45.4)	0.0002
> 7	1,388 (58.7)	10,953 (54.6)	
Behaviour			
Sexual practice (men)			
Heterosexual	1,115 (75.8)	3,751 (85.7)	< 0.0001
MSM	356 (24.2)	626 (14.3)	
Motivation for testing			
Spontaneous, with risk perception	1,455 (56.2)	1,329 (8.0)	< 0.0001
Spontaneous, relative to prevention	585 (22.6)	4,051 (24.2)	
Suggested by health service/professional	386 (14.9)	2,888 (17.3)	
Utilization of public health service	163 (6.3)	10,431 (62.5)	
Origin of clientele (discovery of VCT service)			
Health service/professional	624 (27.0)	15,378 (77.5)	< 0.0001
NGO, Friends or VCT client's	1,035 (44.8)	3,065 (15.4)	
Disclosure in all media	650 (28.2)	1,410 (7.1)	
Use of drugs last year			
Yes	29 (1.1)	231 (1.1)	0.95
No	2,595 (98.9)	19,872 (98.9)	
Sharing needles (lifetime)			
Yes	46 (1,8)	88 (0.4)	< 0.0001
No	2,578 (98.2)	20,015 (99.6)	
No. sexual partners past year			
0	466 (17.8)	1,318 (6.6)	< 0.0001
1	1,134 (43.2)	15,181 (75.5)	
2-4	669 (26.6)	3,029 (15.0)	
>4	325 (12.4)	575 (2.9)	
Condom use: frequent partner			
Always	329 (24.1)	1,373 (7.6)	< 0.0001
More than half exposes	52 (3.8)	2 (0.0)	
Less than half exposes	345 (25.2)	6,113 (33.8)	
Never	640 (46.9)	10,578 (58.6)	
Condom use: casual partner			
Always	339 (36.4)	1,029 (26.8)	< 0.0001
More than half exposes	30 (3.2)	2 (0.0)	
Less than half exposes	248 (26.6)	1,432 (37.4)	
Never	315 (33.8)	1,372 (35.8)	
Clinical			
STD last year			
Yes	155 (5.9)	1,613 (8.0)	0.0002
No	2,469 (94.1)	18,490 (92.0)	
Overall	2,624 (100)	20,103 (100)	-

Figure2.CHAID tree of VCT (A)

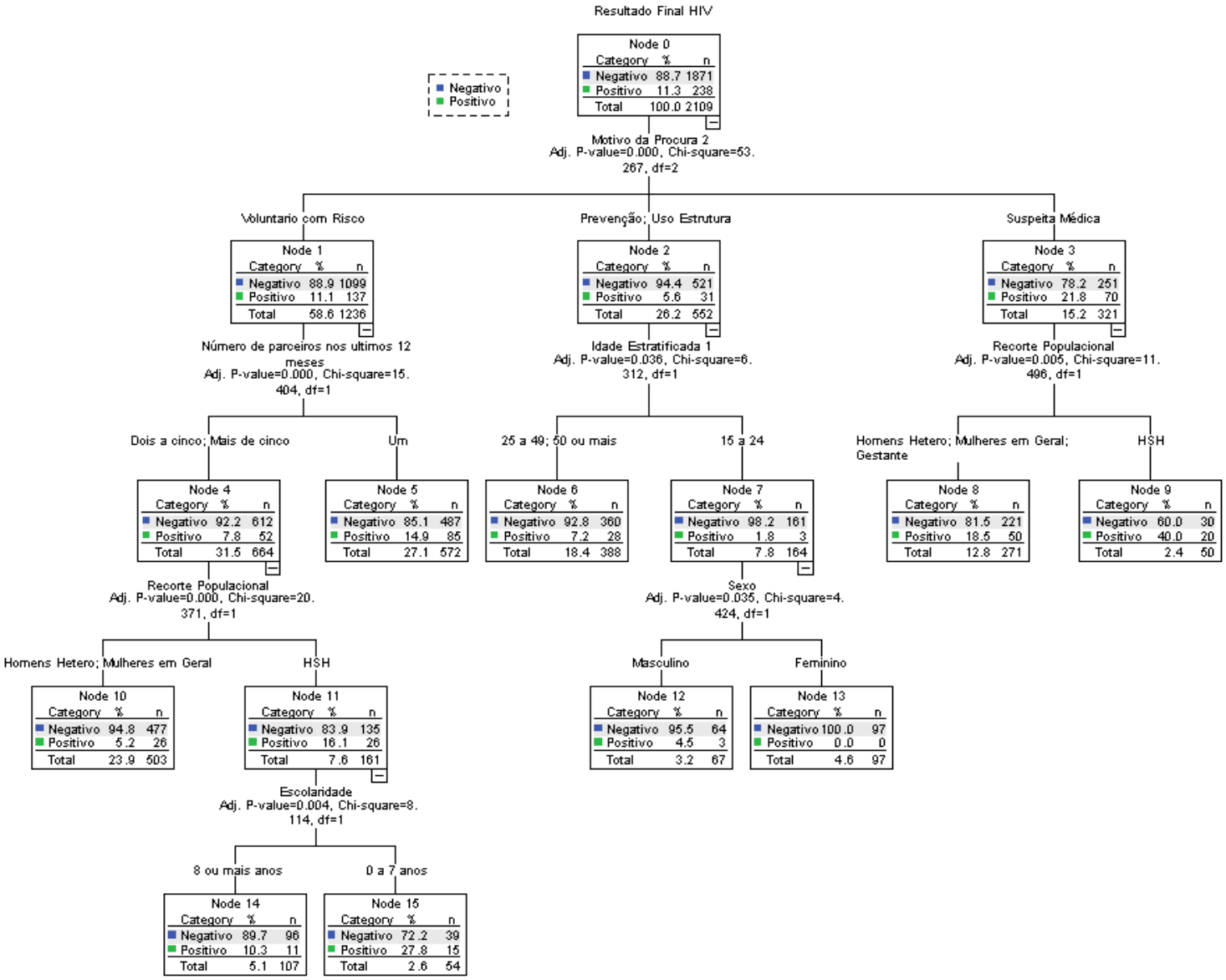


Figure 3. CHAID tree for users tested after health professional suggestion in VCT (B)

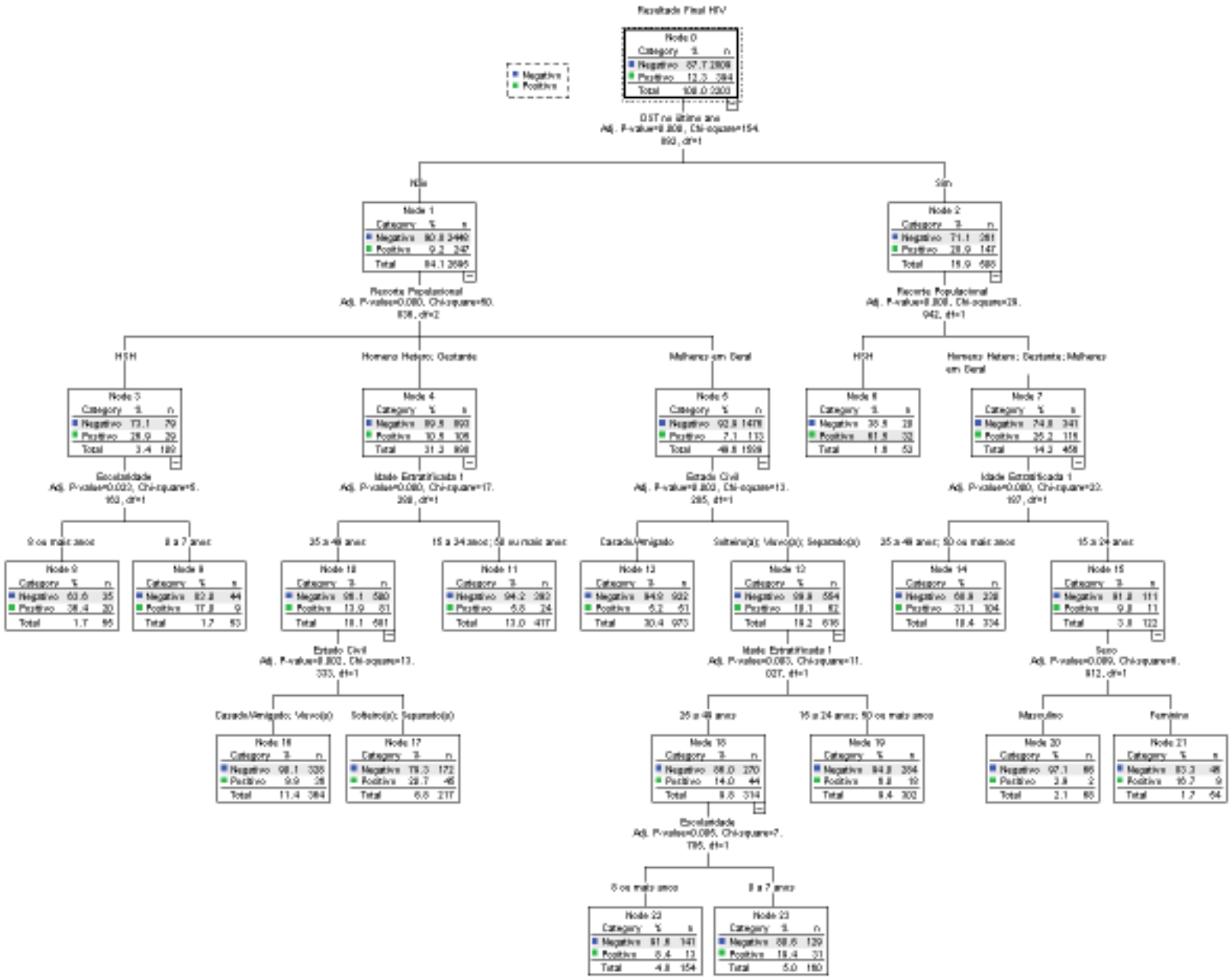


Figure 4. CHAID tree for volunteers with risk perception in VCT (B)

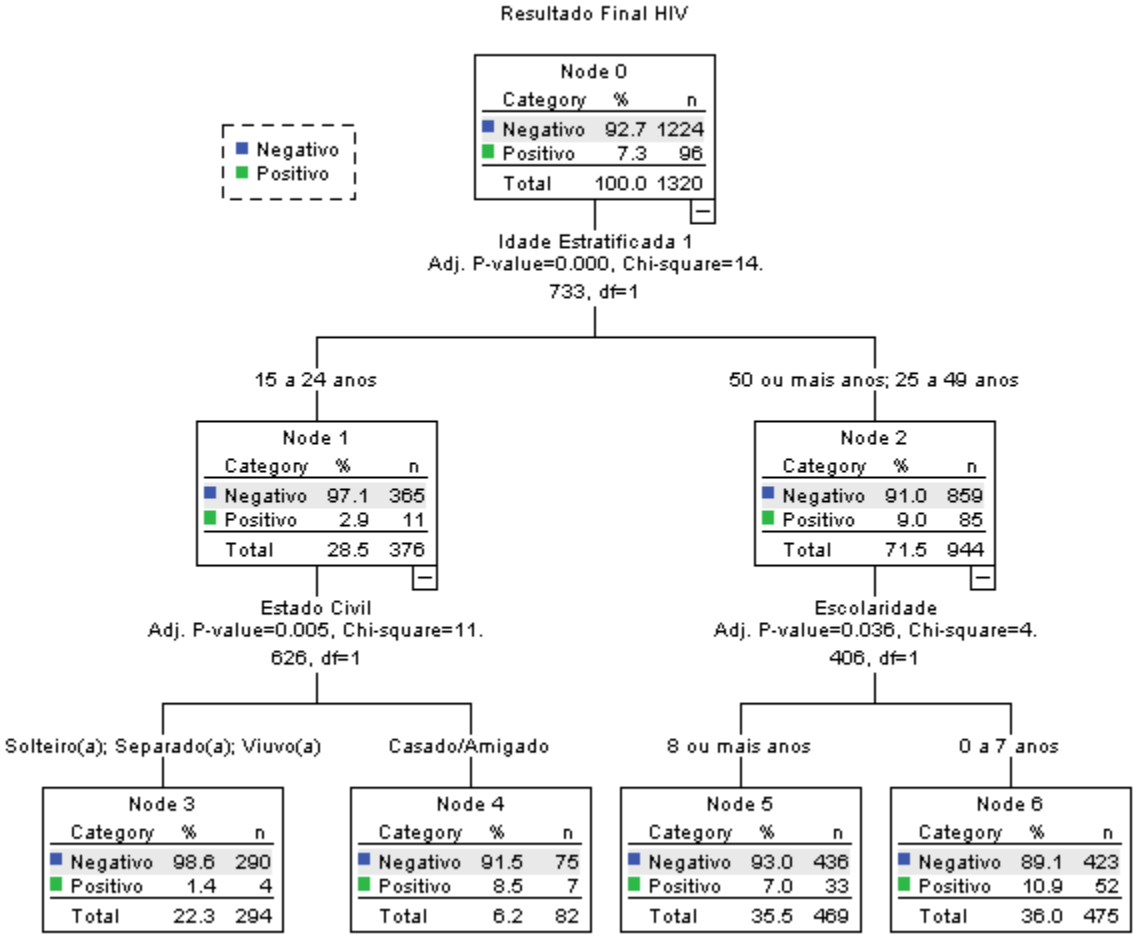


Figure 5. CHAID tree for volunteers with spontaneous testing relative to prevention in VCT (B)

