Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
Department of Biomedical Sciences

Unit of Virology,
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Search for host genetic factors associated with neurological complications (HAM/TSP) in Peruvian HTLV-1 infected individuals

Dissertation for the degree of Doctor in Biomedical Sciences
at the University of Antwerp to be defended by

Michael John TALLEDO ALBUJAR

Promoters

Prof. Dr. Guy Van Camp

Prof. Dr. Guido Vanham

Local Promoter

Prof. Dr. Jorge Arevalo

Antwerp, 2016

Cover:

Man performing in Cusco, Peru, dressing traditional outfit. In the highland there is a predominance of quechua ancestry. The Peruvian poulation is an admixed population although there are registered 77 ethnics groups according to Instituto Nacional de Desarrollo de Pueblo Andinos, Amazónicos y Afroperuano (INDEPA). The admixture has generated a variety of meals, dances, and dressing that makes Peru a very diverse country but also creates complexity in the search of genetic factors associated with diseases.

Zoektocht naar gastheer genetische factoren in verband met neurologische complicaties (HAM/TSP) van Peruaanse HTLV-1 geïnfecteerde individuen

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CURRICULUM VITAE

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University studies

20015-to date PhD student, University of Antwerp Belgium.

1998-2001 Studies at the sciences Master Program
Post Grade school "Victor Alzamora Castro", Universidad Peruana Cayetano Heredia

1989-1995 Sciences bachelor degree in Biology at Universidad Nacional Federico Vilarreal, (Lima-Perú)

Work experience

- September 2010- up to date, coordinator of basic sciences, HTLV-1 group. Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia.
- August 2005 – Up to date, assistant research, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia.
- October 2011– Up to date, Head of molecular diagnostic lab. Bio Links SA
- Julio 1996-Julio 2005, Head of Paternity Lab. Bio Links SA.

Publications

1. Rosado J, Morales S, Lopez G, Clark D, Verdonck K, Gotuzzo E, Van Camp G, Talledo M. The FAS-670 AA genotype is associated with high proviral load in Peruvian HAM/TSP patient. J Med Virol. 2016 Sep 7. doi: 10.1002/jmv.24681.
2. Menezes SM, Decanine D, Brassat D, Khouri R, Schnitman SV, Kruschewsky R, López G, Alvarez C, **Talledo M**, Gotuzzo E, Vandamme AM, Galvão-Castro B, Liblau R, Weyenbergh JV. CD80+

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5. **Talledo M**, López G, Huyghe J, Verdonck K, González E, Clark D, Vanham G, Gotuzzo E, Van Camp G, and Van Laer. Possible implication of NFKB1A and NKG2D genes in susceptibility to HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis in Peruvian patients infected with HTLV-1. J Med Virol. 2012 Feb;84(2):319-26.
6. Adaui V, Maes I, Huyse T, Van den Broeck F, **Talledo M**, Kuhls K, De Doncker S, Maes L, Llanos-Cuentas A, Schönian G, Arevalo J, Dujardin JC. Multilocus genotyping reveals a polyphyletic pattern among naturally antimony-resistant Leishmania braziliensis isolates from Peru. Infect Genet Evol. 2011 Dec;11(8):1873-80.
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9. **Talledo M**, López G, Huyghe JR, Verdonck K, Adaui V, González E, Best I, Clark D, Vanham G, Gotuzzo E, Van Camp G, Van Laer L.Evaluation of host genetic and viral factors as surrogate markers for HTLV-1-associated myelopathy/tropical spastic paraparesis in Peruvian HTLV-1-infected patients. J Med Virol. 2010 Mar;82(3):460-6.
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11. Contreras CA, Ochoa TJ, Lacher DW, DebRoy C, Navarro A, **Talledo M,** Donnenberg MS, Ecker L, Gil AI, Lanata CF, Cleary TG. Allelic variability of critical virulence genes (eae, bfpA and perA) in typical and atypical enteropathogenic Escherichia coli in Peruvian children. J Med Microbiol. 2010 Jan;59(Pt 1):25-31.
12. Montoya, Y., Leon, C., **Talledo, M**., Nolasco, O., Padilla, C., Muñoz-Najar, U. and Barker, D.C. (1997). Recombinant antigens for specific and sensitive serodiagnosis of Latin American tegumentary Leishmanisis. Transactions of the Royal Society of Tropical Medicine and Hygiene 91, 674 -676

Research Projects Participation

1. PI. Evaluación de los efectos anti-inflamatoria y anti-viral del veneno de Apis mellifera en linfocitos infectados con el virus linfotrópico de celulas T humanas tipo 1. Programa Nacional de Innovación Agraria, Lima-Perú. 2016-2018
2. Coordinator of the Peruvian HTLV-1 Component, project" Development a Rapid Diagnostic Test for HTLV-1 infection. 2014-2016. DGD
3. Coordinator of the Peruvian component in the project about "Identification of biomarkers and therapeutic targets for human T-lymphotropic virus 1-associated myelopathy/tropical spastic paraparesis in Peru". Institute of Tropical Medicine "Alexander von Humboldt" Universidad Peruana Cayetano Heredia – Katholieke Universiteit Leuven, Rega Institute for Medical Research, Clinical and Epidemiological Virology, Belgium. Vlaamse Interuniversitaire Raad. (VLIR). 2010-2015
4. Responsible for the genetic part in Peru: Identification of genetic polymorphisms involved in human susceptibility to HTLV-1 infection in children from endemic area. Institute of Tropical Medicine "Alexander von Humboldt" Universidad Peruana Cayetano Heredia INSERM U550, Laboratoire de Génétique Humaine des Maladies. Association pour la recherche sur le cancer France 2009-2011.
5. PhD student. Host genetic, immune and viral factors in transmission and diseases expression of Human T-Lymphotropic Virus type 1 (HTLV-1) in Peru. Institute of Tropical Medicine "Alexander von Humboldt" Universidad Peruana Cayetano Heredia – Antwerp University – Institute of Tropical Medicine Antwerp-Belgium. Vlaamse Interuniversitaire Raad. (VLIR).

International Courses

1. "Computational Approaches to Analyzing Microarray Data". June 20-24, 2012. Madison-Wiconsin USA
2. Human Participants Protection Education for Research Teams online course, sponsored by the National Institutes of Health (NIH), on 01/26/2006
3. Genetic epidemiology of Complex Diseases. 22-26 August 2005. Erasmus Medical Center, Rotterdam, The Netherlands.
4. Searching Genes in Complex Disorders. 15-19 August 2005. Erasmus Medical Center, Rotterdam, The Netherlands.
5. Case Control Studies. 15-19 August 2005. Erasmus Medical Center, Rotterdam, The Netherlands.
6. Principles of Genetic Epidemiology. 8-12 August 2005. Erasmus Medical Center, Rotterdam, The Netherlands.
7. Theoretical and practical course: "I Curso Internacional de Genética Forense – Haplotipos del cromosoma Y" Medellín-Colombia, 25-28th March 2003
8. Theoretical and practical course "Nuevas Metodologías de Análisis de ADN en Estudios de Paternidad In Vivo y Post Morten" 24-28th Buenos Aires - Argentina
9. Theoretical and practical course "Uso de Perfiles Genéticos en Forénsica y Paternidad Implicaiones Eticas y Legales" Caracas-Venezuela
10. Theoretical and practical course "Yeast Molecular Genetics" 26-30th May 1997 Trieste, Italia
11. Theoretical and practical course "Transfection and Expression In Parasites" Buenos Aires-Argentina

Congress and Scientific Mettings

1. American Association for Cancer Research Annual Meeting. 31 Marzo – 4 Abril. 2012
2. 17th International Symposium on Human Identification October 9-12, 2006, Nashville-USA
3. 15th International Symposium On Human Identification. October 4-7 2004. Phoenix-USA
4. Parentage and Mixture Statistics Workshop October 4, 2004. Phoenix-USA
5. Seminario Internacional recuperando Identidades: Antropología y Genética Forense en las Graves Violaciones a los Derechos Humanos. Universidad Nacional Mayor der San Marcos. 16-17 October 2003. Lima-Perú
6. Diversidad Genómica en Humanos y patógenos. Universidad Nacional San Antonio Abad del Cusco 6-9 de October 2003. Cusco-Perú
7. I Foro Internacional Sobre Identificación Humana por ADN en Criminalística. 8-10 July 2002. Lima Peru

8. 12th International Symposium on Human Identification. 9-12 October 2001 Biloxi, Mississippi. USA.
9. Primera Jornada Iberoamericana de Genética Forense. Sociedad Argentina de Genética Forense. 11 December 2000.Buenos Aires - Argentina
10. Curso Internacional de " Toxicología Genética". Universidad Nacional Agraria La Molina. 6 -11 November 2000. Lima-Perú
11. "Análisis de la Variabilidad Genética y sus Aplicaciones Actuales". Universidad Peruana Cayetano Heredia. 22-26 March 1999

Courses (member of the organization committee)

1. XIII Curso y Taller de Adiestramiento en Técnicas de Biología Molecular Aplicadas a Enfermedades Infecciosas y Tropicales. Universidad Peruana Cayetano Heredia. February 2016
2. XII Curso y Taller de Adiestramiento en Técnicas de Biología Molecular Aplicadas a Enfermedades Infecciosas y Tropicales. Universidad Peruana Cayetano Heredia. February 2015
3. XI Curso y Taller de Adiestramiento en Técnicas de Biología Molecular Aplicadas a Enfermedades Infecciosas y Tropicales. Universidad Peruana Cayetano Heredia. February 2014
4. X Curso y Taller de Adiestramiento en Técnicas de Biología Molecular Aplicadas a Enfermedades Infecciosas y Tropicales. Universidad Peruana Cayetano Heredia. February 2013
5. IX Curso y Taller de Adiestramiento en Técnicas de Biología Molecular Aplicadas a Enfermedades Infecciosas y Tropicales. Universidad Peruana Cayetano Heredia. February 2012
6. VIII Curso y Taller de Adiestramiento en Técnicas de Biología Molecular Aplicadas a Enfermedades Infecciosas y Tropicales. Universidad Peruana Cayetano Heredia. February 2011
7. VII Curso y Taller de adiestramiento en Técnicas de Biología Molecular Aplicadas a Enfermedades Infecciosas y Tropicales (PCR, Real Time-PCR y Secuenciamiento). February 2010

Oral presentations in International and National meetings

14th International Conference on Human Retrovirology: HTLV and related retroviruses. Salvador, Bahia-Brazil. July 1st to July 4th 2009

1. KIR genes and HLA-C subgroup frequencies in Peruvian HAM/TSP patients and healthy HTLV-1 carriers
2. Evaluation of candidate genes and viral markers for HTLV-1-associated myelopathy/tropical spastic paraparesis in Peruvian HTLV-1-infected individuals

Primera Conferencia de HTVL en el Perú. Lima-Peru 27 y 28 de Julio del 2009.

1. Aspectos de Genética Humana en la infección por HTLV-1

American Phytopathology Society Annual Meeting. August 7-11, 2010, Charlotte USA.

1. **Talledo M.,** Morales S., Trinidad E., Arévalo J., Trelles A., Montoya Y. Description of two Phytomonas genotypes associated to oil palm diseases in Peru: Marchitez Sorpresiva and a new disease manifestation - Marchitez Lenta

XXI CONGRESO PERUANO DE FITOPATOLOGIA Tarapoto-Perú, del 05 al 10 de Setiembre del 2010

Abstract and oral presentation

1. Morales S., **Talledo M.,** Trinidad E., Trelles A, Tirado H., Arévalo J., Montoya Y. Malezas e insectos: posibles reservorios y vectores de Phytomonas asociadas a enfermedades en palma aceitera.