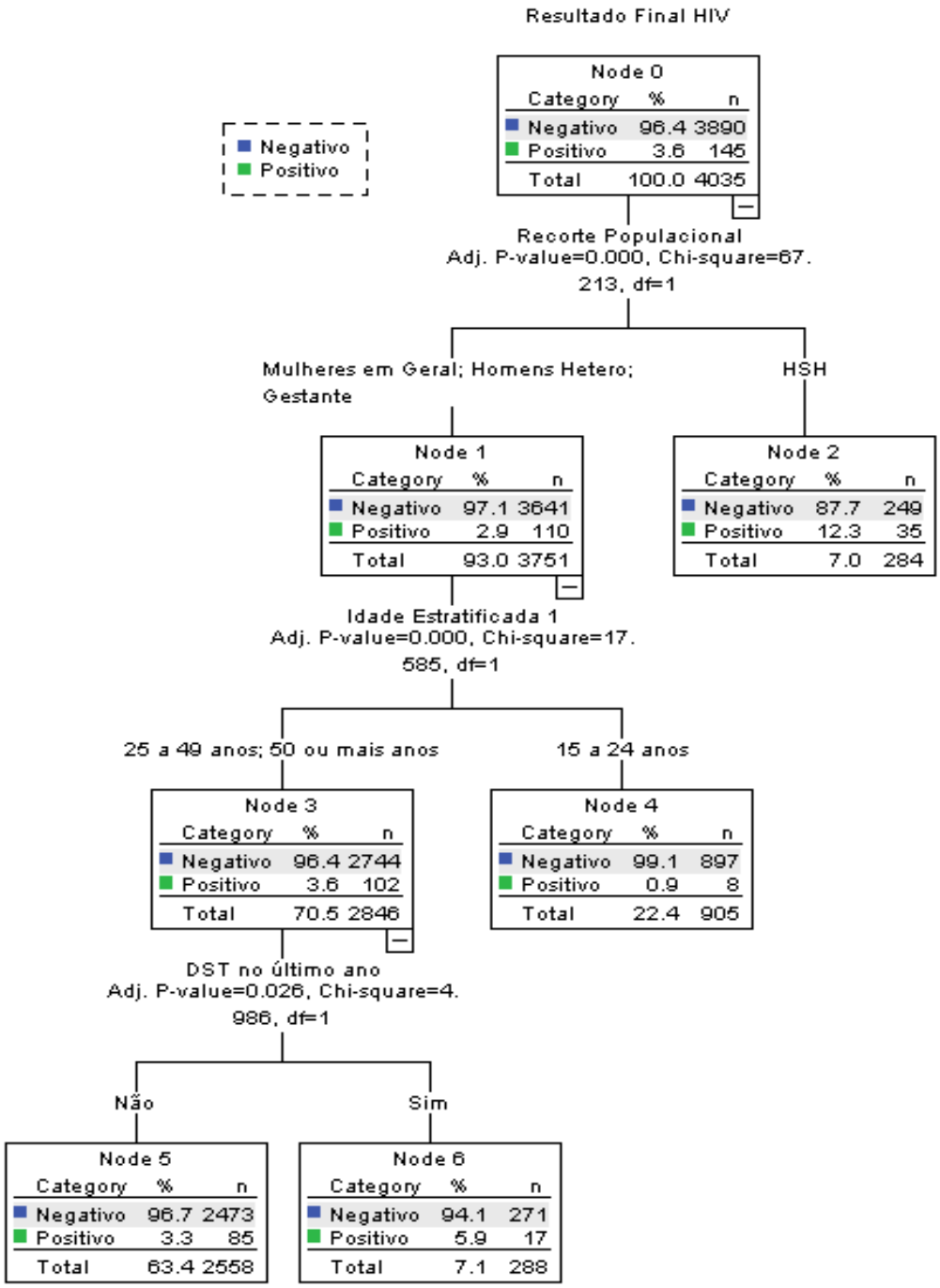


Figure 6. CHAID tree for the group that use the public health services for testing in VCT (B)

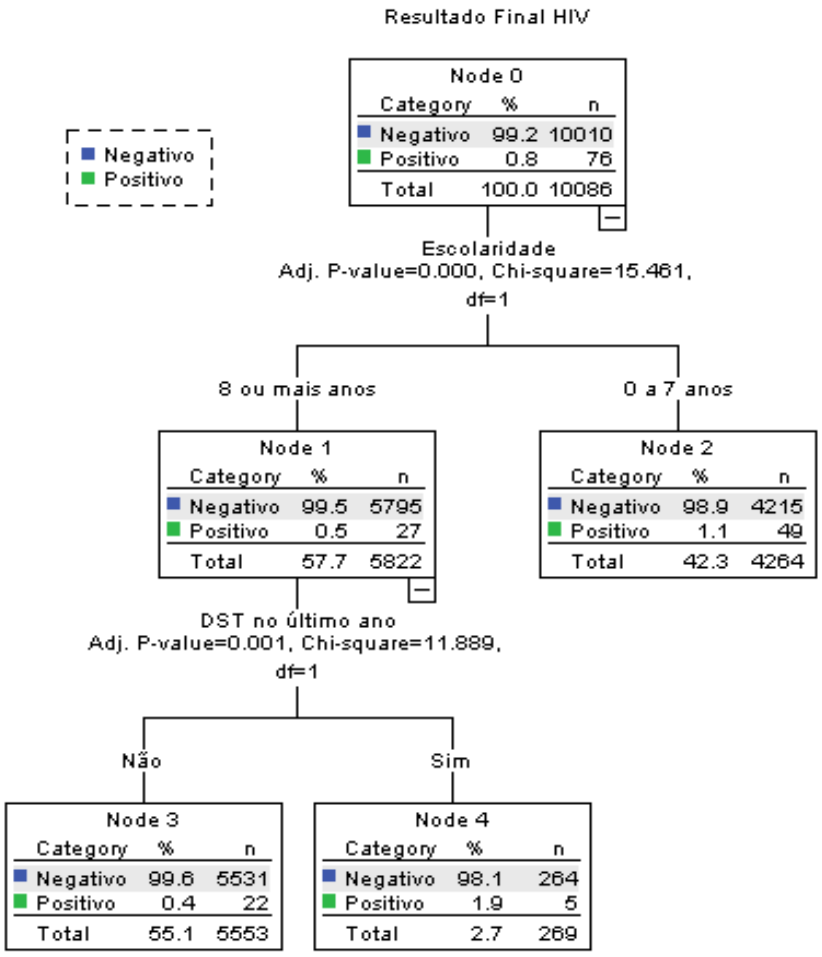


Table 2. Prevalence of HIV infection in populations of VCT (A), obtained by CHAID analysis

Population Segments (N = 2,109)	HIV Positive %	Population in each group
Suggested by health professional	21.8	321 (15.2%)
MSM	40.0	50 (2.4%)
Heterosexual men, Women (pregnant or not)	18.5	271 (12.8%)
Volunteers with risk perception	11.1	1,236 (58.6%)
MSM with more than 1 partner and education lower than 8 ys	27.8	54 (2.6%)
MSM with more than 1 partner and education more than 7 ys	10.3	107 (5.1%)
Heterosexual men, Women (not pregnant) with more than 1 partner	5.2	503 (23.9%)
One Partner	14.9	572 (27.1%)
Spontaneous, relative to prevention or utilization of public health services	5.6	552 (26.2%)
Volunteers more than 24 ys old	7.2	388 (18.4%)
Men from 15 to 24 ys old	4.5	67 (3.2%)
Women from 15 to 24 ys old	0.0	97 (4.6%)

Table 3. Prevalence of HIV infection in populations of VCT (B), obtained by CHAID analysis

Population Segments (N = 18,657)	HIV Positive %	Population in each group
Suggested by health professional	**12.3**	**3,208 (15.2%)**
Without DST in the year before the sample collection	*9.2*	*2,700 (14.5%)*
Heterosexual men and pregnant women from 15 to 24 ys old or older than 49 ys.	5.7	419 (2.2%)
Heterosexual men and pregnant women from 25 to 49 ys old, single or divorced.	20.7	217 (1.2%)
Heterosexual men and pregnant women from 25 to 49 ys old, married or widowed.	9.8	366 (2.0%)
Women – not pregnant from 15 to 24 ys old or older than 49 years, single or divorced or widowed.	6.3	303 (1.6%)
Women – not pregnant from 25 to 49 ys old, single or divorced or widowed and education lower than 8 ys.	19.4	160 (0.9%)
Women – not pregnant from 25 to 49 ys old, single or divorced or widowed and education above than 7 ys.	8.4	154 (0.8%)
Women – not pregnant, married.	5.2	973 (5.2%)
MSM with 1 partner.	10.9	55 (0.3%)
MSM with more than 1 partner.	43.4	53 (0.3%)
Episode of DST in the year before the sample collection	*28.9*	*508 (2.7%)*
MSM	61.5	52 (0.3%)
Women (pregnant or not) from 15 to 24 ys old.	16.7	54 (0.3%)
Heterosexual men from15 to 24 ys old.	2.9	68 (0.4%)
Heterosexual men, Women (pregnant or not) older than 24 ys with 1 to 5 partners.	27.3	275 (1.5%)
Heterosexual men, Women (pregnant or not) older than 24 ys without or more than 5 partners.	49.2	59 (0.3%)
Volunteers with risk perception	**7.3**	**1,321 (7.1%)**
Volunteers from 15 to 24 ys old, single or divorced or widowed.	1.4	294 (1.6%)
Volunteers from 15 to 24 ys old, married.	8.5	82 (0.4%)
Volunteers older than 24 ys old with education lower than 8 ys.	11.1	476 (2.6%)
Volunteers older than 24 ys old with education above than 7 ys.	7.0	469 (2.5%)
Spontaneous, relative to prevention	**3.6**	**4,039 (21.6%)**
MSM	12.3	284 (1.5%)
Heterosexual men, Women (pregnant or not) from 15 to 24 ys old.	0.9	908 (4.9%)
Heterosexual men, Women (pregnant or not) older than 24 ys with STD.	5.9	288 (1.5%)
Heterosexual men, Women (pregnant or not) older than 24 ys without STD.	3.4	2,559 (13.7%)
Utilization of public health services	**0.8**	**10,089 (54.1%)**
Volunteers with education lower than 8 ys.	1.1	4,266 (22.9%)
Volunteers with education more than 7 ys, with STD.	1.9	269 (1.4%)
Volunteers with education more than 7 ys, without STD and with 1 to 5 partners.	0.3	5,460 (29.3%)
Volunteers with education more than 7 ys, without STD and without or more than 5 partners .	3.2	94 (0.5%)

Table 4. Number of recent infections for HIV-1 in each general subgroup creates by CHAID analysis in VCT (A).

Population Segments	Overall / Recent	P value
Motivation for testing		
Volunteers with risk perception	1,307 / 30	***0.01***
Spontaneous, relative to prevention or utilization of public health services	703 / 5	
Suggested by health professional	431 / 14	
Volunteers with risk perception		
With 1 partner	573 / 15	0.57
With more than 1 partner	669 / 14	
Volunteers with risk perception and more than 1 partner		
MSM	162 / 7	***0.05***
Heterosexual men, Women (not pregnant)	507 / 7	
MSM with risk perception and more than 1 partner		
Education lower than 8 ys	54 / 4	0.10
Education more than 7 ys	107 / 2	
Spontaneous, relative to prevention or utilization of public health services		
15 to 24 ys old	217 / 0	0.33
More than 24 ys old	486 / 5	
Suggested by health professional		
MSM	50 / 1	1.00
Heterosexual men, Women (pregnant or not)	381 / 13	

Table 5. Number of recent infections for HIV-1 in each general subgroup creates by CHAID analysis in VCT (B).

Population Segments	Overall / Recent	P value
Motivation for testing		
Volunteers with risk perception	1,329 / 18	***< 0.01***
Spontaneous, relative to prevention	4,051 / 30	
Utilization of public health services	10,103 / 12	
Suggested by health professional	3,217 / 50	
Suggested by health professional		
STD (at least 1 episode in the year before the test)	510 / 18	***< 0.01***
no STD (none episode in the year before the test)	2,707 / 32	
Suggested by health professional, no STD		
MSM	108 / 4	***0.01***
Heterosexual men and pregnant women	1,004 / 15	
Woman not pregnant	1,595 / 13	
Suggested by health professional, MSM, no STD		
Education lower than 8 ys	53 / 3	0.35
Education more than 7 ys	55 / 1	
Suggested by health professional, heterosexual men and pregnant women, no STD		
15 to 24 ys old	421 / 5	0.60
More than 24 ys old	583 / 10	
Suggested by health professional, heterosexual men and pregnant women, no STD older than 24 ys		
Married or widowed	364 / 6	1.00
Single or divorced	217 / 4	
Suggested by health professional, women not pregnant, no STD		
Married	975 / 6	0.27
Single or divorced or widowed	618 / 7	
Suggested by health professional, women not pregnant, no STD, single or divorced or widowed		
From 25 to 49 ys	315 / 5	0.45
From 15 to 24 ys or more than 49 ys	303 / 2	
Suggested by health professional, women not pregnant, no STD, single or divorced or widowed, 25 to 49 ys		
Education lower than 8 ys	160 / 4	0.37
Education more than 7 ys	154 / 1	
Suggested by health professional, with episode of STD		
MSM	52 / 4	0.10
Heterosexual men, women (pregnant or not)	458 / 14	
Suggested by health professional, heterosexual men, women (pregnant or not) with episode of STD		
15 to 24 ys	69 / 1	0.77
More than 24 ys	54 / 2	
Suggested by health professional, with episode of STD, 15 to 24 ys		
Heterosexual men	69 / 1	0.58
Women (pregnant or not)	54 / 2	
Volunteers with risk perception		
15 to 24 ys	377 / 2	0.12
More than 24 ys	952 / 16	

Table 5. Number of recent infections for HIV-1 in each general subgroup creates by CHAID analysis in VCT (B).

Population Segments	Overall / Recent	P value
Volunteers with risk perception from 15 to 24 ys	83 / 1	0.39
Married	294 / 1	
Single or divorced or widowed		
Volunteers with risk perception and more than 24 ys	476 / 10	0.45
Education lower than 8 ys	469 / 6	
Education more than 7 ys		
Spontaneous, relative to prevention	284 / 13	***< 0.01***
MSM	3,767 / 17	
Heterosexual men, women (pregnant or not)		
Spontaneous, relative to prevention, heterosexual men, women (pregnant or not)	911 / 1	0.14
15 to 24 ys	2,856 / 16	
More than 24 ys		
Spontaneous, relative to prevention, heterosexual men, women (pregnant or not), more than 24 ys	290 / 5	***0.02***
STD (at least 1 episode in the year before the test)	2,566 / 11	
no STD (none episode in the year before the test)		
Utilization of public health services	4,266 / 8	0.16
Education lower than 8 ys	5,823 / 4	
Education more than 7 ys		
Utilization of public health services and education more than 7 ys	269 / 2	***< 0.01***
STD (at least 1 episode in the year before the test)	5,554 / 2	
no STD (none episode in the year before the test)		

8 – Anexo III: Resultados complementares referentes ao artigo 4

Tabela 1: Prevalência e incidência estimada com respectivos intervalos de confiança – 95%, obtidos em populações testadas entre 2005 e 2008 em dois CTAs da região metropolitana do Rio de Janeiro. Análise em função do total geral de indivíduos, por gênero, pela presença de gestação em mulheres, prática sexual entre os homens e idade categorizada.

Variáveis	CTA	N	Positivas	Prevalência % (95% IC)	BED (%)	Infecções Recentes (%)	Incidência estimada [a] %/ano (95% IC)
	A	2.624	321	12,2 (11,8 – 12,7)	290 (90,4)	49 (16,9)	5,40 (3,89 – 6,91)
Todos	B	20.103	746	3,7 (3,7 – 3,8)	730 (97,9)	116 (15,9)	1,43 (1,17 – 1,69)
Gênero							
Feminino	A	1.153	104	9,0 (8,5 – 9,5)	94 (90,4)	14 (14,9)	3,42 (1,63 – 5,21)
	B	15.726	379	2,4 (2,4 – 2,4)	374 (98,7)	62 (16,6)	0,96 (0,72 – 1,20)
Masculino	A	1.471	217	14,7 (14,0 – 15,5)	196 (90,4)	35 (17,8)	7,02 (4,70 – 9,35)
	B	4.377	367	8,4 (8,1 – 8,6)	356 (97,0)	54 (15,1)	3,22 (2,36 – 4,07)
Gestante (mulheres)							
Sim	A	116	2	1,7 (1,4 – 2,0)	2 (100)	1 (50,0)	2,04 (0,00 – 6,05)
	B	8.727	64	0,7 (0,7 – 0,7)	62 (96,8)	12 (19,3)	0,34 (0,15 – 0,53)
Não	A	1.037	102	9,8 (9,2 – 10,4)	92 (90,2)	13 (14,1)	3,57 (1,63 – 5,50)
	B	6.999	315	4,5 (4,4 – 4,6)	312 (99,0)	50 (16,0)	1,76 (1,27 – 2,25)
Prática sexual (homens)							
Heterosexuais	A	1.115	145	13,0 (12,2 – 13,8)	135 (93,1)	24 (17,8)	6,07 (3,64 – 8,50)
	B	3.751	246	6,6 (6,4 – 6,8)	241 (98,0)	31 (12,9)	2,10 (1,36 – 2,84)
HSH	A	356	72	20,2 (18,1 – 22,3)	61 (84,7)	11 (18,0)	10,22 (4,18 – 16,25)
	B	626	121	19,3 (17,8 – 20,8)	115 (95,0)	23 (20,0)	10,68 (6,32 – 15,05)
Idade em anos							
15 a 24	A	719	53	7,4 (6,8 – 7,9)	49 (92,4)	11 (22,4)	4,12 (1,69 – 6,56)
	B	7.006	102	1,5 (1,4 – 1,5)	98 (96,1)	27 (27,6)	0,95 (0,59 – 1,31)
25 a 49	A	1.604	231	14,4 (13,7 – 15,1)	205 (88,7)	32 (15,6)	6,00 (3,92 – 8,08)
	B	11.066	560	5,0 (5,0 – 5,2)	548 (97,9)	80 (14,6)	1,82 (1,42 – 2,21)
> 49	A	301	37	12,3 (11,7 – 12,9)	36 (97,3)	6 (16,6)	5,35 (1,07 – 9,64)
	B	2.031	84	4,1 (4,0 – 4,3)	84 (100)	9 (10,7)	1,08 (0,38 – 1,79)

Tabela 2: Prevalência e incidência estimada com respectivos intervalos de confiança – 95%, obtidos em populações testadas entre 2005 e 2008 em dois CTAs da região metropolitana do Rio de Janeiro. Análise em função da motivação para testagem, origem da clientela, estado civil, raça e educação.

Variáveis	CTA	N	Positivas	Prevalência % (95% IC)	BED (%)	Infecções Recentes (%)	Incidência estimada [a] %/ano (95% IC)
Motivação para testagem							
Espontânea, com percepção de Risco	A	1.455	172	11,8 (11,2 – 12,4)	148 (86,0)	30 (20,3)	6,20 (3,98 – 8,42)
	B	1.329	97	7,3 (6,9 – 7,7)	93 (95,8)	18 (19,4)	3,53 (1,90 – 5,15)
Espontânea relative a Prevenção	A	585	42	7,2 (6,6 – 7,8)	41 (97,6)	4 (9,8)	1,76 (0,04 – 3,49)
	B	4.051	146	3,6 (3,5 – 3,7)	143 (98,0)	30 (21,0)	1,83 (1,18 – 2,49)
Sugestão de serviço ou professional de saúde	A	431	99	23,0 (20,1 – 25,1)	95 (96,0)	14 (14,7)	9,84 (4,69 – 14,99)
	B	3.217	395	12,3 (11,9 – 12,7)	390 (98,7)	50 (12,8)	4,14 (2,99 – 5,29)
Utilisação de estrutura pública	A	118	4	3,4 (2,8 – 4,0)	3 (75,0)	1 (25,0)	2,72 (0,00 – 8,04)
	B	10.103	76	0,8 (0,7 – 0,8)	73 (96,1)	12 (16,4)	0,32 (0,15 – 0,50)
Origem da clientela							
Profissional/Serviço de saúde	A	624	104	16,7 (15,4 – 18,0)	91 (87,5)	13 (14,3)	6,51 (2,97 – 10,05)
	B	15.378	509	3,3 (3,2 – 3,4)	500 (98,2)	75 (15,0)	1,20 (0,93 – 1,47)
ONG, Amigos, usuários CTA	A	1.035	105	10,1 (9,5 – 10,8)	95 (90,5)	19 (20,0)	5,18 (2,85 – 7,51)
	B	3.065	146	4,8 (4,6 – 4,9)	142 (97,3)	32 (22,5)	2,62 (1,71 – 3,53)
Campanhas – todas as mídias	A	650	68	10,5 (9,7 – 11,3)	60 (88,2)	12 (20,0)	5,36 (2,33 – 8,39)
	B	1.410	81	5,7 (5,4 – 6,0)	79 (97,5)	8 (10,1)	1,44 (0,44 – 2,44)
Estado civil atual							
Casados	A	911	103	11,3 (10,6 – 12,0)	90 (87,4)	12 (13,3)	3,92 (1,70 – 6,14)
	B	12.937	351	2,7 (2,7 – 2,8)	344 (98,0)	58 (16,7)	1,10 (0,82 – 1,38)
Divorciados	A	186	24	12,9 (11,1 – 14,8)	22 (91,7)	2 (9,1)	3,12 (0,00 – 7,45)
	B	1.310	76	5,8 (5,5 – 6,1)	75 (98,7)	12 (16,0)	2,29 (1,00 – 3,59)
Solteiros	A	1.187	141	11,9 (11,2 – 12,6)	125 (88,7)	25 (20,0)	6,15 (3,74 – 8,57)
	B	5.215	268	5,1 (5,0 – 5,3)	260 (97,0)	42 (16,1)	2,04 (1,42 – 2,66)
Viúvos	A	84	12	14,3 (11,2 – 17,3)	12 (100)	5 (41,7)	15,12 (1,87 – 28,37)
	B	602	47	7,8 (7,2 – 8,4)	47 (100)	4 (8,5)	1,68 (0,03 – 3,33)
Raça							
Brancos	A	663	79	11,9 (11,0 – 12,8)	74 (93,7)	17 (23,0)	7,06 (3,70 – 10,42)
	B	4.486	138	3,1 (3,0 – 3,2)	134 (97,1)	26 (19,4)	1,44 (0,89 – 1,99)
Pardos	A	196	35	17,9 (15,4 – 20,4)	33 (94,3)	7 (21,2)	10,30 (2,67 – 17,93)
	B	4.700	151	3,2 (3,1 – 3,3)	148 (98,0)	28 (18,9)	1,47 (0,92 – 2,01)
Negros	A	169	30	17,8 (15,1 – 20,4)	30 (100)	5 (16,7)	8,13 (1,00 – 15,25)
	B	2.479	101	4,1 (3,9 – 4,2)	98 (97,0)	14 (14,3)	1,42 (0,68 – 2,16)
Educação em anos							
0-7	A	976	144	14,8 (13,8 – 15,7)	127 (88,2)	23 (18,1)	7,12 (4,21 – 10,03)
	B	9.086	416	4,6 (4,5 – 4,7)	411 (98,8)	57 (13,9)	1,55 (1,15 – 1,96)
> 7	A	1.388	135	9,7 (9,2 – 10,2)	121 (89,6)	21 (17,4)	4,31 (2,47 – 6,15)
	B	10.953	329	3,0 (2,9 – 3,1)	318 (96,7)	59 (18,6)	1,34 (1,00 – 1,69)

Tabela 3: Prevalência e incidência estimada com respectivos intervalos de confiança – 95%, obtidos em populações testadas entre 2005 e 2008 em dois CTAs da região metropolitana do Rio de Janeiro. Análise em função de episódio de DST, uso de drogas e parcerias e compartilhamento de seringas.