Poster presentation

1. Melo Omar, Gotuzzo Eduardo, López Giovanni, **Talledo Michael**. KIR genes and adult T-cell leukemia/ lymphoma in patients infected with HTLV-1. Retrovirology 2015 12(Suppl 1):P16. 17th International Conference on Human Retroviruses: HTLV and Related Viruses Trois Ilets, Martinique. 18-21 June 2015
2. Rúa Jorge, Rosado Jason, López Giovanni, Alvarez Carolina, Clark Daniel, Gotuzzo Eduardo, **Talledo Michael**. IL8 Gene Polymorphism SNP rs4073 analysis between HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis and HTLV-1 Carriers. Retrovirology 2015 12(Suppl 1):O36. 17th International Conference on Human Retroviruses: HTLV and Related Viruses Trois Ilets, Martinique. 18-21 June 2015
3. Bernia Cynthia, Rosado Jason, López Giovanni, Gotuzzo Eduardo, **Talledo Michael**. A NKG2D Thr72Ala polymorphism is not associated with HAM/TSP and proviral load values in Peruvian HTLV-1 infected patients with HAM/TSP and asymptomatic carriers. Retrovirology 2015 12(Suppl 1):O37. 17th International Conference on Human Retroviruses: HTLV and Related Viruses Trois Ilets, Martinique. 18-21 June 2015
4. **Michael Talledo**, Jason Rosado, Carolina Alvarez, Daniel Clark, Eduardo Gotuzzo. Differential expression in genes involved in the NF-κβ pathway among asymptomatic HTLV-1 carriers and HAM/TSP patients in Peru**.** Conference on Human Retrovirology: HTLV and Related Viruses held in Montreal on June 26-30, 2013.
5. Morayma Temoche, Jason Rosado, Carolina Alvarez, Daniel Clark, Eduardo Gotuzzo, **Michael Talledo**. Proteomic profiles among asymptomatic HTLV-1 carriers and HAM/TSP patients in Peru.

Conference on Human Retrovirology: HTLV and Related Viruses held in Montreal on June 26-30, 2013.

6. Jason Rosado, Carolina Alvarez, Daniel Clark, Eduardo Gotuzzo, **Michael Talledo**. Differential miRNA expression profiles in Peruvian HTLV-1 carriers. 16th International Conference on Human Retrovirology: HTLV and Related Viruses held in Montreal on June 26-30, 2013.
7. Carolina Alvarez, Kristien Verdonck, Martín Tipismana, **Michael Talledo**, Jason Rosado, Daniel Clark, Johan Van Weyenbergh, Anne-Mieke Vandamme, Eduardo Gotuzzo. HAM/TSP in relatives of HAM/TSP cases and in relatives of asymptomatic HTLV-1 carriers**.** 16th International Conference on Human Retrovirology: HTLV and Related Viruses held in Montreal on June 26-30, 2013.
8. **Michael Talledo,** Giovanni López, Kristien Verdonck, Elsa González, Martin Tipismana, Eduardo Gotuzzo,Daniel Clark. Pattern mRNA expression similarities between ATL patients and patients coinfected with HTLV-I-Strongyolides stercoralis. Retrovirology 2011 8(Suppl 1):A165.
9. **Michael Talledo,** Giovanni López, Jeroen R Huyghe, Kristien Verdonck, Elsa González, Martin Tipismana, Daniel Clark, Guido Vanham, Eduardo Gotuzzo, Guy Van Camp, Lut Van Laer. Host genetic factors associated to proviral load in Peruvian HTLV-1 infected. Retrovirology 2011 8(Suppl 1): A147.
10. Sandra Morales, Jason Rosado, Giovanni López, Jeroen Huyghe, Kristien Verdonck, Elsa González,Martin Tipismana, Daniel Clark, Guido Vanham, Eduardo Gotuzzo, Guy Van Camp, Lut Van Laer, **Michael Talledo.** HLA-G 14 bp insertion/deletion polymorphism is not associated to proviral load levels and presence of HAM/TSP in Peruvian HTLV-1 infected individuals
11. Britta Moens, Daniele Decanine, Ricardo Khouri, Giovanni Lopez, **Michael Talledo,** Eduardo Gotuzzo,Françoise Bex, Bernardo Galvão Castro, Anne-Mieke Vandamme, Johan Van Weyenbergh. Ascorbic acid has superior antiviral and antiproliferative effects over IFN-alpha in HAM/TSP PBMC ex vivo. Retrovirology 2011 8(Suppl 1):A61.
12. **Michael Talledo,** Giovanni López, Jeroen R. Huyghe, Kristien Verdonck, Elsa González, Daniel Clark, Guido Vanham, Eduardo Gotuzzo, Guy Van Camp and Lut Van Laer. Host genetic factors and susceptibility to HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis in Peruvian HTLV-1 Infected

Table of Contents

Abbreviations

AC	Asymptomatic carriers of HTLV-1
AIMs	Ancestry-informative markers
AP-1	Activated protein 1
ATF	Activating transcription factors
CREB	Cyclic adenosine monophosphate (cAMP) response element binding
CNS	Central nervous system
ATL	Adult T-cell leukaemia/lymphoma
BhLH	Basic helix-loop-helix
CTL	Cytotoxic T cells
DC-SIGN	DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HAM/TSP	HTLV-1-associated myelopathy/tropical spastic paraparesis
HBZ	HTLV-1 bZIP factor
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
hn-RNP	Heterogeneous nuclear ribonucleoproteins
HSPG	Heparan sulphate proteoglycans
HTLV-1	Human T-lymphotropic virus 1
IFN-γ	Interferon gamma
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-15	Interleukin 15
IMTAvH	Instituto de Medicina Tropical Alexander von Humboldt
KIR	Killer-cell immunoglobulin-like receptors
LFA-1	Leukocyte function-associated antigen-1
LTR	Long terminal repeat
MDDCs	Monocyte-derived dendritic cells
MHC	Major histocompatibility complex
MMP-9	Matrix metalloproteinase-9
MTOC	Microtubule-organizing centre
NF-kB	Nuclear factor kappa B
NRP1	Neuropillin-1
PBMC	Peripheral blood mononuclear cells
PTLV	Primate T-cell lymphotropic Virus

PVL	Proviral load
SDF	Stromal cell-derived factor-1
SNP	Single nucleotide polymorphism
SRF	Serum responsive factor
STLV	Simian T-cell lymphotropic virus
TNF-α	Tumour necrosis factor-alpha
TRE	Tax-response element
UTR	Untranslated region

Summary

Search for host genetic factors associated with neurological complications (HAM/TSP) in Peruvian HTLV-1 infected individuals

Infection by the human T-cell leukemia virus type 1 (HTLV-1) retrovirus is widely spread throughout the world. This infection is also endemic in Peru with an estimated 150,000-450,000 infected individuals. Between 1 and 10% of HTLV-1 infected individuals develops a disease manifestation associated with this infection. HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) is the major neurodegenerative complication of HTLV-1 infection and is characterized by difficulties in the movement of lower limbs. With regard to it's pathogenesis, no differences in viral genotypes have been associated with HAM/TSP disease. A high proviral load has consistently been associated with HAM/TSP, but there is important overlap with asymptomatic carriers (AC). In view of these findings, it is hypothesized that that host genetic factors play a role in the etiology of HAM/TSP.

This thesis therefore aims at finding genetic factors associated with HAM/TSP in Peruvian HTLV-1 infected individuals. In a first approach we tried to confirm previous findings of SNPs associated with HAM/TSP in Japan. However, no replication was found in Peruvian HTLV-1 infected individuals. In a second study we focused on natural killer genetic markers and analyzed variants in *KIR* genes, but found no differences in allele frequencies between HAM/TSP and AC. In a third study we evaluated the frequency distribution of SNPs belonging to several candidate genes between cases and controls. These included various innate immunity functions, genes encoding cytokines, chemokines and human leukocyte antigens (HLA), and genes involved in cell adhesion. In addition, ancestry Informative markers were used to control for population stratification. Variants in two genes, *NKG2D* and *NFKBIA*, were associated with HAM/TSP disease. In a final study we studied the expression of the *NFKBIA* gene, and found it to be under-expressed in HAM/TSP compared to AC. The *NFKBIA* encodes the protein IkBα, which is an inhibitor of the NF-kB pathway and prevents the entrance of NF-kB into the nucleus. On the basis of these findings we hypothesize that HAM/TSP patients have less IkBα protein

than AC, exerting less inhibition. This might be one of the reasons why HAM/TSP patients show more activation of the NF-kB pathway than AC.

Samenvatting

Zoektocht naar gastheer genetische factoren in verband met neurologische complicaties (HAM/TSP) van Peruaanse HTLV-1 geïnfecteerde individuen

Infectie met het humaan T-cel leukemie virus type 1 (HTLV-1) retrovirus is wijd verspreid over de hele wereld. Deze infectie is ook endemisch in Peru met een raming van 150.000-450.000 besmette personen. Tussen 1 en 10% van de HTLV-1 geïnfecteerde individuen ontwikkelt een klinische manifestatie van de ziekte. HTLV-1 geassocieerde myelopathie / tropische spastische paraparese (HAM/TSP) is een belangrijke complicatie van HTLV-1 infectie. Het is een neurodegeneratieve ziekte die problemen in de motoriek van de onderste ledematen veroorzaakt. Er werden geen verschillen in virale genotypes gevonden tussen HAM/TSP en asymptomatische dragers (AC). Een hoge provirale lading werd wel geassocieerd met HAM/TSP, maar er is een belangrijk overlap met AC. Gezien deze bevindingen, is onze basishypothese dat humane genetische factoren een rol spelen in de etiologie van HAM/TSP.

Dit doctoraal proefschrift stelt zich tot doel om genetische factoren te identificeren, die samenhangen met HAM/TSP in een cohorte van Peruaanse HTLV-1 besmette personen. In een eerste benadering probeerden we een associatie tussen bepaalde SNPs en HAM/TSP, die eerder in Japan gevonden was, te bevestigen, maar dit bleek niet te kloppen in de Peruaanse HTLV-1 cohorte. In een tweede studie werden varianten in KIR genen geanalyseerd, maar we vonden geen verschillen in allel-frequenties tussen HAM/TSP en AC. In een derde studie werd de frequentieverdeling van SNPs behorend tot meerdere kandidaat-genen tussen HAM/TSP cases en AC controles geëvalueerd. Afkomst informatieve markeringen (AIM) werden gebruikt om te corrigeren voor de etnische stratificatie van de bevolking. Varianten in twee genen, NKG2D en NFKBIA, bleken geassocieerd te zijn met HAM/TSP. In een laatste studie onderzochten we de expressie van het NFKBIA-gen, en vonden dat het minder tot expressie kwam in HAM/TSP vergeleken met AC. De NFKBIA codeert het eiwit IkBα, die een inhibitor is van de NF-kB pathway en zo voorkomt dat NF-kB naar de celkern transloceert. Op basis van deze observatie veronderstellen we dat HAM/TSP patiënten minder IkBα eiwit aanmaken en dus minder remming uitoefenen op de NF-kB pathway, wat uiteindelijk leidt tot een verhoogde pro-inflammatoire activiteit bij HAM/TSP in vergelijking met AC.

Publications

1. Menezes SM, Decanine D, Brassat D, Khouri R, Schnitman SV, Kruschewsky R, López G, Alvarez C, **Talledo** M, Gotuzzo E, Vandamme AM, Galvão-Castro B, Liblau R, Weyenbergh JV. CD80+ and CD86+ B cells as biomarkers and possible therapeutic targets in **HTLV**-1 associated myelopathy/tropical spastic paraparesis and multiple sclerosis. J Neuroinflammation. 2014 Jan 29;11:18. doi: 10.1186/1742-2094-11-18.
2. Moens B, Pannecouque C, López G, **Talledo** M, Gotuzzo E, Khouri R, Bittencourt A, Farré L, Galvão-Castro B, Vandamme AM, Van Weyenbergh J. Simultaneous RNA quantification of human and retroviral genomes reveals intact interferon signaling in **HTLV**-1-infected CD4+ T cell lines. Virol J. 2012 Aug 23;9:171. doi: 10.1186/1743-422X-9-171.
3. Moens B, Decanine D, Menezes SM, Khouri R, Silva-Santos G, Lopez G, Alvarez C, **Talledo** M, Gotuzzo E, de Almeida Kruschewsky R, Galvão-Castro B, Vandamme AM, Van Weyenbergh J. Ascorbic acid has superior ex vivo antiproliferative, cell death-inducing and immunomodulatory effects over IFN-α in **HTLV**-1-associated myelopathy. PLoS Negl Trop Dis. 2012;6(7):e1729. doi: 10.1371/journal.pntd.0001729. Epub 2012 Jul 24.
4. **Talledo** M, López G, Huyghe JR, Verdonck K, González E, Clark D, Vanham G, Gotuzzo E, Van Camp G, Van Laer L. Possible implication of NFKB1A and NKG2D genes in susceptibility to **HTLV**-1-associated myelopathy/tropical spastic paraparesis in Peruvian patients infected with **HTLV**-1. J Med Virol. 2012 Feb;84(2):319-26. doi:10.1002/jmv.22255.
5. Adaui V, Maes I, Huyse T, Van den Broeck F, **Talledo** M, Kuhls K, De Doncker S, Maes L, Llanos-Cuentas A, Schönian G, Arevalo J, Dujardin JC. Multilocus genotyping reveals a polyphyletic pattern among naturally antimony-resistant Leishmania braziliensis isolates from **Peru**. Infect Genet Evol. 2011 Dec;11(8):1873-80. doi: 10.1016/j.meegid.2011.08.008. Epub 2011 Aug 17.
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Chapter 1 Introduction

Human T-lymphotropic virus 1 (HTLV-1), a single-stranded RNA virus, belongs to the Retroviridae family, together with the human immunodeficiency virus type I (HIV-I), which was originally named HTLV-III (Popovic et al., 1984). Worldwide, an estimated 5 to 10 million individuals are infected with HTLV-1 (Gessain and Cassar, 2012). The virus gives rise to a lifelong infection. The majority of HTLV-1-infected subjects remain asymptomatic, but up to 10% of the carriers develop serious complications. HTLV-1 infection has been associated with malignant diseases such as adult T-cell leukaemia/lymphoma (ATL), inflammatory diseases including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), Sjögren's syndrome, uveitis, polymyositis, arthropathy, thyroiditis, infective dermatitis and several "opportunistic" infectious diseases such as strongyloidiasis, tuberculosis and scabies (Table 1) (Proietti FA, 2005). Clearly, HTLV-1 causes serious health problems in those areas of the world where the infection is prevalent. The consequences may be very severe (i.e. death from ATL or chronic disability from HAM/TSP). The high proviral load is the only consistent risk factor associated with HAM/TSP but does not explain fully the disease appearance, and the treatment options for this disease are very limited. Moreover, although HTLV-1 has not been included in the list of neglected tropical diseases (http://www.who.int/neglected_diseases/diseases/en/), it shares several characteristics with these conditions: the infection primarily affects people in

developing countries, it promotes poverty because it often causes chronic and disabling complications that affect several members of the same family, and health interventions and research are insufficient (Casseb, 2009).

Table 1. Diseases associated with HTLV-1 infection

Factor, condition	**Strength of evidence in favour of an association**	**Subclass**
Adult T-cell leukaemia (ATL)	++++	Neoplasia
HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)	++++	Inflammatory syndrome
Uveitis	++++	Inflammatory syndrome
Tuberculosis	++	Infectious complication
Strongyloidiasis	++++	Infectious complication
Scabies	++	Infectious complication
Infective dermatitis	+++	Infectious complication
Polymyositis, inclusion body myositis	++	Inflammatory syndrome
HTLV-1-associated arthritis	++	Inflammatory syndrome
Pulmonary infiltrative pneumonitis	++	Infectious complication
Sjögren's syndrome	+	Inflammatory syndrome

++++, proven association;
+++, probable association;
++, likely association;
+, possible association
Adapted from Proietti et al (Proietti FA, 2005)

1. Origin and spread

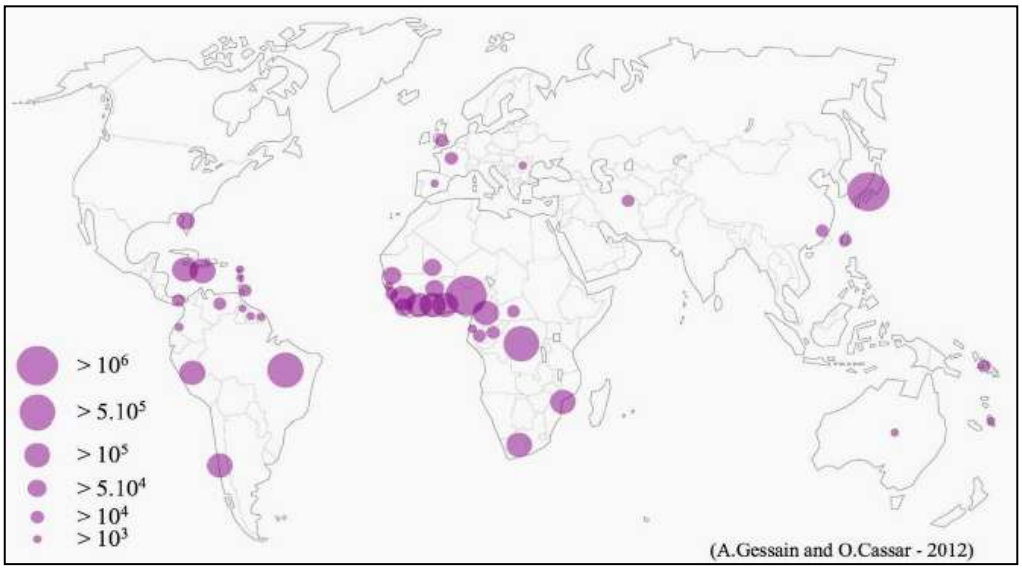
The primate T-cell lymphotropic viruses (PTLV) include human (HTLV) and simian (STLV) T-lymphotropic viruses. Epidemiological and phylogenetic data suggest that these viruses originated in African monkeys. Some of the subtypes were transmitted to humans and disseminated to many parts of the world. Four types of HTLV have been described, of which HTLV-1 and HTLV-2 are much more prevalent than HTLV-3 and HTLV-4 (Calattini S, 2005; Kalyanaraman et al., 1982; Mahieux and Gessain, 2009; Wolfe et al., 2005). Whereas HTLV-1 is clearly pathogenic, the clinical consequences of HTLV-2 infection are less prominent. Nevertheless, HTLV-2 has been linked with some cases of HAM/TSP, pneumonia, bronchitis, arthritis, and increased mortality (Roucoux DF, 2004). Whether HTLV-3 and HTLV-4 are associated with human diseases remains unknown.

HTLV-1 is the most studied of these viruses. Although it has a very low sequence variation, seven subtypes have been described (a-g), the cosmopolitan subtype HTLV-1a, the Australo-Melanesian subtype HTLV-1c and the five central African subtypes HTLV-1b, HTLV-1d, HTLV-1e, HTLV-1f, and HTLV-1g. The cosmopolitan subtype HTLV-1a is divided into different subgroups: Transcontinental "A or TC", Japanese "B or Ajp", West African "C or Awa", North African "D or Ana" and Peruvian Black "E" (Gessain and Cassar, 2012; Van Dooren et al., 1998).

Twenty years ago, it was estimated that 15 to 20 million people were infected with HTLV-1 worldwide (de Thé G, 1993). This figure is likely to be imprecise, mainly because in large parts of the world, prevalence studies of the general population have never been done. Most estimations are based on studies of blood donors, pregnant women, indigenous people, people with sexual risk behaviour and other selected population groups (Hlela C, 2009 ; Proietti FA, 2005). In addition, the diagnostic methods and criteria varied widely among these studies. It is clear however, that HTLV-1 is not evenly distributed across the world. Regions with a high prevalence in the general population include the South of Japan (6%), the Melanesian islands (14%), the Caribbean (Jamaica and Trinidad and Tobago; 6%), Africa (Guinea-Bissau, Cameroon and Benin; 5%), and particular areas in South America, with a prevalence up to 2% in Argentina, Brazil, Peru and Colombia; (Gonçalves DU, 2010; Proietti and Carneiro-Proietti, 2006; Yanagihara R, 1990).

In most of these countries, the distribution of HTLV-1 is not homogeneous, with pockets of high prevalence in certain regions. In areas with a low prevalence (<1%) such as Europe and North-America, HTLV-1 infection is mainly found in immigrants coming from endemic regions, in intravenous drug users and in close relatives of these population groups (Gonçalves DU, 2010). Overall, a systematic review performed by Gessain and Cassar (Gessain and Cassar, 2012) have estimated that there are between 5 and 10 million HTLV-1-infected individuals worldwide (Figure 1).

Figure 1.-Prevalence of HTLV-1 infection in different countries (Gessain and Cassar, 2012).



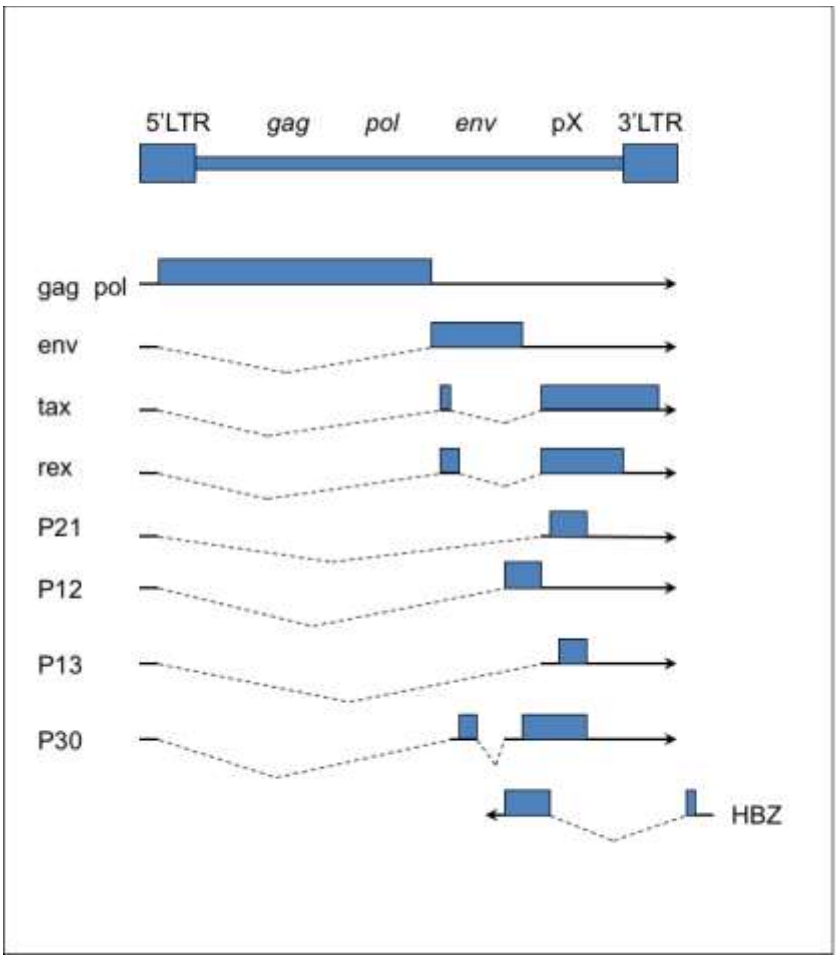
Worldwide distribution of HTLV-1-infected individuals. The proportions of individuals infected were estimated mainly from studies with blood donors, pregnant women and patients not representative of the population.

2. Virological aspects and interaction with host cell metabolism

HTLV-1 was the first human oncogenic retrovirus discovered in 1980 (Poiesz, 1980). Its single stranded RNA is 9 Kb in size. The mature virion is spherical, enveloped and has a diameter of 110 to 140 nm. Similar to other retroviruses, the genome of HTLV-1 contains *gag* which encodes structural proteins that constitute the viral core, *pol* encoding replication enzymes and *env* encoding envelope proteins flanked by long terminal repeat (LTR) sequences at both ends (Seiki, 1983) (Figure 2). The pX region between *env* and the 3'-LTR is characteristic of HTLV-1. This pX region encodes several regulatory proteins: p40 (*tax*), p27 (*rex*), p12, p13, p30 and p21. The minus strand of the pX region

encodes an additional regulatory protein: the HTLV-1 basic zipper factor (*hbz*) (Figure 2) (Gaudray, 2002).

Figure 2. HTLV-1 genomic structure. The known HTLV-1 genes are indicated.



Genomic structure of HTLV-1 showing the gag, pol and env genes flanked by the long terminal repeats (LTRs). The tax, Rex, p21, p12, p13 and p30 proteins are located at the 3`portion inside a region called pX. The HBZ protein is encoded in the minus strand RNA and is synthesized from the 3` LTR.