Variáveis	CTA	N	Positivas	Prevalência % (95% IC)	BED (%)	Infecções Recentes (%)	Incidência estimada [a] %/ano (95% IC)
DST no último ano							
Sim	A	155	29	18,7 (15,8 – 21,7)	25 (86,2)	1 (4,0)	2,14 (0,00 – 6,35)
	B	1.613	207	12,8 (12,2 – 13,5)	203 (98,1)	33 (16,2)	5,48 (3,61 – 7,35)
Não	A	2.469	292	11,8 (11,4 – 12,3)	265 (90,8)	48 (18,1)	5,56 (3,99 – 7,14)
	B	18.490	539	2,9 (2,9 – 3,0)	527 (97,8)	83 (15,7)	1,11 (0,87 – 1,35)
Uso de drogas no último ano							
Sim	A	29	7	24,1 (15,4 – 33,0)	6 (85,7)	1 (16,6)	11,75 (0,00 – 34,79)
	B	231	23	10,0 (8,7 – 11,2)	22 (95,6)	3 (13,6)	3,49 (0,00 – 7,44)
Não	A	2.595	314	12,1 (11,6 – 12,6)	284 (90,4)	48 (16,9)	5,33 (3,82 – 6,84)
	B	19.872	723	3,6 (3,6 – 3,7)	708 (97,9)	113 (16,0)	1,41 (1,15 – 1,67)
Compartilhou seringas (vida)							
Sim	A	46	9	19,6 (13,9 – 25,2)	8 (88,9)	0	0,00
	B	88	5	5,7 (4,5 – 6,9)	5 (100)	0	0,00
Não	A	2.578	312	12,1 (11,6 – 12,6)	282 (90,4)	49 (17,4)	5,48 (3,95 – 7,01)
	B	20.015	741	3,7 (3,6 – 3,8)	725 (97,8)	116 (16,0)	1,48 (1,18 – 1,70)
Nº. Parcerias sexuais ultimo ano							
0	A	466	74	15,9 (14,4 – 17,3)	73 (98,6)	2 (2,7)	1,21 (0,00 – 2,89)
	B	1.318	95	7,2 (6,8 – 7,6)	95 (100)	3 (3,1)	0,58 (0,00 – 1,23)
1	A	1.134	147	13,0 (12,2 – 13,7)	131 (89,1)	26 (19,8)	6,73 (4,14 – 9,31)
	B	15.181	432	2,8 (2,8 – 2,9)	422 (97,7)	72 (17,1)	1.17 (0,90 – 1,44)
2-4	A	669	63	9,4 (8,7 – 10,1)	55 (87,3)	16 (29,1)	6,88 (3,51 – 10,25)
	B	3.029	156	5,2 (5,0 – 5,3)	152 (97,4)	27 (17,8)	2,25 (1,40 – 3,09)
Acima de 4	A	325	37	11,4 (10,2 – 12,6)	31 (83,8)	5 (16,1)	4,76 (0,59 – 8,94)
	B	575	63	11,0 (10,0 – 11,9)	61 (96,8)	14 (23,0)	6,44 (3,06 – 9,81)
Uso preservativo c/ parceiro fixo							
Sempre	A	329	29	8,8 (7,9 – 9,8)	23 (79,3)	3 (13,0)	2,93 (0,00 – 6,24)
	B	1.373	127	9,2 (8,8 – 9,7)	121 (95,3)	20 (16,5)	3,89 (2,19 – 5,60)
Mais da metade das vezes	A	52	7	13,5 (9,8 – 17,1)	6 (85,7)	2 (33,3)	11,51 (0,00 – 27,46)
	B	2	0	-	-	-	-
Menos da metade das vezes	A	345	45	13,0 (11,7 – 14,4)	34 (75,5)	2 (5,9)	2,06 (0,00 – 4,91)
	B	6.113	185	3,0 (2,9 – 3,1)	183 (98,9)	29 (15,8)	1,16 (0,74 – 1,58)
Nunca	A	640	70	10,9 (10,1 – 11,8)	63 (90,0)	8 (12,7)	3,61 (1,11 – 6,10)
	B	10.578	295	2,8 (2,7 – 2,8)	287 (97,3)	50 (17,4)	1,17 (0,85 – 1,49)
Uso preservativo c/ parceiro casual							
Sempre	A	339	35	10,3 (9,2 – 11,4)	29 (82,9)	4 (13,8)	3,67 (0,07 – 7,27)
	B	1.029	61	5,9 (5,6 – 6,3)	58 (95,1)	8 (13,8)	2,03 (0,62 – 3,43)
Mais da metade das vezes	A	30	1	3,3 (2,1 – 4,5)	1 (100)	0	0,00
	B	2	0	-	-	-	0,00
Menos da metade das vezes	A	248	33	13,3 (11,6 – 15,0)	29 (87,9)	5 (17,2)	6,04 (0,75 – 11,34)
	B	1.432	98	6,8 (6,5 – 7,2)	97 (99,0)	14 (14,4)	2,47 (1,17 – 3,76)
Nunca	A	315	36	11,4 (10,2 – 12,7)	33 (91,7)	3 (9,1)	2,72 (0,00 – 5,81)
	B	1.372	70	5,1 (4,8 – 5,4)	67 (95,7)	11 (16,4)	2,06 (0,84 – 3,27)

Figura 1. Prevalência e incidência estimada de acordo com a idade estratificada nas populações que buscaram testagem para HIV em dois CTAs localizados na região metropolitana do Rio de Janeiro entre 2005 e 2008.

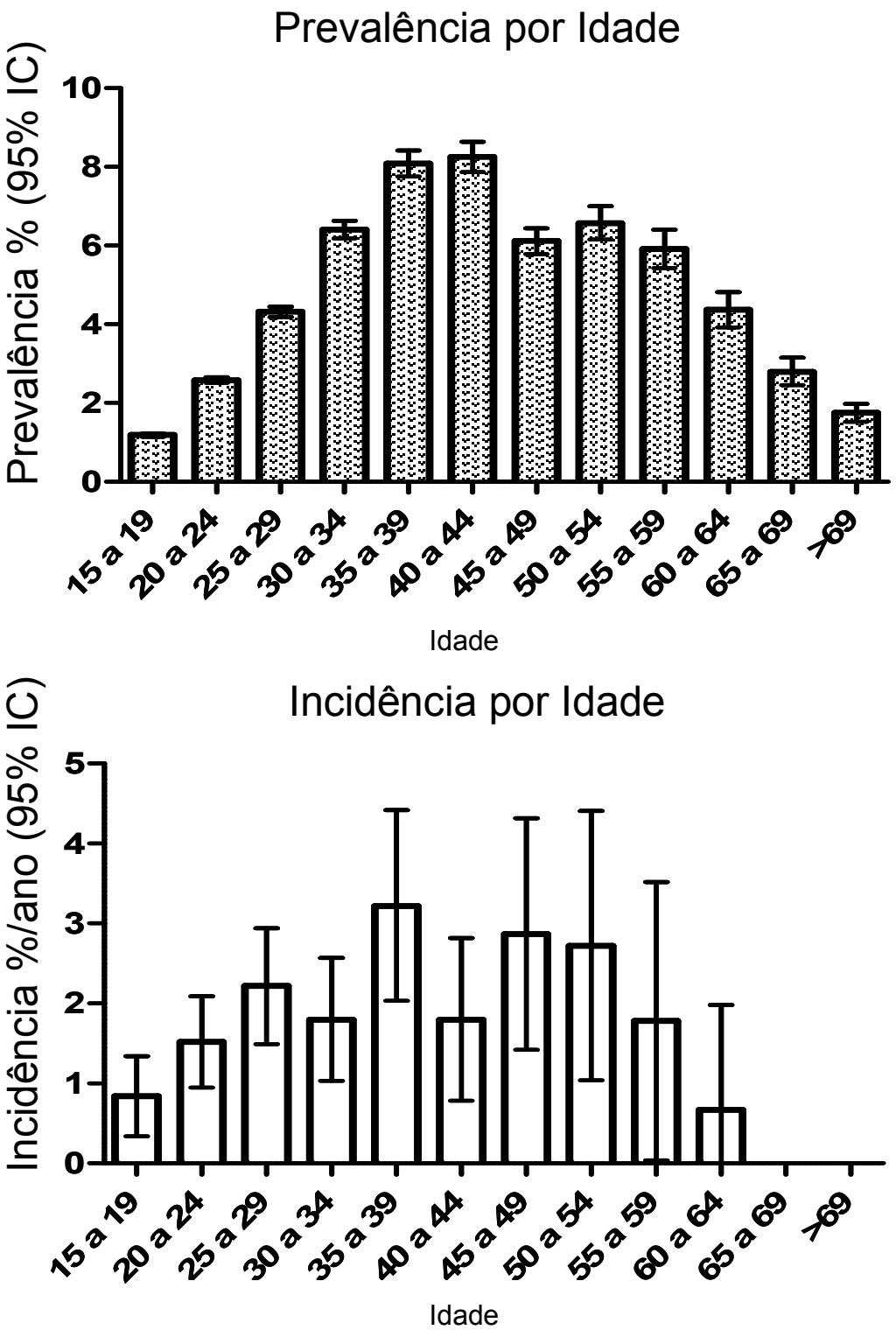


Figura 2. Tendências na prevalência (gráficos à esquerda) e incidência estimada (gráficos à direita) em populações que buscaram testagem para HIV em dois CTAs localizados na região metropolitana do Rio de Janeiro entre 2005 e 2008. CTA (A) na cidade do Rio de Janeiro e CTA (B) na cidade de Nova Iguaçu.