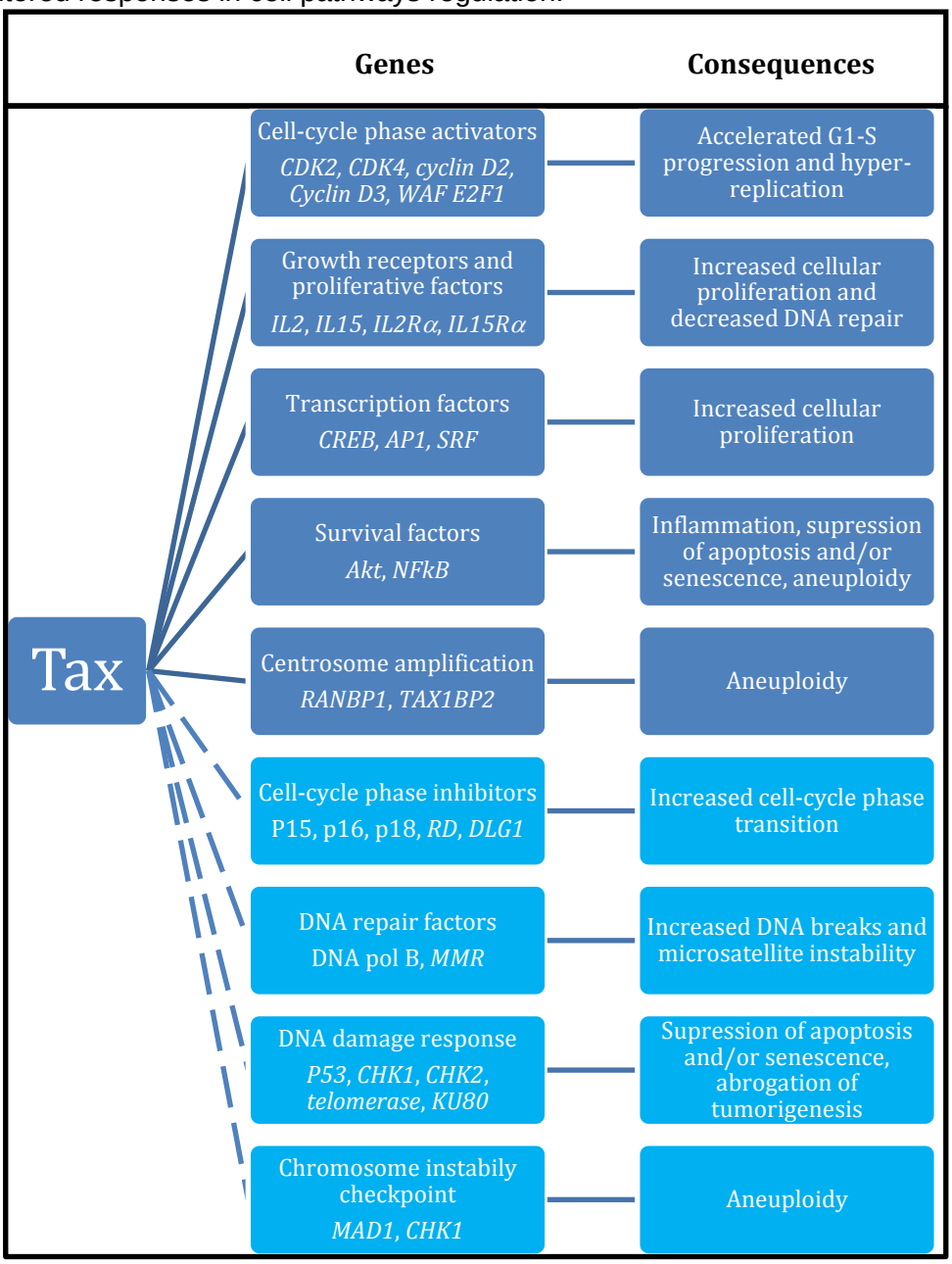
Tax is a pleiotropic viral oncoprotein that plays an important role in viral gene transcription and replication. Besides binding to the Tax response element 1 (TRE-1) located in the 5'-LTR of the proviral DNA in the host cells, which promotes viral transcription, Tax also interacts with many cellular transcription factors. This eventually leads to the expression of host proteins, which stimulates proliferation of HTLV-1-infected cells (Beimling, 1989; Yoshida, 2001). Thus, Tax transactivates genes that encode cytokines, transcription factors, apoptotic inhibitors, cell adhesion molecules and nuclear proteins known to inhibit DNA repair, and it increases DNA replication and cellular proliferation (Matsuoka, 2007). Tax also activates transcriptional pathways involving nuclear factor kappa B (NF-kB), serum responsive factor (SRF), activated transcription factors and cyclic AMP response element-binding protein (ATF/CREB), activated protein 1 (AP-1) as well as basic helix-loop-helix (bHLH) proteins such as c-Myc and MyoD. The NF-kB pathway is tightly regulated in normal T cells but is constitutively active in HTLV-1-associated diseases as well as in many human cancers (Currer et al., 2012). All these Tax effects eventually lead to cell cycle perturbation, cell transformation, and inhibition of apoptosis (Baydoun et al., 2007; Currer et al., 2012; Kashanchi and Brady, 2005) (Figure 3). Clearly, in combination with other, not yet fully identified factors, Tax can promote cell transformation and oncogenesis.

HBZ, a gene encoded by the minus strand, can exert activities as mRNA and as a protein. HBZ antagonizes many activities of Tax, suggesting that they have distinct roles in the HTLV-1 pathogenesis (Ma et al., 2016). The HBZ

mRNA abundance correlates with proviral load in AC, HAM/TSP and ATL patients (Andrade et al., 2013; Saito et al., 2009). The *HBZ* gene has two isoforms an unspliced (usHBZ) form and a spliced (sHBZ) form, which differ in their 5`UTR and the first several amino acids (Gaudray, 2002).

The HBZ mRNA promotes T-cell proliferation (Satou et al., 2006). The spliced form of HBZ (sHBZ) is transcribed in most ATL cases and induces T-cell proliferation (Ma et al., 2016; Yoshida et al., 2008). Therefore, the role of HBZ may be to maintain ATL transformation.

Figure 3.- Summary of Tax interactions with host factors and induction of altered responses in cell pathways regulation.



Genes activated by Tax (solid lines), genes inactivated by Tax (- - - - - dashed lines). *AP1*, activator protein 1; *CDK*, cyclin-dependent kinase; *CHK1*, checkpoint kinase 1; *CHK2*, checkpoint kinase 2; *CREB*, cyclic AMP responsive element binding protein; *DLG1*, discs large homologue 1; IL, interleukin; *IL15Rα*, interleukin 15 receptor α; *IL2Rα*, interleukin 2 receptorα; *MAD1*, mitotic arrest deficiency protein 1; *MMR*, mismatch repair; *NER*, nuclear excision repair; *NFκB*, nuclear factor κB; *PCNA*, proliferating cell nuclear antigen; *RANBP1*, Ran-binding protein 1; *RB*, retinoblastoma; *SRF*, serum response factor;*TAX1BP2*, Tax-binding protein 2. Adapted from (Matsuoka, 2007).

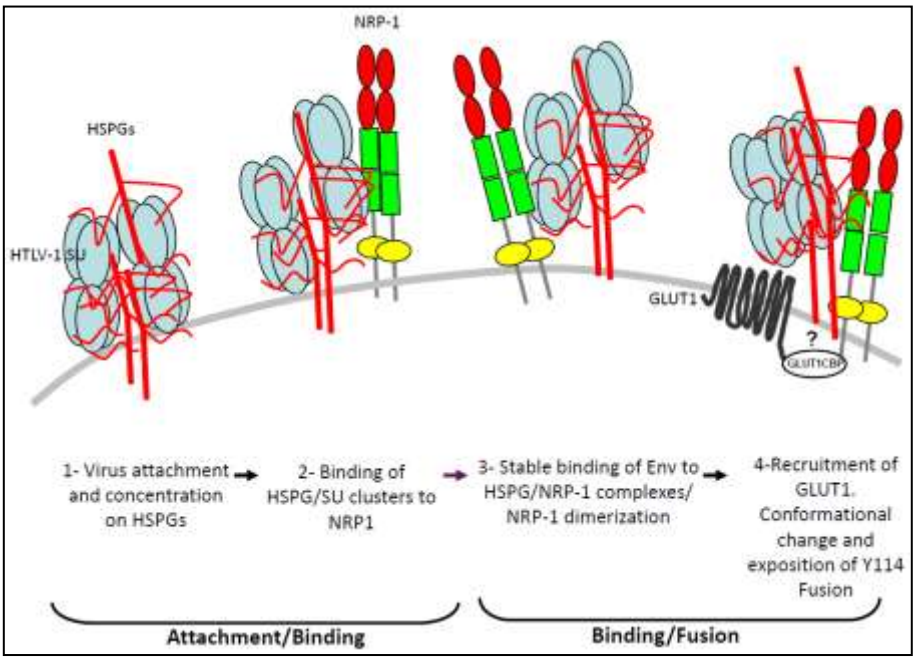
2.1. Viral cycle

The virus spread in vivo both by proliferation of the infected cells and by re-infection of new cells through direct cell-to-cell contact. After HTLV-1 infection by sexual intercourse or breastfeeding, dendritic cells will be naturally infected. The DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN) expressed in dendritic cells facilitates HTLV-1 binding and the entrance of HTLV-1 to these cells (Dutartre et al., 2016; Macatonia et al., 1992; Martin-Latil et al., 2012). HTLV-1 Infected-dendritic cells and could then pass the virus to CD4+ T cells contributing to the viral dissemination (Alais et al., 2015). The monocyte-derived dendritic cells (MDDCs) are more susceptible to HTLV-1 infection than lymphocytes, which suggest that infection of MDDC would be one of the first steps for the process of transmission mechanisms (Alais et al., 2015).

Three others molecules appear to be implicated in the process of HTLV binding and entry into the cell: glucose transporter 1 (GLUT1) (Manel N, 2003), Neuropilin-1 (NRP-1) (Ghez D, 2006) and heparin sulphate proteoglycans (HSPG) (Pinon JD, 2003). The virally encoded glycoproteins (Env) include the surface subunit (SU) gp46 and the transmembrane unit gp21. HSPG interacts with SU, which leads to the concentration of virions at the cell surface and facilitates the recruitment of SU to NRP-1, forming a ternary complex (Env-HSPG-NRP-1). The interaction of SU with NRP-1 is stabilized by HSPG and allows the subsequent binding of SU with GLUT-1, resulting in unmasking the transmembrane fusion peptide of gp21 (Figure 4)

(Ghez D, 2010). The C-terminus of HTLV-1 SU binds to the CD4+ cells with a higher efficiency than SU from HTLV-2, (Jones et al., 2006). HTLV-1 and HTLV-2 both use NRP-1 and GLUT1 during entry, but in *in vitro* assays the removal of HSPG reduces the binding of HTLV-1 but not HTLV-2 (Jones et al., 2006). It is known that HTLV-1 and HTLV-2 bind to activated, but not naïve T cells (Nath et al., 2003). Activated CD4+ T cells show a high HSPG/low GLUT1 phenotype while activated CD8+ T cells show a low HSPG/high GLUT1 phenotype (Jones et al., 2006). This difference may explain that HTLV-1 infects predominantly CD4+ cells while HTLV-2 infects predominantly CD8+ T cells.

Figure 4.- HTLV-1 receptors in the host cell.



Hypothetical model of the HTLV receptor complex and HTLV entry:
1) HSPG interaction with SU allows the initial attachment and concentration of the virions at the cell surface.
2) HSPG facilitates the recruitment of SU to NRP-1.
3) SU interacts with NRP-1, this interaction is stabilized by HSPG.
4) SU-NRP-1 interaction triggers a conformational change within the SU allowing exposure of the tyrosine 114 that is critical for binding to GLUT1. Interaction between GLUT1 and the SU triggers a conformational change allowing the unmasking of the transmembrane fusion peptide (not depicted). The small adaptor protein GLUT1CBP might form a link between NRP1 and GLUT1 and stabilize the receptor complex.
Adapted from Ghez D(Ghez D, 2010),

Abbreviations:	HSPG	:Heparan sulphate proteoglycans
	SU	: Surface subunit (gp46)
	GLUT1	: Glucose transporter 1
	NRP-1	: Neuropilin-1
	HSPG	: heparin sulphate proteoglycans

HTLV-1 infection by free virions is inefficient *in vitro* and almost no cell-free virus particles have been detected in the serum (Bangham, 2003). Clearly, HTLV-1 infection of target cells takes place by direct passage through cell-to-cell contact, via a structure called "virological synapse" (Figure 5, Figure 6).