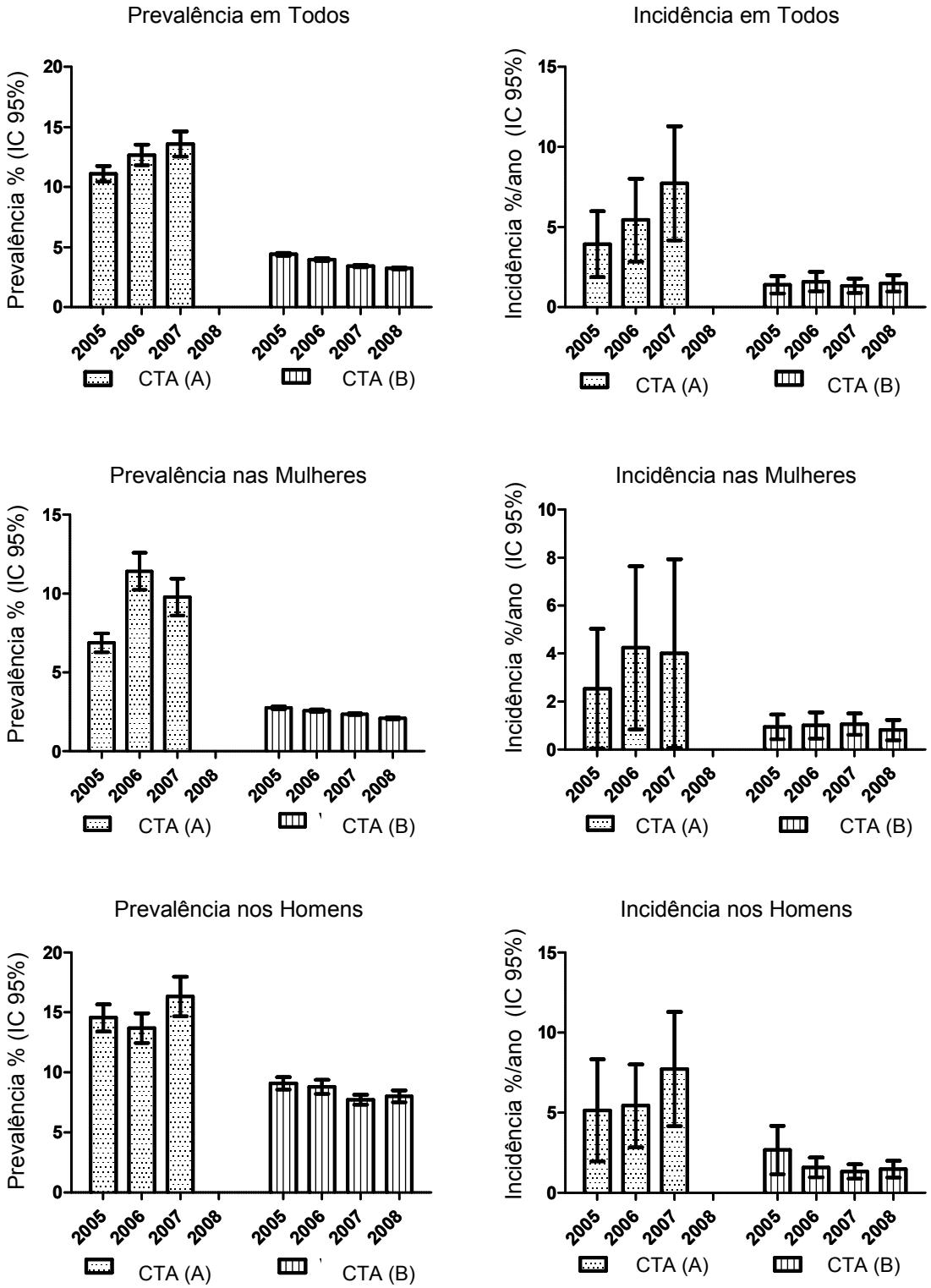
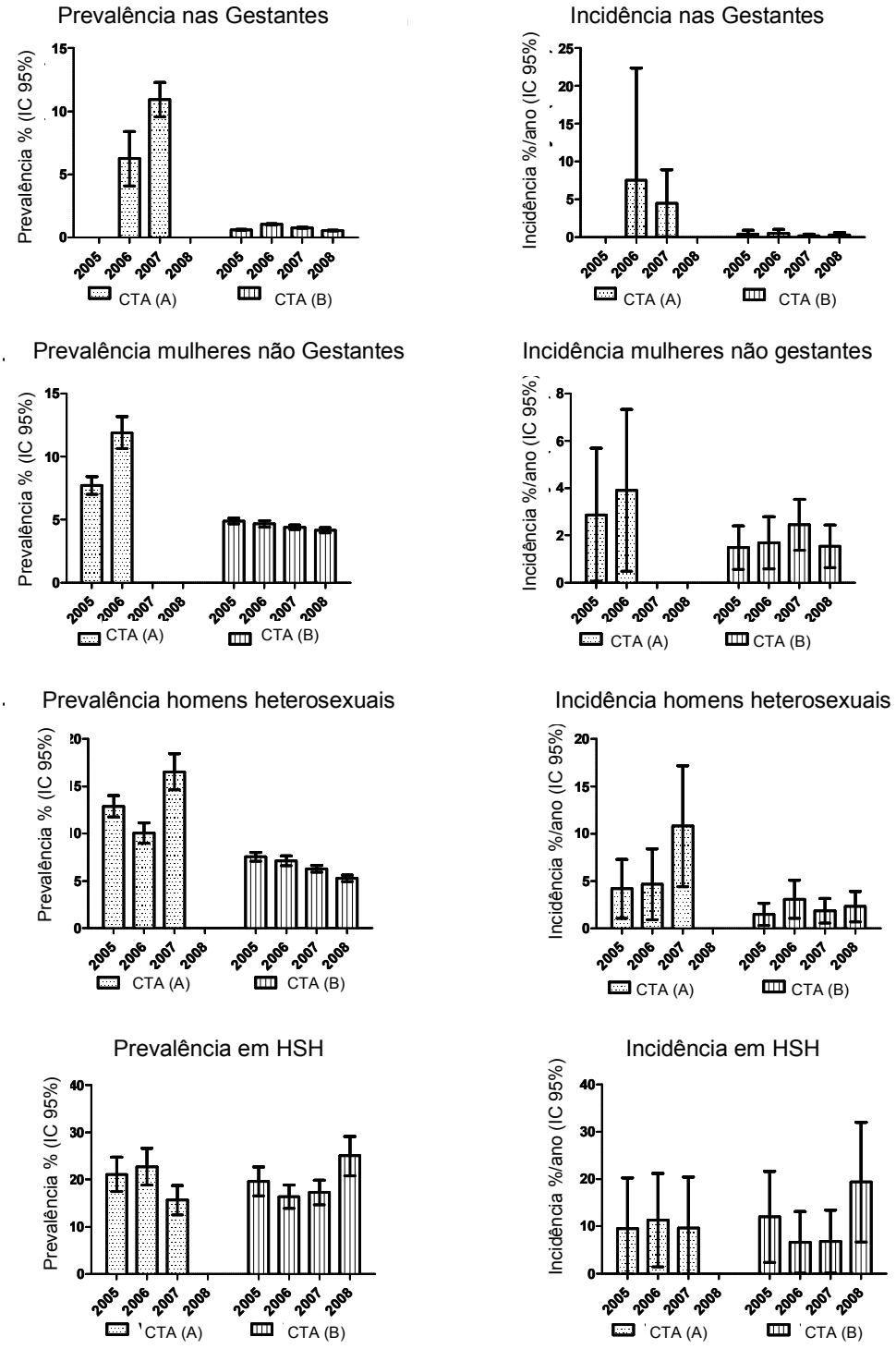


Figura 3. Tendências na prevalência (gráficos à esquerda) e incidência estimada (gráficos à direita) em populações que buscaram testagem para HIV em dois CTAs localizados na região metropolitana do Rio de Janeiro entre 2005 e 2008. CTA (A) na cidade do Rio de Janeiro e CTA (B) na cidade de Nova Iguaçu.



9 – Anexo IV: Produção relacionada com a temática da tese

Neste artigo utilizamos algumas ferramentas anteriormente descritas e relacionadas ao cerne da presente tese (BED-CEIA, AI e β2-M).

"Immune activation and antibody responses in non-progressing elite controller individuals infected with HIV-1"

Este artigo, foi publicado no periódico *J Med Virol.* 2009 Oct;81(10):1681-90.

8) Avaliar a reatividade e a avidez de anticorpos através do uso de métodos utilizados para a identificação de infecção recente pelo HIV-1, em indivíduos com perfis distintos de progressão para a AIDS.

9) Avaliar a reatividade e a avidez de anticorpos através do uso de métodos utilizados para a identificação de infecção recente pelo HIV-1, em indivíduos não progresores de longo termo.

Journal of Medical Virology 81:1681–1690 (2009)

Immune Activation and Antibody Responses in Non-Progressing Elite Controller Individuals Infected With HIV-1

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An extremely rare subset of patients infected with HIV-1 designated as "non-progressing elite controllers" appears to be able to maintain stable CD4+ T-cell counts and a median plasma viremia below the detection limit of current ultrasensitive assays (10 years in the absence of antiretroviral therapy. Lymphocyte subsets (CD4+, CD8+), immune activation markers (HLA-DR+, CD38+, Beta-2-microglobulin), and HIV-specific antibody responses were longitudinally examined in four non-progressing elite controllers over more than 5 years. Two control groups of seronegative healthy individuals and untreated patients infected with HIV-1 presenting detectable viremia were also included. None of the non-progressing elite controllers displayed the high T-cell activation levels generally seen in the seropositive individuals, keeping them within the normal range. Three non-progressing elite controllers showed no significant immune system abnormalities when compared to seronegative individuals, displaying a low proportion of HIV-1-specific binding antibodies and low avidity index, similar to those observed for individuals infected recently with HIV-1. One non-progressing elite controller exhibited CD8+ T-cell counts and β2-M levels above normal ranges and developed a low but "mature" (high-avidity) HIV-1-specific antibody response. Thus, the non-progressing elite controllers are able to maintain normal T-cell activation levels, which may contribute to prevent, or greatly reduce, the damage of the immune system typically induced by the HIV-1 over time. They are, however, immunologically heterogeneous and very low levels of antigen exposure seem to occur in these patients, sufficient for sustaining a low, but detectable, HIV-1-specific immunity. ***J. Med. Virol. 81:1681–1690, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HIV controllers; long-term non-progressors; chronic activation; binding antibodies; antibody avidity

INTRODUCTION

In the absence of antiviral therapy, the median time from human immunodeficiency virus type 1 (HIV-1) infection to AIDS (asymptomatic period) is estimated to be around 8–11 years for adults [Collaborative Group on AIDS Incubation and HIV Survival, 2000]. Whether all individuals infected with HIV-1 will eventually develop AIDS is still unclear.

Since the early 1990s, cohort studies have identified a small percentage (~5–10%) of HIV-1-infected people, called long-term non-progressors, who remain asymptomatic and maintain a relative high $CD4^+$ T-cell count (>500 cells/μl) for >8–10 years without antiviral therapy [Buchbinder and Vittinghoff, 1999]. Despite the absence of clinical symptoms for many years, most of those individuals display many hallmarks of HIV-1 infection including: low but persistent viral replication [Lefrere et al., 1997; Barker et al., 1998; Rodes et al., 2004], rapidly evolving viral quasispecies [Wolinsky et al., 1996; Delwart et al., 1997; Shioda et al., 1997], and evidence of chronic immune activation [Lifson et al., 1991; Sheppard et al., 1993; Ferbas et al., 1995; Lefrere et al., 1997; Barker et al., 1998; Zaunders et al., 1999]. After prolonged infection, many long-term non-progressors had evidence of immunologic damage and suffered a transition from the non-progressor to the progressor

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state, suggesting that most of them are actually slow progressors rather than true non-progressor patients [Lefrere et al., 1997; Learmont et al., 1999; Rodes et al., 2004].

More recently reports have identified another special subset of individuals infected with HIV-1, called "elite controllers" (also known as "HIV controllers," "elite suppressors," or "natural viral suppressors") [Lambotte et al., 2005; Bailey et al., 2006; Deeks and Walker, 2007; Sajadi et al., 2007], who naturally control viral replication below the detection limit of current ultrasensitive assays (<50–500 copies HIV RNA/ml of plasma). These elite controllers display very homogeneous HIV-1 quasispecies and little or no evidence of ongoing viral evolution over time [Wang et al., 2003; Bello et al., 2005, 2007; Bailey et al., 2006]. Despite efficient control of viral replication and evolution, some of them display augmented expression of activation markers on $CD8^+$ T cells [Greenough et al., 1999; Wang et al., 2002; Hunt et al., 2008], low $CD4^+$ T-cell counts (<500 cells/μl) [Greenough et al., 1999; Lambotte et al., 2005; Bailey et al., 2006; Hunt et al., 2008], and progress to AIDS [Hunt et al., 2008]; showing that immunological damage and disease progression can still occur in the setting of undetectable viral replication.

Some factors, however, could have been limiting the identification of subjects with a true non-progressing disease in previous studies. First, elite controllers were only defined on the basis of their natural ability to suppress viral replication below the detection limit, regardless of their $CD4^+$ T-cell counts; and some of them are not long-term non-progressors according to their immunological status [Lambotte et al., 2005; Bailey et al., 2006; Hunt et al., 2008]. Second, in some studies the duration since HIV diagnosis was not part of the elite controller definition [Bailey et al., 2006; Hunt et al., 2008]. It has been estimated that ~5–9% of HIV-1-infected patients are able to spontaneously suppress RNA viral load <50–400 copies/ml during the first 5 years after infection; however, <1% of them appear to be able to maintain such suppression for longer times (>10 years) [Lefrere et al., 1999; Lambotte et al., 2005; Madec et al., 2005a,b]. This suggests that elite controllers with short-term versus long-term viral suppression should be considered as separate subgroups. Finally, most of the previous studies were based on cross-sectional analyses of the immunologic profile of the elite controllers [Lambotte et al., 2005; Bailey et al., 2006; Hunt et al., 2008; Pereyra et al., 2008].

In this study, we performed a longitudinal analysis of the immunologic profile of an extremely rare subset of patients that fulfill the definitions of both long-term non-progressors and elite controllers, defined as "non-progressing elite controllers," assessing the T-lymphocyte subsets, immune activation markers, and HIV-1-specific humoral immunity. Two additional groups, including healthy individuals and untreated HIV-1-seropositive patients presenting detectable viremia, were evaluated as controls with the goal of identifying immunologic characteristics associated with the long-term control of HIV-1 replication in the context of stable $CD4^+$ T-cell counts.

PATIENTS AND METHODS

Patients

Four persons infected with HIV-1 subtype B, two men who have sex with men (Patients 44 and 46) and two heterosexual women (Patients 42 and 52) were studied. They fulfill the criteria of non-progressing elite controllers and were identified in a cohort of ~1,700 seropositive individuals followed at the Instituto de Pesquisa Clínica Evandro Chagas (IPEC) - FIOCRUZ, in Rio de Janeiro, Brazil. Non-progressing elite controllers were defined as subjects with a documented HIV-1 infection for >10 years, no AIDS-related conditions in the absence of antiretroviral treatment, a median plasma HIV-1 RNA load <80 copies/ml, and for whom >90% of the $CD4^+$ lymphocyte count measurements were ≥500 cells/μl. Two additional groups were included as controls. One of them with 45 HIV-1-seronegative healthy individuals and the other containing 45 HIV-1-seropositive untreated patients presenting detectable viremia (range, 200–2.6×10^6 copies/ml), and a variable number of $CD4^+$ T cells (range, 120–1,077 cells/μl). All patients were included in the study after signing an informed consent form according to protocols approved by the local ethical committee.

Sample Collection

Whole-blood samples from non-progressing elite controllers were collected every 4–12 months over an 8-year period (from 1999 to 2007); while blood samples from control groups were collected at a single time point. Plasma and peripheral blood mononuclear cell (PBMC) fractions were separated and processed immediately or stored at −70°C until testing.

Plasma HIV-1 RNA Load

Plasma HIV-1 RNA levels were quantified using either the Nuclisens test kit (lower limit of detection 80 copies HIV RNA/ml; Organon Teknika, Durham, NC), or the branched-DNA Versant HIV-1 RNA 3.0 Assay (lower limit of detection 50 copies HIV RNA/ml; Siemens Healthcare Diagnostics, Inc., Tarrytown, NY) according to the manufacturer's instructions.

Lymphocyte Subsets

Immunophenotyping was performed using freshly collected, EDTA-anticoagulated whole blood. For $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T-cell counts, single- or dual-platform methods were utilized over time. In the dual-platform method, 100 μl of whole blood was incubated for 30 min at room temperature in the dark with 10 μl of TriTEST three-color monoclonal antibodies (CD4-FITC/CD8-PE/CD3-PerCP) (BD Biosciences, Franklin Lakes, NJ) and lysed automatically and fixed using a Multi-Q-Prep system, according to the manufacturer

(Coulter Corp., Healeah, FL). T-cell subset determinations were carried out using an EPICS XL-MCL Flow Cytometer (Coulter Corp.). In the single-platform method, 20 µl of single-color antibodies (CD4-FITC, CD8-PE, and CD3-PerCP) and 50 µl of whole blood were added to bead-containing TruCount tubes (BD Biosciences). These were incubated for 15 min at room temperature before 450 µl of FACS lysing solution™ was added. Samples were analyzed after 1 hr on a FACS calibur flow cytometer (BD Biosciences). For T-cell activation analyses, 5 µl of dual-colors antibodies (CD8-FITC/CD38-PE, and CD3-FITC/HLA-DR-PE) (BD Biosciences) and 50 µl of whole blood were incubated for 30 min at room temperature in the dark. The lysis and fixation were performed using a Multi-Q-Prep system or by adding 450 µl of FACS lysing solution™. Samples were analyzed after 1 hr on a EPICS XL-MCL flow cytometer or a FACS calibur flow cytometer.

Beta-2-Microglobulin (β^2-M)

The concentration of serum β^2-M (expressed in µg/ml) was measured on −70°C stored plasma samples using a enzyme-linked fluorescent assay (Vidas® β2-Microglobulin, BioMérieux, Marcy L'Etoile, France) according to the manufacturer's instructions.

Humoral Immune Responses

Plasma samples stored at −70°C were also used to perform the following assays.

Binding antibodies. The proportion of HIV-1-specific binding antibodies against a peptide derived from the gp41 protein in total IgG was measured using the Calypte® HIV-1 BED Incidence EIA (Calypte Biomedical Corporation, Portland, OR). Briefly, in this assay, once plasma is added, anti-HIV-1 IgG and non-anti-HIV-1 IgG populations are captured on goat-anti-human IgG-coated wells, and the relative amount of anti-HIV-1 IgG captured is measured. Samples with an optical density (OD) <0.8 of calibrator OD are classified as recent infections (<6 months since infection).

Antibody avidity index. The antibody avidity index (AI) against HIV-1 Env and Gag recombinant proteins was calculated using a previously discussed approach [Suligoi et al., 2002, 2003]. Briefly, two aliquots of each sample were subjected to a pretest 1:10 dilution: one aliquot was diluted in phosphate-buffered saline (PBS), and the other one in 1 M guanidine (G). After incubation at room temperature for 10 min, both aliquots were subjected to the AxSYM HIV1/2 group O immunoassay (Abbott Laboratories, Wiesbaden-Delkenheim, Germany), following the manufacturer's instructions. Sample/cutoff (S/CO) ratios of both aliquots were calculated, and the AI of HIV antibodies was obtained using the following formula: AI = (S/CO G aliquot/S/CO PBS aliquot). Samples with an AI ≤0.85 are classified as recent infections (<6 months since infection).

Neutralizing antibodies. Heat inactivated plasma samples from the different individuals were tested for their capacity to neutralize the reference X4 HIV-1 III^B and SF162 strains expanded in the PBMCs, as previously described [Bongertz et al., 2007]. Briefly, neutralization was tested using pre-activated normal human PBMCs (10^5 cells/well), and a multiplicity of infection (MOI) of 0.001–0.005 (10–50 infective units per well). At least five threefold plasma dilutions, using 1:10 as the first step, were used. Quantitation of the HIV-1 p24 antigen was carried out on the seventh day of cell culture, according to the manufacturer's instruction (HIV-1 p24 ELISA; Zeptometrix Corporation, Buffalo, NY). Serum dilutions at which 90% of the viral input was neutralized (IC^{90}) were derived from linear regression curves or directly from the neutralization curve.

RESULTS

Viral Replication

Plasma HIV-1 RNA load was periodically assessed with current ultrasensitive assays (detection limit, 50–80 copies/ml) in all non-progressing elite controllers since 1999, with a median of two viral load determinations per year for each patient (Fig. 1). Patients 44 and 52 maintained HIV-1 RNA levels below the limits of quantitation in all determinations (16 viral load measurements) throughout follow-up. Patients 42 and 46, however, displayed infrequent (20–40%) episodes of plasma viremia (also known as "blips") in the low but detectable range (90–1,300 copies/ml). In up to three consecutive viral load measurements, values for Patient 42 were below the detection limit, and up to three consecutive blips. Patient 46 displayed up to seven consecutive viral load determinations below the detection limit, and no more than two consecutive blips. Detailed analysis of the virus diversity and evolution in these patients was described elsewhere [Bello et al., 2007].

Peripheral $CD4^+$ and $CD8^+$ T-Cell Counts

HIV-1 infection is characterized by a decline in the number of $CD4^+$ T cells and an increase in the number of $CD8^+$ lymphocytes, leading to inversion of the $CD4^+/CD8^+$ ratio (value <1.0) that in most cases occurs soon after seroconversion. The number of $CD4^+$ and $CD8^+$ T cells was periodically assessed since 1992 for Patient 46, 1996 for Patient 42, 1997 for Patient 52, and 1999 for Patient 44. Cross-sectional analyses of $CD4^+$ and $CD8^+$ T-cell counts in the groups of healthy controls and untreated individuals infected with HIV-1 presenting detectable viral load were also performed. All non-progressing elite controllers displayed stable $CD4^+$ T-cell counts within the normal range over the entire follow-up period (Fig. 1), with a mean $CD4^+$ T-cell count comparable to the HIV-negative control group (Fig. 2a). The $CD8^+$ T-cell counts showed more variation among patients (Fig. 1). Patients 44 and 52 displayed stable $CD8^+$ T-cell counts usually within the normal range during follow-up. Patient 42 also showed a $CD8^+$ T-cell level within the normal range, but with a clearly increasing trend over time. Patient 46 exhibited persistently

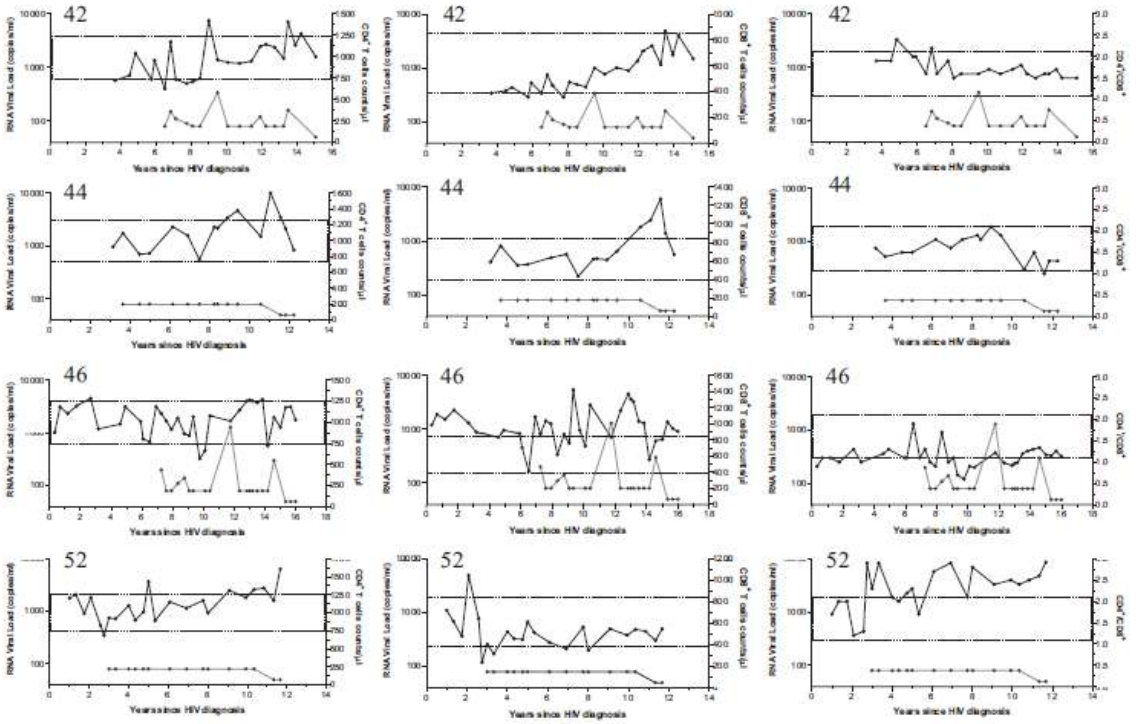

Fig. 1. Changes in $CD4^+$ and $CD8^+$ T-cell counts and plasma viremia over time in non-progressing elite controllers. Open circles: Plasma HIV-1 RNA (measurements below the detection limit were arbitrarily set at 80 or 50 copies/ml). Filled circles: Absolute $CD4^+$ T-cell count (**left**), absolute $CD8^+$ T-cell count (**middle**), and $CD4^+/CD8^+$ ratio (**right**). Dashed rectangles represent the 10th–90th percentiles range estimated in a group of 45 individuals negative for HIV-1.

high $CD8^+$ T-cell counts above the normal range. Patients 42, 44, and 52 had a mean $CD8^+$ T-cell count similar to the HIV-negative control group, and lower than Patient 46 and the HIV-1-positive control group (Fig. 2b). The $CD4^+/CD8^+$ mean ratio was above 1 in all non-progressing elite controllers (Fig. 2c); although Patient 46 exhibited an inverted (<1) ratio at several time points (Fig. 1).

Immune Activation

Chronic activation of the immune system is another hallmark of HIV-1 infection. The percentages of activated peripheral T lymphocytes ($CD3^+$ $HLA\text{-}DR^+$ and $CD8^+$ $CD38^+$ phenotypes), and the level of the serological activation marker β2-M were measured, at six to nine different time points during follow-up in non-progressing elite controller patients, and at single time points for the two control groups. All non-progressing elite controllers displayed percentages of $CD3^+$ $HLA\text{-}DR^+$ and $CD8^+$ $CD38^+$ T cells within the normal range during the studied period (Fig. 3), comparable to those seen in HIV-negative individuals and lower than the corresponding values obtained for the HIV-1-positive control group (Fig. 2d,e). The level of β2-M also remained within the normal range over the studied period for Patients 42, 44, and 52; but was persistently high for Patient 46 correlating with the high number of $CD8^+$ T cell seen in this patient (Fig. 3). Patients 42, 44, and 52 had a mean level of β2-M similar to that of the HIV-negative group, and lower than Patient 46 and the HIV-1-positive control group (Fig. 2f). In transient viremic patients 42 and 46, the level of immune system activation at those time points with or without detectable viral load was comparable.