Upon meeting an uninfected T-cell, the microtubule-organizing centre (MTOC) of the HTLV-1-infected cell becomes polarized toward the uninfected cell. Complexes of HTLV-1 core (Gag) and the RNA genome of the virus accumulate at the point of cell contact and are then transferred to the uninfected cell. The "virological synapse" and the cell-to-cell transmission process depend on the expression of leukocyte function-associated antigen-1 (LFA-1), LFA-3 and intracellular adhesion molecule-1 (ICAM-1) (Figure 5, Figure 6). The whole process is stimulated by Tax, because Tax promotes the up-regulation of ICAM-1 and transactivates CREB. Tax proteins are observed close to the MTOC, which, together with the activation of the CREB pathway is required to induce the polarization of MTOC (Matsuoka, 2007; Nejmeddine M, 2005).

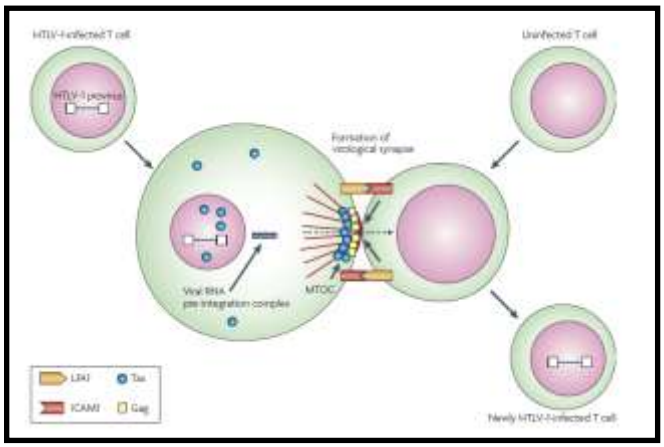
When a new cell gets infected, the viral single-stranded RNA is converted by reverse transcriptase to double-stranded proviral DNA, which subsequently is transported into the nucleus. The HTLV-1 provirus integration into the host genome takes place with the assistance of the viral integrase (Seiki, 1983).

The cell-to-cell transmission through a "virological synapse" is important at the early infection stage (Nejmeddine and Bangham, 2010). HTLV-1 increases its copy number mainly by proliferation of infected cells (clonal expansion) and much less by infection of new target cells. These infected cells replicate by mitosis and can survive for several years (Wattel, 1995). Clearly, HTLV-1 initially spreads from cell to cell to give rise to different clones and, in a second step, cellular proliferation produces clonal expansion, replicating the integrated

provirus (Pique and Jones, 2012). In practice, this replication and survival strategy of HTLV-1 results in life-long infection of the host and is responsible of the chronic infection by maintaining the proviral load after reaching an equilibrium with the immune system. The exact site of viral insertion in the human DNA plays an important role (Gillet et al., 2011; Nejmeddine and Bangham, 2010). Clonal abundance is related to integration in genomic regions with active transcription, which may promote cell proliferation. Remarkably, integration in a gene or active genomic regions was more frequently observed in AC compared with HAM/TSP independent of proviral load (Niederer et al., 2014). The risk of ATL can be related to the number of clones: larger number of clones increases the chance of malignant transformation (Cook et al., 2014).

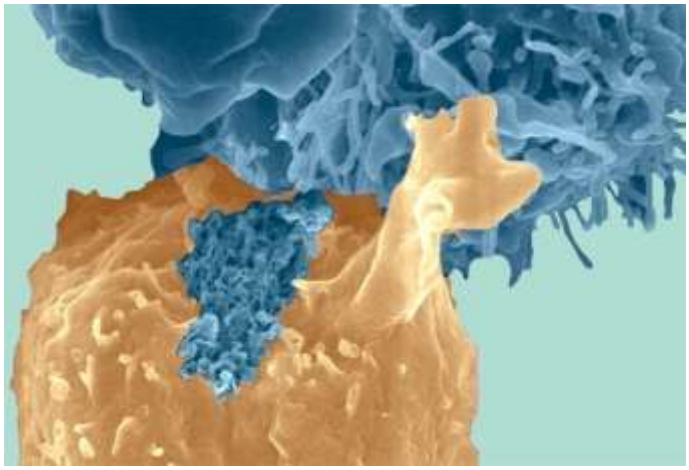
Conversely, the contribution of reverse transcriptase-based proliferation and ongoing cellular infection via free viruses is very small; the mutation rate caused by errors of reverse transcriptase is very low. This contrasts with HIV, which uses the error-prone reverse transcriptase to increase its mutation rate (Mansky, 2000).

Figure 5.-Cell to cell HTLV-1 transmission through virological synapse



Cell-to-cell contact through a virological synapse is required for efficient transmission of HTLV-1 genome from an infected cell to a new host cell.
Binding of Lymphocyte function-associated antigen 1 (LFA1) and the intercellular adhesion molecule 1 (ICAM1) is crucial for the cell to cell contact and to polarize the microtubule organizing centre (MTOC)(Matsuoka, 2007).

Figure 6.-Virological Synapse. Scanning electron micrograph revealing the biofilm-like structures (dark blue) formed by HTLV-1 particles adhering to the surface of infected T cells (blue) creating a viral synapse with a target cell (yellow).



HTLV-1-infected T cells transiently store HTLV-1 particles on their cell surface as carbohydrate-rich "pools" with structure, composition, and function resembling that of bacterial biofilms. These assemblies attach to the cell surface by extracellular matrix components (e.g., collagen and agrin) and cellular linker proteins (e.g. tetherin and galectin-3), and they rapidly adhere to other cells, facilitating virus transfers to target cells(Thoulouze, 2011).

3. Transmission between humans

HTLV-1 is present in the human host as a provirus, integrated into the DNA of the host's lymphocytes. Infected cells are required for transmission of the virus (Yamamoto, 1982). HTLV-1 is transmitted through three main routes: (i) transmission from mother to child, mainly through breastfeeding, (ii) parenteral transmission through whole blood transfusion or through the exchange of needles and syringes among intravenous drug users and (iii) sexual transmission (Khabbaz et al., 1992; Okochi and Sato, 1986; Proietti FA, 2005; Roucoux, 2005).

In places where HTLV-1 is endemic, breastfeeding seems to be the most frequent route of transmission. HTLV-1 transmission from mother to child is estimated to occur in about 20% of offspring of infected mothers, mainly by infected lymphocytes in the breast milk (Gotuzzo, 2007). Intrauterine or peripartum mother-to-child transmission is less frequent: it has been reported to occur in less than 5% of children of infected mothers (Fujito T, 2000).

The duration of breastfeeding has a very clear effect on the risk of transmission. In Peru, it was shown that among children of seropositive mothers, the proportion of infection increased with the duration of breastfeeding: 4% for those who received breastfeeding for less than 6 months, 15% for a breastfeeding duration of 6 to 12 months, 28% for 12 to 24 months and 33% for those who received breastfeeding for more than 24 months (Gotuzzo, 2007). In addition, in this study the presence of maternal HAM/TSP and strongyloidiasis was associated with increased HTLV-1

infection in the children (Gotuzzo, 2007). It has been suggested that this gradual increase in transmission during prolonged breastfeeding may be related to the fact that the maternal, protective HTLV-1 antibodies in the child decrease over time and disappear after about nine months (Hisada M, 2002; Wiktor, 1997). However, given the cell-associated nature of HTLV-1, the activity of the antibody-associated protection is questionable anyhow. In fact, besides a higher proviral load (PVL) in breast milk, high antibody titres of the mother have also been associated with an increased risk of mother-to-child transmission (Li, 2004).

Interestingly, HTLV-1 transmission via breastfeeding has been correlated with mother-to-child HLA class I type concordance in a dose-dependent way (Biggar RJ, 2005). Because HTLV-1 is passed from lymphocytes via synapses, the cell-to-cell HTLV-1 transmission could be enhanced when maternal cells show HLA-class I (HLA-A, B and C) allele concordance with cells from the child. Up to six HLA-class I alleles may be similar between the mother's cells and the child's cells. The more HLA-class I alleles are shared between mother and child, the higher the risk of infection in the child. In this case, maternal cells are recognized as being "self" by the child's immune system, resulting in less immune responses and hence contributing to an increase of HTLV-1 transmission. A similar behaviour has been observed in HIV transmission (Polycarpou et al., 2002).

In Japan, pregnant women undergo HTLV-1 screening, in order to reduce mother-to-child transmission. Seropositive women are then urged to bottle-

feed their children. The general reduction in the number of mothers breastfeeding together with a shortening of breastfeeding duration contributed to a decrease in HTLV-1 prevalence in the general population of Okinawa from 9% in the 1968-1970 to 6% in 1996-1998 (Kashiwagi, 2004). Although it is an established fact that bottle feeding prevents transmission, this may be difficult to implement in developing countries, where insufficient support to get artificial feeding and the lack of access to clean water might result in consequences worse than the consequences of HTLV-1 infection *per se.*

Parenteral transmission occurs when infected blood cells are passed through blood transfusion or through the exchange of needles and syringes among intravenous drug users. The risk of infection after an HTLV-1-contaminated blood transfusion is very high, ranging from 40 to 60% (Manns et al., 1992; Okochi et al., 1984). In endemic areas for HTLV-1, the screening of candidate blood donors is an important measure to reduce HTLV-1 transmission (Gonçalves DU, 2010). HTLV-1 can also be transmitted via organ transplantation, leading to a rapid virus spread in the recipient (Cook et al., 2016; Mateos AV, 2005).

Finally, HTLV-1 can be transmitted during sexual intercourse. The incidence of HTLV-1 transmission among discordant couples is about 1 per 100 person years (Roucoux, 2005). Although early reports suggested that transmission is more common from male to female than vice versa, better designed cohort studies show that female-to-male transmission plays an important role as well (Roucoux, 2005). A high PVL in peripheral blood mononuclear cells (PBMC)

appears to be associated with a higher risk of sexual transmission (Li, 2004). HTLV-1 infection has been linked to unprotected sex, many lifetime sexual partners, and the presence of other sexually transmitted infections (Murphy EL, 1989; Roucoux, 2005). In a series of studies in Peru, it was found that HTLV-1 is frequent among female sex workers and that the frequency increased with the duration of prostitution: from 4% (less than 3 years) over 9% (between 3 and 6 years) and up to 16% (more than 6 years) (Gotuzzo E, 1994). Moreover, two of these studies showed that condom use protects against HTLV-1 infection among female sex workers (Gotuzzo E, 1994; Trujillo L, 1999).

4. Immune responses to HTLV-1

4.1. Adaptive immune response

Although the immune response against HTLV-1 infection is strong, its contribution to protect from infection or to control the equilibrium of PVL in infected subjects remains a matter of debate. However, there is a clear conceptual difference between the role of antibodies, CD4+ T cells and CD8+ T cells in pathogenesis and protection.

Since the virus spread results mainly from cell-to-cell contact and mitosis of infected cells, there is little evidence that antibodies actually interfere with the virus (Bangham, 2000). Antibody detection and specificity are mainly of diagnostic importance, while CD4+ T cells (and to a lesser degree CD8+ T cells) constitute the main HTLV-1 host cells in peripheral blood (Andrade et al., 2013; Hanon, 2000, Melamed 2015). Tax expression drives mitosis of the

infected CD4+ T cells and contributes to maintenance of the PVL. The frequency of HTLV-1-specific CD4+ T cells appears to be significantly greater in patients with the inflammatory condition HAM/TSP than in ACs with the same PVL. This observation raises the possibility that such CD4+ T cells might contribute to the pathogenesis of the inflammatory disease (Goon PK, 2003).

CD8+ T cells play a critical role in limiting virus replication in most acute viral infections. However, apparently CD8+ T cells are unable to destroy all HTLV-1-infected cells. The efficiency of the CTL response to control the PVL varies between individuals. This variation is likely to be influenced by the class I MHC composition (Jeffery, 1999; Vine, 2002). Individuals heterozygous at all three HLA class I loci had a lower PVL than homozygous individuals. The most likely explanation for this association is that the variability in HLA loci broadens the range of HTLV-1-derived epitopes to be recognized by the CTL. This leads to a better recognition of infected cells and an increased efficiency to kill these cells (Jeffery, 2000).