HIV-Specific Antibody Responses

In therapy-naive subjects the titer, proportion, and avidity of anti-HIV-1 antibodies present in plasma gradually increase over time reaching a plateau around 1 year post-infection. Based on these kinetics several testing algorithms have been developed to distinguish between recent (<6 months) and long-standing HIV-1 infections in a single serum sampling [Parekh and McDougal, 2005]. In the present study, two of these assays were used to evaluate the relative amount (proportion) of anti-gp41-binding antibodies, and the AI against Env and Gag proteins at five to six different time points in the four

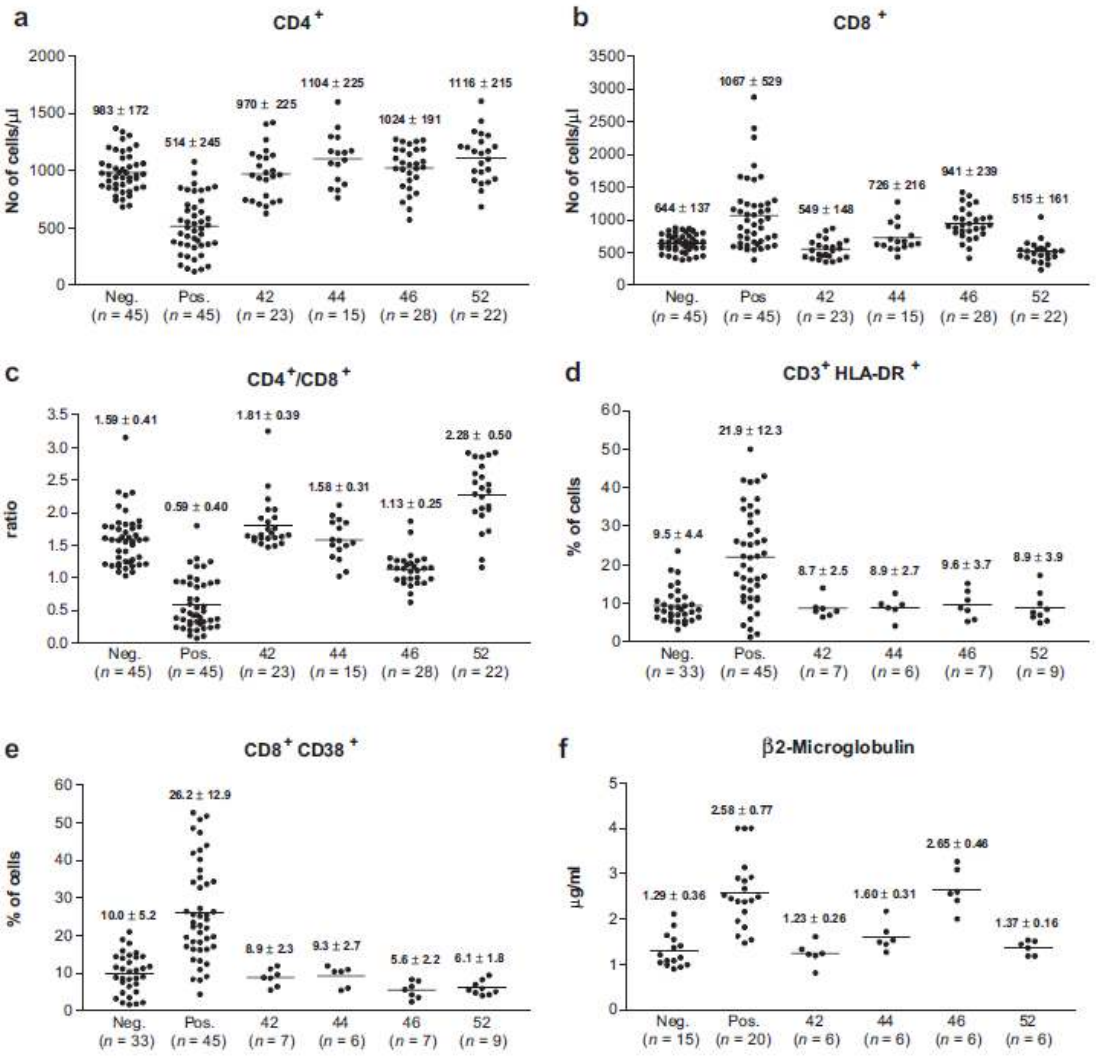

Fig. 2. T-cell counts and general immune activation markers in non-progressing elite controllers (42, 44, 46, and 52) compared to HIV-1-seronegative (Neg.) and viremic-seropositive (Pos.) control groups. a: $CD4^+$ T-cell counts. b: $CD8^+$ T-cell counts. c: $CD4^+/CD8^+$ ratio. d: HLA-DR^+ expression on $CD3^+$ T cells. e: $CD38^+$ expression on $CD8^+$ T cells. f: Plasma level of β2-M. The horizontal bars denote mean values. Numbers above each column are the mean ± standard deviation. For each assay, the number of subjects studied in the Neg. and Pos. control groups, and the number of samples analyzed from each non-progressing elite controller subject are shown in the bottom.

non-progressing elite controller patients, and at a single time point in a randomly selected subgroup of 16 viremic untreated HIV-1-seropositive patients. Despite being measured between 5 and 15 years after the first HIV-positive test, plasma from all non-progressing elite controllers displayed a very low proportion of anti-gp41-binding antibodies, similar to that seen in patients with <6 months of infection and lower than those observed in patients with chronic HIV-1 infection (Figs. 4 and 5a). Analysis of the AI at the same time points revealed that Patients 42 and 44 displayed very low levels comparable to patients infected recently. Patient 52 exhibited a value around to the cut-off, while Patient 46 displayed high AI comparable to patients chronically infected with HIV-1 (Figs. 4 and 5b). Plasma samples from non-progressing elite controllers were also tested for the presence of neutralizing antibodies against heterologous HIV-1 strains SF162 and IIIB at three to five time points during follow-up, exhibiting variable titers against both isolates, with IC^{90} neutralization titers ranging from

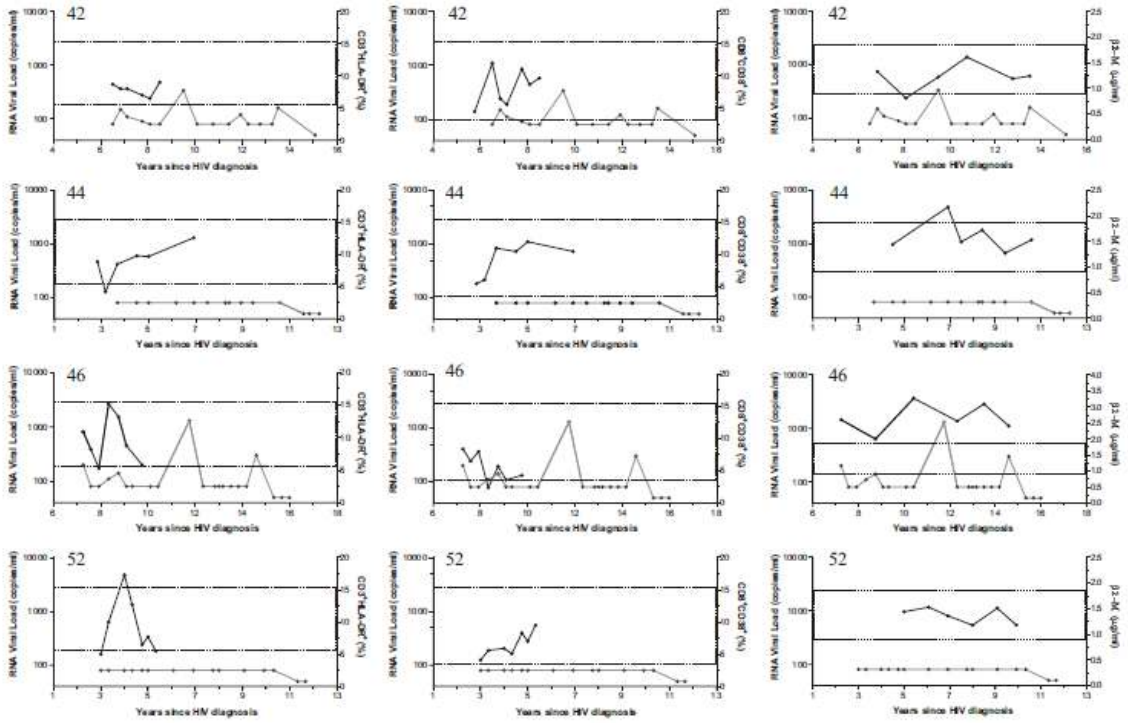

Fig. 3. Changes in general immune activation markers and plasma viremia over time in non-progressing elite controllers. Open circles: Plasma HIV-1 RNA (see Fig. 1). Filled circles: Percent of $CD3^+$ T cells expressing $HLA\text{-}DR^+$ (**left**), percentage of $CD8^+$ T cells expressing $CD38^+$ (**middle**), and plasma β2-M level (**right**). Dashed rectangles represent the 10th–90th percentiles range estimated in a group of 15–33 individuals negative for HIV-1.

very low (<1:10) to fairly high (>1:100) within each patient (Fig. 4).

DISCUSSION

In this study, a longitudinal analysis was undertaken of the immunologic profile of four patients infected with HIV-1 who naturally controlled viral replication to levels below the detection limit at all or most time points and also maintained high and stable $CD4^+$ T-cell counts for >12 years without antiviral therapy.

It has been demonstrated that elite controller individuals have higher $CD8^+$ T-cell activation levels than subjects without HIV-1, and this was associated with lower $CD4^+$ T-cell counts [Hunt et al., 2008]. Declining $CD4^+$ T-cell counts and increased levels of activated $CD8^+$ T cells were also observed in an elite controller infected with *nef*-deleted forms of HIV-1 [Greenough et al., 1999]. This suggests that abnormally high T-cell activation may contribute to progressive $CD4^+$ T-cell loss in the setting of undetectable viral replication. Notably, three out of four non-progressing elite controller subjects with stable $CD4^+$ T-cell counts analyzed in the present study displayed no significant immune system abnormalities when compared with HIV-1-seronegative individuals. The fourth patient (Patient 46), who also maintained stable $CD4^+$ T-cell counts, exhibited elevated levels of $CD8^+$ T cells and β2-M, but percentages of $CD3^+$ $HLA\text{-}DR^+$ and $CD8^+$ $CD38^+$ T cells within the normal range. Normal levels of peripheral T-cell subsets and $CD8^+$ T-cell activation are extremely rare during natural HIV-1 infection and, to our knowledge, have been described previously in only three elite controllers infected with *nef*-attenuated HIV-1 strains [Zaunders et al., 1999]. These results demonstrate that some of these subjects are able to maintain T-cell activation levels within normal range (even in the presence of occasional viremic blips), which may prevent, or greatly reduce, the progressive damage of the immune system typically induced by HIV-1 infection.

Recent studies also showed that samples from some elite controller patients chronically infected could be misclassified as recent infections by the low proportion [Hayashida et al., 2008] and titer [Laeyendecker et al., 2008; Hatano et al., 2009] of HIV-1-specific antibodies. By contrast, it has been suggested that these individuals have a mature binding avidity and no samples from this group were misclassified as recent infection by the avidity assay [Laeyendecker et al., 2008]. Consistent with this previous finding, one non-progressing elite

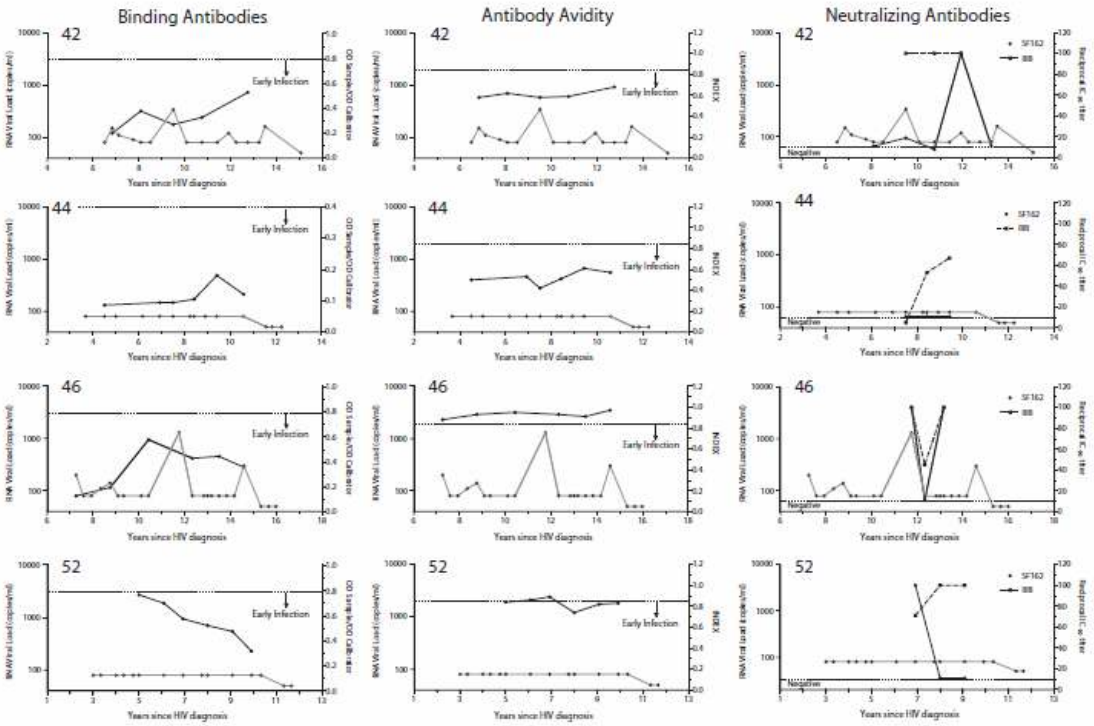

Fig. 4. Changes in serum HIV-1-specific antibody responses and plasma viremia over time in non-progressing elite controllers. Open circles: Plasma HIV-1 RNA (see Fig. 1). Filled circles: Proportion of anti-gp41-binding antibodies (**left**), avidity index to Env and Gag proteins (**middle**), and reciprocal neutralizing antibody IC^{90} titers against the HIV-1 SF162 isolate (**right**). Asterisk indicates the reciprocal neutralizing antibody IC^{90} titers against the HIV-1 III^B isolate. Horizontal dashed lines represent the conventional binding antibody (0.80) and avidity index (0.85) cut-off values below which a sample is classified as recent infection (<6 months of seroconversion), and the neutralizing antibody assay detection limit (*reciprocal dilution* = 10). Titers <1/10 were considered negative and arbitrary set at 1/5. Titers >1/100 were arbitrary set at 1/100. OD, optical density.