4.2. Role of natural killer cells

Natural killer (NK) cells are part of the innate immune system, which plays an important role by killing virally infected and cancer cells, without the need for prior sensitization. Their antiviral function is performed by cytotoxic activity and through the release of cytokines (Vilches and Parham, 2002).

NK cell activity is mainly regulated by NK receptors, which include killer cell immunoglobulin-like receptors (KIR) and lectin-like receptors. KIR are encoded by a family of 16 genes which are part of the leukocyte receptor complex on chromosome 19 (Trowsdale, 2001). KIR recognise the loss of MHC class I antigens located at the cell surface. These MHC class I molecules are important for NK cells to distinguish "healthy" from "unhealthy" cells. Cells showing altered or lacking particular MHC class I molecules can be recognized as unhealthy cells by the NK cell and can subsequently be destroyed.

Two types of KIR receptors have been described: activating KIR (aKIR) and inhibitory KIR (iKIR). The 16 KIR genes are distributed in 8 iKIR, 6 aKIR and 2 pseudogenes. The 2 pseudogenes are not transcribed (www.ebi.ac.uk/ipd/kir/introduction.html). The aKIR are *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL15*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, while the iKIR are *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5* and *KIR3DS1*, and the two-pseudo genes are *KIR2DP1* and *KIR3DP1*. The first digit corresponds to the number of extracellular domains (D). An "S", "L" or "P" can follow the D. "S" refers to a short cytoplasmic tail; "L" to a long cytoplasmic tail and the P refers to a pseudogene. The last number indicates the gene number (Natarajan et al., 2002). In addition, sometimes an asterisk and a number are added, indicating the number of alleles for every gene.

The number and nature of KIR genes varies amongst individuals. As the KIR genes are located close to each other within a 150 Kb genomic stretch, the content of KIR genes in each individual is indicated by the term 'haplotype'.

Four KIR genes are common in almost all KIR haplotypes: *KIR2DL4*, *KIR3DL1*, *KIR3DL3* and the pseudogene *KIR3DP1*; they are called "framework genes". KIR haplotypic diversity adds to the well-known wide variability of HLA allotypes. NK cells express different numbers and combinations of KIR genes, which contribute an additional level of complexity to the NK cell repertoires.

A fine balance in the interactions between KIR and HLA regulates the activity of NK cells. In case of viral infection or cell transformation, the HLA content at the cell surface is down regulated. As a consequence, the iKIR do not engage to an HLA molecule and no inhibitory signal is given inside the NK cell, causing the lysis of the cell with the decreased expression of HLA molecules.

Conversely, the "activating" KIR (aKIR) are thought to bind modified HLA molecules. In case of an altered HLA, aKIR binds this HLA and this interaction provides an activation signal to the NK cell, allowing this NK cell to destroy the target cell. Some tumours alter the expression of ligands for aKIR, thus overcoming the inhibitory signals and promoting the NK-mediated killing of those tumoral cells. Clearly, a complex balance between inhibitory and activating receptor-ligand interactions manages the activity of the NK cells (Williams et al., 2005).

Some KIR-HLA combinations have been associated with susceptibility or resistance to specific infectious diseases. For example, individuals with *KIR2DL3*, *KIR2DL3* / *HLAC1*, *HLAC1* resolve hepatitis C virus infection more efficiently than individuals without this combination (Khakoo et al., 2004). HIV-infected individuals with the KIR3DS1-HLA-Bw4^{Ile80} combination may be

protected against opportunistic infections, which delays the appearance of AIDS (Qi et al., 2006).

In the case of HTLV-1 infection, a decreased activity of NK cells has been described in HAM/TSP patients compared with AC (Yu et al., 1991). Saito et al reported a low frequency of CD94/NKG2A+ T lymphocytes in HAM/TSP patients compared to AC (Saito et al., 2003). Seich reported that *KIR2DL2* enhances the association between *HLA-Cw*08* and proviral load in ACs, a synergy with T-cells is proposed (Seich Al Basatena et al., 2011).

Taking together all this information, it is tempting to speculate that NK cells and KIR genes could indeed determine the course and complications of HTLV-1 infection.

5. Diagnosis

After HTLV-1 infection, the human body produces antibodies against viral antigens. The first antibodies are directed against Gag, followed by antibodies against gp21 and finally by antibodies against Tax. The diagnosis of HTLV-1 infection is based mainly on serological assays that trace HTLV-1-specific antibodies. Enzyme immunoassays and agglutination tests are used for screening. A chemiluminescent enzyme immunoassay (CLEIA) test is also used for HTLV-1 diagnosis; CLEIA will generate less than 0.5% of false positive results, a lower rate as compared to agglutination tests (Koga et al., 2010). Clearly, nowadays, the enzyme-linked immune-sorbent assay (ELISA) is the most commonly used format. In case of a positive screening result, it is recommended to perform a confirmatory test with another method that also

discriminates between HTLV-1 and HTLV-2. The most commonly used confirmatory tests are Western blot, after positive screening results. In some cases, atypical HTLV profiles with inconclusive results are observed, and sometimes it is difficult to discriminate between HTLV-1 and HTLV-2.

False-negative results remain a challenge, and indeterminate results may occur in recent infections when seroconversion is not yet complete (Namen-Lopes, 2009). For transfusion-transmitted HTLV-1 where a large inoculum is passed, the window period varies between 41 and 65 days or longer (Manns et al., 1991; Okochi and Sato, 1986). The window period is even longer when the inoculum of HTLV-1 is low as is the case for other routes of transmission. This delayed seroconversion hampers the diagnosis of HTLV-1 infection.

Although improvements have been made to immunological assays for HTLV-1 detection, HTLV-1 indeterminate results continue to be frequent. In those cases, to clarify indeterminate or non-typeable results, a polymerase chain reaction using a conserved region of the *tax* gene as target is used (Mangano AM, 2004). Several PCR approaches have been developed for HLTV-1 and HTLV-2 diagnosis (Brunetto et al., 2014), and a diagnostic algorithm considering PCR as screening test has been proposed (Canepa et al., 2015; Ishihara et al., 2014). Multiplex PCR assays have been developed to detect, genotype and quantify HTLV-1 and HTLV-2 (Moens et al., 2009) and to differentiate and quantify simultaneously HTLV-1, HTLV-2 and HTLV-3 (Besson and Kazanji, 2009).

6. The clinical significance of proviral load

PVL is expressed as HTLV-1 copy number per fixed amount of PBMC or CD4+ T cells. The PVL is measured by quantitative PCR. The PVL tends to remain constant over time in each infected individual but may vary between HTLV-1-infected people more than 10,000-fold. In fact, the PVL reaches a stable equilibrium “set point” several years after infection (Kubota, 1993). The factors that determine this individual “set point” are not well understood. However, the individual differences in the rate at which cytotoxic T cells (CTL) kill HTLV-1-infected cells that express viral peptides are likely to be important. This CTL efficiency might be determined by genetic host factors e.g. diversity in HLA and T cell receptor genotype, determining the efficiency of recognition of HTLV-derived antigenic peptides.

High PVL has been consistently correlated with HAM/TSP disease in several populations of HTLV-1-infected subjects (Adaui, 2006; Kira J, 1991; Nagai, 1998; Sabouri AH, 2005). For example, in Japan, the median PVL in PBMC is 5% in patients with HAM/TSP compared to 0.3% in asymptomatic carriers (AC) (Nagai, 1998). In Iran and in Peru, the difference seems to be less pronounced. Reports from these countries show that the median PVL in HAM/TSP patients is on average only two-fold higher than in AC) (Adaui, 2006; Sabouri AH, 2005). However, in all studies there is an overlap between the PVL of patients with HAM/TSP and AC: some HAM/TSP patients have a low PVL and some AC have a high PVL (Adaui, 2006; Furtado Mdos et al.,

2012), indicating that a high PVL is not the only factor that determines the development of HAM/TSP.

7. HTLV-1 associated diseases

Up to 10% of HTLV-1-infected individuals develop complications. These HTLV-1-associated diseases can be divided in three groups: malignancies including ATL and cutaneous T-cell lymphoma, inflammatory syndromes such as HAM/TSP, uveitis, polymyositis, Sjögren's syndrome, thyroiditis, arthropathy, polyneuropathy and T-lymphocyte alveolitis and infectious complications, including strongyloidiasis, scabies, leprosy, and tuberculosis (Verdonck, 2007). The two most important and best-studied HTLV-1 associated diseases are HAM/TSP and ATL. The main focus of this thesis is to identify genetic factors associated with HAM/TSP disease and therefore this disease will be more extensively discussed.

7.1. HAM/TSP

7.1.1. Clinical characteristics

In 1985 in Martinique patients with a chronic neuromyelopathy called Tropical Spastic Paraparesis (TSP) were positive to antibodies to HTLV-1 (Gessain et. al. 1985). In 1986 in Kagoshima-Japan patients with similar symptoms were described, with high titres of HTLV-1 antibodies, they proposed the term HTLV-1 Associated Myelopathy (HAM) (Osame et. al. 1986). HAM/TSP is an inflammatory disease that is characterized by parenchymal infiltration of mononuclear cells into the grey and white matter of the thoracic spinal cord,

resulting in severe lesions in the central nervous system, leading to progressive spasticity and weakness of the lower extremities, with tendency to stumble and fall, hyperreflexia in the lower limbs, abnormal gait, back pain, bladder dysfunction, sexual dysfunction and constipation. The diagnosis of HAM/TSP is made according to De Castro-Costa and colleagues guidelines (De Castro-Costa et al., 2006) requiring the demonstration of HTLV-1 infection and the exclusion of other causes of myelopathy (Table 2).

Table 2. Guidelines for the diagnosis of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) according to De Castro-Costa (De Castro-Costa et al., 2006).

Definite

1. A non-remitting progressive spastic paraparesis with sufficiently impaired gait to be perceived by the patient. Sensory symptoms or signs may or may not be present. When present, they remain subtle and without a clear-cut sensory level.

Urinary and anal sphincter signs or symptoms may or may not be present.

2. Presence of HTLV-I antibodies in serum and cerebro-spinal fluid (CSF) confirmed by Western blot and/or a positive PCR for HTLV-I in blood and/or CSF.

3. Exclusion of other disorders that can resemble HAM/TSP

Probable

1. Monosymptomatic presentation: spasticity or hyperreflexia in the lower limbs or isolated Babinski sign with or without subtle sensory signs or symptoms, or neurogenic bladder only confirmed by urodynamic tests.

2. Presence of HTLV-I antibodies in serum and/or CSF confirmed by Western blot and/or a positive PCR for HTLV-I in blood and/or CSF.

3. Exclusion of other disorders that can resemble HAM/TSP

Possible
1. Incomplete clinical presentation.
2. Presence of HTLV-I antibodies in serum and/or CSF confirmed by Western blot and/or a positive PCR for HTLV-I in blood and/or CSF.
3. Disorders that can resemble HAM/TSP have not been excluded.

7.1.2. Epidemiology

HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a progressive and chronic neurologic disease affecting 1-5% of HTLV-1-infected subjects. Although some cases of HAM/TSP have been observed in childhood, HAM/TSP is usually a disease of adults and in general, the first signs appear at age 40-50 years (Orland et al., 2003; Yamano and Sato, 2012). HAM/TSP is more frequently observed in women than in men (Kaplan, 1990). Remarkably, several cases of HAM/TSP have been reported after blood transfusion and organ transplantation with a short incubation period of the disease, ranging from a few months to two years (Domingues et al., 1997; Osame et al., 1990; Shoeibi et al., 2013). Clearly, the immunosuppression of these patients as well as the greater inoculum of the virus transmitted may favour the rapid increase of the HTLV-1 PVL, which possibly leads to a rapid development of disease (Toro et al., 2003).

7.1.3. Pathogenesis

Although HAM/TSP was first recognized as an HTLV-1-associated disease in the mid-1980s, the pathogenic mechanisms are not yet fully understood. Moreover, the fact that not all infected subjects develop a disease suggests that HTLV-1 infection itself is not sufficient to cause HAM/TSP and environmental factors and/or individual differences in genetic predisposition may have a part in disease development. A number of studies on the relationship between HAM/TSP, HTLV-1 PVL, Tax expression, *tax* genotype and cellular immune responses against Tax and other HTLV-1 proteins have yielded a complex and not always very consistent picture.

A high level of Tax expression and a low CD8+ T-cell efficiency have been independently associated with high PVL (Asquith, 2000). In addition, Tax expression was significantly higher in HAM/TSP patients compared to AC. This increment of Tax expression may lead to a chronic activation of HTLV-1-specific CD8+ T cells (Asquith, 2005). An increased frequency of these CTL has been reported, but this does not necessarily equals a highly efficient CTL function.

In this regard, we need to stress that Tax protein constitutes the major target of the CTL response *in vivo*, but CTL activity against other antigens such as Gag, Pol and Env has also been detected. The frequency of Tax-specific CTL is much higher in HAM/TSP than in ACs. The fact that the PVL is also higher in HAM/TSP than in AC suggests that the CTLs of HAM/TSP patients cannot

control the number of infected cells properly, probably due to the less efficient cytolytic activity of the CTL towards infected cells, despite the high Tax-specific CTL frequency (Wodarz, 2001).

Similarly, the presence of infiltrating macrophages and activated CD8+ T cells in the spinal cord lesions, with high levels of HTLV-1-specific CTL in peripheral blood and cerebrospinal fluid in HAM/TSP patients suggests either a) that the HTLV-1-specific CD8+ T cell response, instead of protecting against the infection, is pathogenic and causes bystander tissue damage in the central nervous system (Jacobson, 1990), or b) that in AC the specific CD8+ T cell response is more effective than in HAM/TSP causing a protective effect in AC by reducing PVL and the risk of HAM/TSP development. Although both mechanisms may seem mutually exclusive, in fact, they may coexist at the same time, such that relatively inefficient CTL against HTLV-1-infected cells may get “over-activated” and cause “collateral damage”, by release of their lytic content and pro-inflammatory cytokines (Asquith, 2000).

In addition, HTLV-1 tax gene sequence variation has been associated with the risk of HAM/TSP in Japan: tax subgroup A with four substitutions in positions 7897, 7959, 8208, and 8344 was found more frequently in HAM/TSP (15.5%) compared to AC (7%) (Furukawa, 2000), while an earlier report by Mahieux *et al*. found no association between the tax mutation at position 7959 and HAM/TSP (Mahieux et al., 1995). The exacerbated immune response observed in HAM/TSP patients is characterized by an increase in the frequency of interferon-gamma (IFN-γ) and (tumour necrosis factor-alpha

(TNF-α) producing cells. Conceptually, increased production of these proinflammatory cytokines might contribute to tissue damage of the central nervous system (Best I, 2006; Biddison W, 1997). Clearly, high levels of proinflammatory cytokines, such as TNF-α, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF), have indeed been detected in the cerebrospinal fluid of HAM/TSP patients and can be the cause of the "bystander damage" induced by HTLV-1 (Kuroda et al., 1993; Nishimoto et al., 1990).

7.1.4. HAM/TSP as an autoimmune disease?

Levin and colleagues suggested that HAM/TSP might be an anti-hnRNPA1–mediated autoimmune disease (Levin MC, 1998; Levin MC, 2002). hn-RNPs are heterogeneous nuclear ribonucleoproteins, which form the major component of the nuclear core complex in eukaryotic cells. They are involved in several functions of the cell, including mRNA splicing, transport of mRNA from the nucleus to the cytoplasm and mRNA turnover. hn-RNPA1 proteins, the most abundant type of hn-RNPs, may play a pathogenic role in several autoimmune diseases (Caporali R, 2005). Levin et al. (Levin MC, 2002) found anti-hnRNPA1 antibodies in the cerebrospinal fluid of 13 HAM/TSP patients and suggested that these anti-hnRNPA1 antibodies react with the HTLV-1-tax protein epitope ($tax^{346–353}$). They hypothesized that this molecular mimicry may lead to an autoimmune reaction and thus contribute to the pathogenesis of HAM/TSP in HTLV-1-infected individuals. However, the host protein hnRNP-A1 is not restricted to the central nervous system as it is widely expressed

(Dreyfuss, 1993). In addition, because of its nuclear localisation, most probably it is not accessible to antibody attacks and therefore does not explain the onset of HAM/TSP (Saito M, 2010).

7.1.5. Therapy

Corticosteroids, interferon-α, interferon-β, pentoxifylline, valproate, zidovudine and lamivudine have all been considered in various combinations for the treatment of HAM/TSP. Most of these therapies have been evaluated only in small or uncontrolled studies (Harrington WJ Jr, 1991; Shirabe S, 1997). No therapy has conclusively been shown to modify the long-term disability associated with HAM/TSP. Therefore, up to now, the treatment of HAM/TSP remains a challenge and is largely limited to supportive and symptomatic measures, aiming at relieve of pain and stiffness. There is an urgent need for new and better-controlled studies on both anti-inflammatory and anti-viral reagents. In addition, there are no validated biomarkers to measure the disease activity and to assess the effect of a therapy in HAM/TSP patients.

7.1.6. Host genetics

Similar to clinical manifestations in other infectious diseases, the development of HAM/TSP can be considered as the result of interactions between viral factors on one hand and genetic/immune factors of the host on the other, with a possible implication of environmental factors (Orland, 2003; Sabouri AH, 2005). However, the virus is genetically stable and therefore genetic variation in HTLV-1 can only have a limited contribution, as discussed by Furukawa et

al (Furukawa, 2000). Conversely, in view of the high PVL observed in HAM/TSP patients and the lack of clear differences in HTLV-1 sequence between HAM/TSP and AC, it is believed that individual differences in host genetic factors might be associated with susceptibility to high PVL or HAM/TSP risk in HTLV-1-infected individuals. In this regard, several candidate gene studies have been performed to determine the influence of host genetics factors on HAM/TSP susceptibility (Table 3). Quite a number of potential genetic associations have been studied in the past. In the text below, the most intensively studied ones are discussed first and the less studied are briefly mentioned thereafter. In the summarizing Table 3 page 31, the markers are arranged in alphabetical order.

7.1.6.1. HLA associations

HLA loci class I (HLA-A, -B, -C, -E, -F and -G) and class II (HLA-DR, -DQ, -DM and -DP) have an important role in the antigen presentation to CD8+ and CD4+ T cells respectively. The HLA region on chromosome 6 is one of the most variable regions in the human genome, and HLA genes are important to discriminate self and non-self-molecules.

Heterozygous individuals at all three HLA class I loci were found to have a lower PVL than homozygous individuals at one or more loci, suggesting that class I HLA heterozygosity is beneficial for HTLV-1-infected individuals (Jeffery, 2000). Individuals with two different alleles at each HLA locus present a wider repertoire of antigenic peptides to the CTL than homozygous

individuals do, which may result in a more efficient CTL control of viral replication and therefore may explain the lower risk of disease (Jeffery, 2000). In a similar way, heterozygous individuals for HLA class-I have a slower progression to AIDS and lower mortality when they are infected with HIV (Carrington, 1999).

*HLA-A*02* and *HLA-Cw*08* independently have been associated with a significant reduction in PVL and with a lower risk of HAM/TSP in Japan (Table 3) (Jeffery, 1999; Vine, 2002), suggesting that HLA-A*02-restricted or HLA-Cw*08-restricted CTL are important to control HTLV-1 PVL *in vivo*. For HLA-A*02, the proposed mechanism is recognition of a nine-amino acid peptide of the Tax protein ($Tax^{11–19}$) (Kannagi M, 1992), leading to the destruction of the HTLV-1-infected cell by specific CD8+ T cells. *HLA-A*0206* was clearly associated with low PVL and HAM/TSP protection (Jeffery, 1999). Other *HLA-A*02* subtypes such as *A*0201*, *A*0203*, *A*0207*, and *A*0210* also seemed to be associated with low PVL, but the number of individuals with these particular HLA types was too small to reach a conclusion on the real effect of these alleles on PVL or HAM/TSP risk (Jeffery, 1999). *HLA-Cw*07* was found to be associated with a higher risk of HAM/TSP in Brazil (Catalan-Soares BC, 2009), while in Japan the allele *HLA-Cw*0702* was found associated with HAM/TSP risk (Jeffery, 1999).

*HLA-DRB1*0101* has been associated with increased susceptibility to HAM/TSP in HLA-A*02-negative (Jeffery, 1999), in Japanese as well as in Iranian populations (Sabouri AH, 2005). However, *HLA-DRB1*0101* was not

associated with a higher PVL in Iranian HTLV-1-infected subjects. Intriguingly, *HLA-B*5401* was associated with an increased susceptibility to HAM/TSP in Japan (Jeffery, 1999; Jeffery, 2000) but not in Iran (Table 3) (Sabouri AH, 2005). In Jamaica the *HLA-DQB1*0602* was found associated with HAM/TSP protection (Goedert JJ, 2007). It has been shown that asymptomatic carriers have HLA class I alleles that bind HBZ more strongly than HAM/TSP patients. This strong binding is associated independently with both: low proviral load and a reduction in HAM/TSP risk (Macnamara et al., 2010).

In summary, it is clear that, as expected, HLA polymorphism has a role in (protection against) HAM/TSP, but the contribution of individual HLA alleles is complex and apparently partly dependent on the ethnic characteristics of the population studied.

7.1.6.2. TNF-α

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that exerts its effects via binding to its receptors (TNFR1 and TNFR2) and has been implicated in the pathogenesis of chronic inflammatory diseases, including HAM/TSP. TNF-α producing cells have been identified in the cerebrospinal fluid of HAM/TSP patients (Nakamura S, 1993). In addition, HTLV-1-specific CD4+ T cells that produce TNF-α were more frequent in HAM/TSP than in AC (Goon PK, 2003). Therefore, genetic differences in the individual capacity to produce increased levels of TNF-α or its receptors might be associated with the presence of inflammatory diseases.

In a population from Japan, differences in frequency distribution of a *TNF-α* promoter polymorphism at position -857C>T (rs1799724) (rs is an accession number to refer to specific SNP) were observed between HAM/TSP and HTLV-1-negative controls, but no differences were found between HAM/TSP and AC (Nishimura M, 2000). Conversely, Vine (Vine, 2002) found that allele -863A of *TNF-α* (*TNF-α*-863 A>C) increases the risk of HAM/TSP in individuals with a high PVL (Table 3). No differences in the frequency distribution of the *TNFR-1* promotor -383A>C polymorphism were found between HAM/TSP and uninfected controls and between HAM/TSP and AC ($p>0.05$) (Nishimura M, 2000). However, significant differences were observed for *TNFR-2* between HAM/TSP and uninfected controls ($p=0.0043$) but not between HAM/TSP and AC (Nishimura M, 2000).

7.1.6.3. SDF-1

Stromal cell-derived factor-1 (*SDF-1*; CXCL12) is a member of the superfamily of chemokines that regulate the growth, survival, development and migration of multiple cell types, including human hematopoietic stromal stem cells. It is a chemoattractant for lymphocytes. An increase in SDF-1 production due to Tax protein and a migration of HTLV-1-infected cells due to SDF-1 has been reported, suggesting that SDF-1 might alter the distribution of HTLV-1-infected cells and contribute to the infiltration into affected tissues in HAM/TSP (Arai, 1998).

Differences in allele and genotype frequency between HAM/TSP and AC have been reported for *SDF1*-3′UTR +801G>A (rs1801157) (Vine, 2002) (Table 3). The allele +801A showed a protective effect (Vine, 2002), but the exact mechanism how SDF protects against HAM/TSP development is not well known.

7.1.6.4. Perforin

Perforin is a cytolytic molecule that contributes to CTL-mediated killing of virus-infected cells. Polymorphisms in the perforin gene may determine the efficiency of the CTL response in HTLV-1-infected individuals. The frequency of the T allele at position +418*C/T relative to the transcription start site was increased in AC compared to HAM/TSP patients (P=0.026) in a study performed in Iran (table 3). Up to now, no defined function for this allele is known (Rafatpanah H, 2004). Two hypotheses were provided by the authors to account for this association: a) The T allele is associated with lower expression of perforin in CD8+ CTL, leading to viral persistence, increase of PVL and the development of neurologic pathology, b) conversely, the T allele might be associated with higher perforin expression in HTLV-1-specific CTL and this increased cytotoxic activity may result in bystander neural damage, contributing to the appearance of HAM/TSP (Rafatpanah H, 2004). However, these hypotheses implicate that the allele T is a risk factor instead of a protective factor effect of HAM/TSP, which is unproven. Functional analysis is needed to determine the effect of the allele +418T on the perforin expression to explain its effect over HAM/TSP disease.