controller subject analyzed in the present study (Patient 46) displayed a low proportion of anti-gp41-binding antibodies in the setting of a mature binding avidity. The other three subjects, however, displayed a low proportion of anti-gp41-binding antibodies and an immature binding avidity along the years, comparable to that seen in patients recently infected with HIV-1 (<6 months of infection). Differences in the avidity assay and/or inter-subject variability may explain these contradictory results. In either case, these data clearly

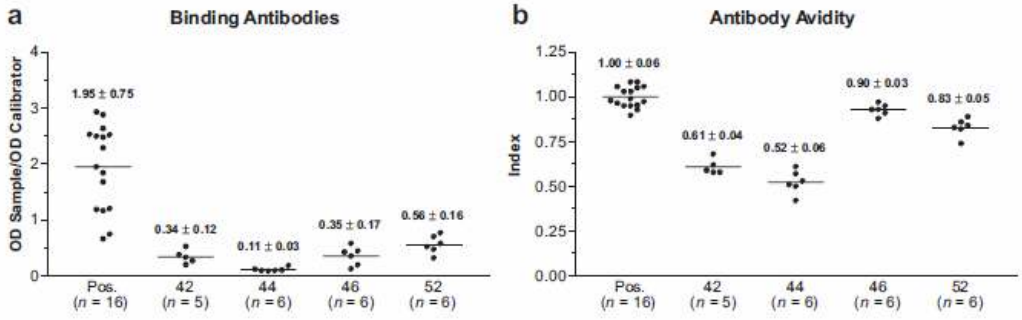

Fig. 5. HIV-1-specific antibody responses in non-progressing elite controller subjects (42, 44, 46, and 52) and viremic individuals infected with HIV-1 (Pos.). **a**: Proportion of anti-gp41-binding antibodies. **b**: Avidity index to Env and Gag proteins. The horizontal bars denote mean values. Numbers above each column are the mean ± standard deviation. For each assay, the number of subjects studied in the Pos. control group and the number of samples analyzed from each non-progressing elite controller subject are shown in the bottom.

reveal the long-term persistence of a weak, and in some cases immature HIV-1-specific humoral immunity in some non-progressing elite controllers, and indicate that actual antibody testing algorithms based on the titer, proportion, or avidity of HIV-specific antibodies cannot be used to discriminate between recent and long-standing infections in these subjects.

Detectable levels of neutralizing antibodies against heterologous HIV-1 strains were observed for the four non-progressing elite controller individuals. Their titers, as measured in sequential plasma samples from each subject, varied from barely detected ($IC^{90} < 1/10$) to fairly high ($IC^{90} > 1/100$), with no detectable correlation to viral load blips, proportion of binding antibodies, or AI. Comparable variation in the titers of neutralizing antibodies against heterologous HIV-1 strains was described previously among distinct elite controller and non-progressing elite controller subjects [Wang et al., 2002; Kloosterboer et al., 2005; Bailey et al., 2006; Verity et al., 2007; Pereyra et al., 2008]. These findings also suggest that very low-level of antigen exposure seems to occur in these patients, sufficient for sustaining a low, but detectable, HIV-1-specific immunity in the setting of undetectable viral load.

The overall low levels of general and specific immune activation seen in the non-progressing elite controllers may reflect the extremely low levels of HIV-1 antigenic stimulation due to the suppression of viral replication, supporting the hypothesis that an antigenic threshold must be reached to sustain high-level chronic immune activation and HIV-1-specific immune responses [Ferbas et al., 1995; Hogervorst et al., 1995; Jin et al., 2000; Bailey et al., 2006; Verity et al., 2007]. Consistent with this model, reduction of viral replication to undetectable levels in patients under HAART also coincides with a decline of general immune activation markers [Autran et al., 1997; Bisset et al., 1998; Bouscarat et al., 1998; Giorgi et al., 1998], and the titers of HIV-1-specific binding and neutralizing antibodies [Morris et al., 1998; Markowitz et al., 1999; Binley et al., 2000; Killian et al., 2006; Bongertz et al., 2007; Hayashida et al., 2008]. At the same time, however, abnormally high $CD8^+$ T-cell activation levels [Greenough et al., 1999; Wang et al., 2002; Hunt et al., 2008] and strong HIV-1-specific humoral responses [Wang et al., 2002; Kloosterboer et al., 2005; Pereyra et al., 2008] have been described in some elite controller and non-progressing elite controller subjects. In this study, one of the four individuals analyzed (Patient 46) exhibited high levels of $CD8^+$ T-cell counts, β2-M, and antibody avidity to HIV-1 proteins. Overall, these results support the notion that non-progressing elite controllers (and elite controllers) are immunologically heterogeneous [Pereyra et al., 2008], and suggest that individual immune responses are not simply driven by the level of plasma viremia.

Because most of these individuals are identified after several years of HIV infection, very little is known about when and how the infection starts to be controlled. Some clues, however, could be obtained from the analysis of antibody avidity. HIV-1 viremia must be sustained initially for at least 1 year to generate a fully mature response, and initiation of HAART during acute/early HIV infection may block the typical evolution of the HIV-1-specific antibody response [Binley et al., 2000; Kassutto et al., 2005; Hare et al., 2006; Killian et al., 2006; Hayashida et al., 2008], and prevents the maturation of antibody avidity [Selleri et al., 2007]. Initiation of HAART during the chronic phase of HIV infection, however, seems to have only minor effects on the AI [Suligoi et al., 2002, 2003]. Thus, the low AI observed in some non-progressing elite controllers may reflect a very early containment of HIV-1 replication, before a fully mature antibody response had sufficient time to develop. The suppression of viremia during the acute phase of HIV infection seen in some HLA-B57 elite controllers [Altfeld et al., 2003], and the finding that most non-progressing elite controllers have homogeneous populations of ancestral proviruses that date close to the patient's seroconversion time [Bello et al., 2005] are consistent with this hypothesis. Data from elite controller macaques also demonstrate that viral replication is quickly brought under control after acute phase viremia [Loffredo et al., 2007]. The high AI observed in Patient 46 may reflect a later initial suppression of viral replication.

In summary, the long-term persistence of normal percentages of $CD3^+$ $HLA\text{-}DR^+$ and $CD8^+$ $CD38^+$ T cells and a low proportion of anti-gp41-binding antibodies clearly distinguish non-progressing elite controllers among the individuals infected with HIV-1. However, these individuals are not immunologically homogeneous. Some of them show no evidence of immune system abnormalities and display an immature HIV-1-specific humoral immune response similar to that of patients infected recently. We suggest that these patients are the most likely candidates to have "true" non-progressive infection. Other non-progressing elite controllers display little evidences of immune activation and HIV-1-specific antibodies with high avidity. Whether such limited chronic immune activation is a prognostic marker of future progressive disease remains to be determined. Understanding the mechanisms that allow the natural persistence of a detectable HIV-1-specific immunity in the absence of chronic activation and damage of the immune system for long time periods may provide unique insights into the development of an effective HIV vaccine.

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10 – Conclusões

- O presente estudo é a avaliação mais abrangente da população que procura testagem para o HIV na área metropolitana do Rio de Janeiro realizado até agora e pode contribuir para a formulação de políticas renovadas com o objetivo de prevenir e tratar pessoas vivendo com HIV/AIDS no Rio de Janeiro.

- Nossos dados destacam que os HSH continua a ser uma população altamente vulnerável e intervenções de prevenção centradas neste grupo devem ser continuamente implementadas.

- Devido ao fato de que nossos dados foram obtidos a partir de uma população que procurou por iniciativa própria o teste de HIV, os resultados não podem ser generalizados para a população em geral ou para o conjunto de homens que fazem sexo com homens que vivem na região metropolitana do Rio de Janeiro e devem ser vistos com a cautela necessária.

- Nos CTAs aqui estudados a prevalência de infecção pelo HIV se mostrou associada à composição da população atendida, e diferenças foram evidenciadas mesmo estando os centros em uma mesma região metropolitana.

- Embora tenham sido encontradas taxas de prevalência consistentemente mais elevadas na capital, o cenário que foi observado na periferia não é muito diferente e o baixo uso de preservativos compõe um cenário propício a novas infecções.

- Campanhas de prevenção devem se concentrar na prevenção de doenças sexualmente transmissíveis em geral e buscar intensificar os esforços na

periferia, em indivíduos com menor escolaridade e enfatizar a importância da retestagem como uma ferramenta para o diagnóstico precoce.

- A aplicação de uma zona cinzenta contribuiu para uma redução significativa da presença de amostras indeterminadas quando BED-CEIA e os testes de AI foram aplicados para a avaliação da infecção recente pelo HIV-1.

- A avaliação do estado imune nas amostras com resultados discordantes - após a etapa de "zona cinzenta" - mostrou-se útil para a identificação de amostras com resultado falso recente.

- Os dados obtidos sugerem que o teste de AI é mais específico do que o teste BED-CEIA.

- O algoritmo aqui proposto não mostrou diferença em cenários com diferentes proporções de amostras inicialmente caracterizadas como incidentes.

- A sensibilidade do algoritmo para reduzir a proporção de amostras indeterminadas associa-se com a proporção de indivíduos com diagnóstico tardio.

- Não foi observada variação importante no padrão de subtipos de HIV-1 quando comparou-se RS e LTS no primeiro ano de estudo.

- O aumento da proporção de amostras recombinantes, bem como uma maior proporção de amostras relacionadas com CRFs, em comparação com URFs foi observado ao longo do tempo e se alinha com dados recentes publicados pela OMS-UNAIDS. Neste estudo esta tendência parece se dever ao aumento da prevalência de amostras caracterizadas como relacionadas a CRFs ao longo do tempo entre os homens, sendo este aumento mais pronunciado entre os heterossexuais do que entre os HSH.

- A abordagem utilizada para este estudo também permitiu a identificação de novos casos de isolados de HIV-1, como genomas recombinantes relacionados ao subtipo K, encontrado pela primeira vez no Rio de Janeiro e uma detecção relativa crescente de amostras recombinantes envolvendo os subtipos A e G entre os indivíduos caracterizados como recém infectados.

- Nossos dados revelam que as taxas globais de resistência primária aos antiretrovirais permaneceram relativamente estáveis ao longo do tempo, entretanto as taxas encontradas relatam um cenário preocupante.

- Entre os HSH a taxa de resistência foi o dobro da registrada em homens heterosexuais, o que sugere uma distribuição desigual em relação à resistencia primária em determinados grupos. Como consequência, recomenda-se que os estudos futuros devem buscar analisar grupos específicos para detectar eventuais padrões.

- Estes resultados alinham nosso estudo com outros realizados no Brasil que têm sugerido um incremento na resistência primária aos antirretrovirais no Brasil, o que sugere a necessidade de intensificar a discussão sobre o custo-benefício da realização do teste de genotipagem antes do início da terapia.

- O aumento da frequência de formas recombinantes e de subtipos que não eram comumente encontrados em nosso meio sinaliza a necessidade de um maior monitoramento de nossos instrumentos de diagnóstico, acompanhamento e tratamento da infecção por HIV-1.

- A longa persistência das baixas proporções de anti-gp41 do HIV-1 sugerem que o teste BED-CEIA pode ser um parâmetro adicional para a triagem de controladores de elite não progressores e que o teste de AI poderia ser utilizado em conjunto para melhorar a especificidade na busca dos LTNPs.

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