7.1.6.5. Matrix Metalloproteinase

Matrix metalloproteinase-9 (MMP-9 type IV collagenase, gelatinase B) is a proteolytic enzyme whose main substrate is collagen IV, a principal component of the basal lamina. Infiltration of T lymphocytes expressing MMP-9 in active lesions of the spinal cord is characteristic for HAM/TSP patients (Umehara, 1993). HTLV-1-infected T-cell lines express higher levels of MMP-9 than uninfected T-cell lines, and Tax protein activates the *MMP-9* promoter and induces MMP-9 expression in T cells (Mori, 2002). In addition, longer d(CA) repeat alleles in the *MMP-9* promoter, associated with higher Tax-mediated transcriptional activity, were more frequently observed in HAM/TSP patients than in ACs ($p<0.01$) (Table 3) (Mori, 2002; Shimajiri, 1999).

The hypothetical pathogenic mechanism is that MMP-9 released by HTLV-1-infected cells attacks the extracellular matrix components in the basal lamina around central nervous system blood vessels. This process subsequently opens the blood brain barrier, thus allowing the influx of inflammatory cells (Kodama D, 2004).

7.1.6.6. Interleukin-6

Interleukin-6 (IL-6) is a cytokine secreted by macrophages, lymphocytes, fibroblasts, adipocytes, and endothelial cells, in response to different types of inflammatory stimuli. IL-6 induces endothelial damage, stimulating the intracellular adhesion molecule-1 (ICAM-1). High levels of IL-6 were observed in both serum and cerebrospinal fluid of HAM/TSP patients (Ohbo K, 1991).

Polymorphisms in the *IL-6* promoter region at positions -174 G>C (rs1800795) and -634 C>G (rs1800796) influence plasma levels of IL-6 (Bonafè M, 2001). The proportion of *IL-6*-634 C homozygotes among patients with HAM/TSP (38.5%) was lower than in AC (61.5%) (P=0.0071) and seronegative controls (62.5%) (p=0.0033) (Table 3), suggesting that the -634 C/C genotype may have a potentially protective role in the development of HAM/TSP. Nevertheless, preliminary assays did not show functional differences between these alleles (Nishimura M, 2002).

7.1.6.7. Vitamin D receptor

The hormone, derived from vitamin D, 1,25-dihydroxyvitamin D3 (1,25-[OH]2 D3) inhibits the activation of Th1 cells, lymphocyte proliferation and cytokine production. The activity of 1,25-[OH]2 D3 is carried out by binding to the vitamin D receptor (VDR), which is overexpressed in HTLV-1-infected individuals (Inoue et al., 1993). *ApaI* polymorphisms in *VDR* have been associated with a reduced risk of HAM/TSP in HTLV-1-infected individuals but this was not associated with PVL (Table 3) (Saito et al., 2005). However, as this single nucleotide polymorphism (SNP) is located in intron 8, it probably does not play a direct role in HAM/TSP pathogenesis, but it could be in linkage disequilibrium with another SNP located elsewhere.

7.1.6.8. Interleukin-10

Interleukin-10 (IL-10) is an immunoregulatory cytokine. Its principal role is to limit the inflammatory response by inducing and maintaining the T-cell

exhaustion phenotype, thus facilitating the viral persistence (Brooks et al., 2006; Ejrnaes et al., 2006). The expression of IL-10 is influenced by three SNPs located in the promoter region of *IL-10* (Reuss, 2002). The allele *IL-10* -592A (rs1800872), was associated with HAM/TSP protection and with a lower PVL in individuals from Kagoshima in Japan (Sabouri AH, 2004) (Table 3). However, this association with HAM/TSP was not replicated in Iran (Shirdel et al., 2013). The *IL-10* -592C allele is transactivated by Tax with more efficiency than the *IL-10* -592A allele. The hypothesis is that the increased expression of IL-10 might lead to an impaired T cell response by reducing the activity or the efficiency of HTLV-1-specific cytotoxic T lymphocytes, causing possibly viral persistence and increasing the risk of HAM/TSP.

7.1.6.9. Interleukin-15

IL-15 promotes the maintenance of high frequency of Tax-specific CD8+ T cells, which can reduce the PVL in HTLV-1-infected individuals. A polymorphism in Interleukin-15 (*IL-15* +191C allele) showed a significant association with lower PVL but not with HAM/TSP (Table 3) (Vine, 2002). How this allele could result in PVL reduction is not known; there is a possibility that another polymorphism will be in linkage disequilibrium with this SNP.

7.1.6.10. ABO groups

ABO groups and HTLV-1 infection were evaluated in HTLV-1-infected blood donors and healthy controls from Iran (Ayatollahi H, 2008). The A group was more frequent in healthy controls suggesting that A+ blood group decreases

the risk of HTLV-1 infection while the AB+ group was more frequent in HTLV-infected carriers (Table 3).

7.1.6.11. Aggrecan

Aggrecan is the major proteoglycan protein expressed in both joint cartilage and the spinal cord. Its function is to maintain the water content in the extracellular matrix and act as barrier against cell migration and as a guide for axonal growth in the central nervous system, playing a role in tissue repair in injured SNC. Variable number of tandem repeat (VNTR) polymorphism in this gene were evaluated in Japanese individuals, and the allele 28 was more frequent in HAM/TSP than AC suggesting the implication of this gene in HAM/TSP susceptibility (Table 3).

Table 3. Genetic determinants implicated in PVL or HAM/TSP risk in HTLV-1-infected individuals.

	HAM/TSP	Proviral load	Population	Reference
ABO[a] Group A+	Protection		Iran	(Ayatollahi H, 2008)
ABO[a] Group AB+	Increased risk		Iran	
Aggrecan (VNTR allele 28)	Increased risk		Japan	(Nobuhara Y, 2006)
HLA-A*02+	Protection	Low	Japan	(Jeffery, 1999)
	Protection	Low	Brazil	(Catalan-Soares BC, 2009)
	Neutral	Neutral	Iran	(Sabouri AH, 2005)
HLA-A-0206	Protection	[c]	Japan	(Jeffery, 1999)
HLA-B*5401	Not found		Iran	(Sabouri AH, 2005)
	Increased risk	High	Japan	(Jeffery, 2000)
	Not found		Brazil	(Catalan-Soares BC, 2009)
***HLA*-Cw*07**	Risk		Brazil	(Catalan-Soares BC, 2009)

***HLA*-Cw*0702**	Risk		Japan	(Jeffery, 1999)
***HLA*-Cw*08**	Protection	Low	Japan	(Jeffery, 1999)
	Neutral	Neutral	Iran	(Sabouri AH, 2005)
	Neutral		Brazil	(Catalan-Soares BC, 2009)
HLA-DRB1*0101	Increased risk[a]		Japan	(Jeffery, 1999; Jeffery, 2000)
	Increased risk[a]	Neutral	Iran	(Sabouri AH, 2005)
HLA-DQB1*0602	Protection		Jamaica	(Goedert JJ, 2007)
IFN-874AT	Increased risk		Brasil	(Rocha-Junior et al., 2012)
IL-6-634 CC	Protection		Japan	(Nishimura M, 2002)
IL-10 -592A	Neutral		Iran	(Shirdel et al., 2013)
	Neutral		Japan	(Nishimura M, 2002)
	Protection	Low	Japan	(Sabouri AH, 2004)
IL-15 +191C	Protection[b]	Low	Japan	(Vine, 2002)
***MMP-9 longer* d(CA) repeat**	Increased risk		Japan	(Kodama D, 2004)
Perforine +418T	Protection		Iran	(Rafatpanah H, 2004)
***SDF-1 +801A* 3_UTR**	Protection		Japan	(Vine, 2002)
TNF _863A	Increased risk		Japan	(Vine, 2002)
VIT D Receptor APaI→ AA	Protection		Japan	(Saito M, 2005)

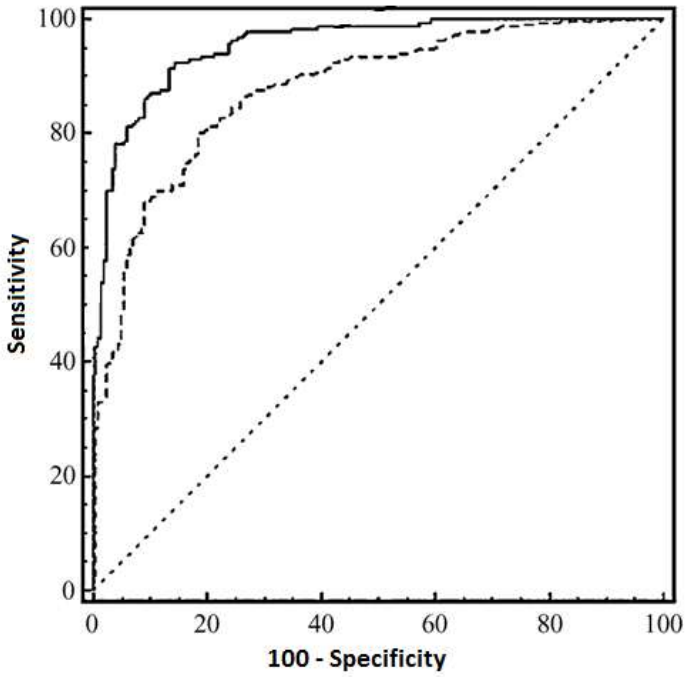
[a] This effect was only observed in Japanese and Iranian *HLA-A*02*-negative but not in *HLA-A*02*-positive, HTLV-1-infected individuals
[b]The effect of *IL-15* disappears in a multivariate analysis when PVL was included.
[c] In HAM/TSP *HLA-A*0206* positive individuals have higher PVL than *HLA-A*0206* negative individuals, but in AC there was an opposite effect

7.1.7. HAMTSP prediction, logistic regression equation

In a candidate gene study in Japan (Jeffery, 1999; Vine, 2002), a best-fit logistic regression equation including the genotype of four loci (*TNF-α-863*, *SDF-1* +801, *HLA-A* and *HLA-C*), age, PVL and viral subgroup (A or B) has been derived (Table 4). This equation calculates the odds that an individual who is infected with HTLV-1 has HAM/TSP (Furukawa, 2000; Vine, 2002).

Nose et al. evaluated if this equation could be used to distinguish HAM/TSP patients from AC. Based on the original study population, they calculated that the optimal cut-off value would be an odds of HAM/TSP of 0.78 (figure 7). They then used this equation in a new population of AC and found an association between high odds of HAM/TSP and clinical signs of latent central nervous system disease (Nose H, 2006).

Figure 7.-Receiver operating characteristic (ROC) curve of HTLV-1 provirus load, and the odds for HAM/TSP calculated by the best-fit logistic regression equation.



The ROC curve was constructed by plotting sensitivity against the false positive rate (1-specificity) over a range of odds for HAM values or HTLV-1 provirus load by using the equation previously reported (Vine *et al*, 2002). The cut-off value to differentiate HAM/TSP and ACs was determined from the ROC curve as 0.78. Odds for HAM/TSP = 0.78 maximizes the sensitivity to diagnose HAM/TSP and minimizes the false-positive rate to misdiagnose ACs as HAM/TSP. Using this value, the sensitivity and specificity of the HAM/TSP odds required to diagnose HAM/TSP are 92.1% and 86.5%, respectively (Nose H, 2006).
————:Odds for HAM/TSP;
– – – – – –: log10 (HTLV-1 Tax copy number per 1×10^4 PBMC).

Table 4**.** Best-fit logistic regression equation for the risk of HAM/TSP in the Kagoshima HTLV-1–infected cohort (Vine *et al*, 2002)

Factor, condition	ln (odds of HAM/TSP)	Odds ratio (P)
Constant	−1.716	
Age	-(0.145 x age)+ (0.003 x age^2)	
Proviral load	+(0.460 × load)+ (0.487 × load^2)	
TNF-α −863A+	+3.057 − (4.616 × load) + (1.476 × load ^{2})	
SDF-1 +801GA	−0.808	0.45(0.042)
SDF-1 +801AA	−1.689	0.18(0.003)
*HLA-A*02+*	−0.638	0.53 (0.043)
*HLA-Cw*08+*	−0.894	0.41 (0.046)
HTLV-1 subgroup B	−1.587	0.20 (0.017)

Example: An HTLV-1–infected individual in Kagoshima, 60 years old, with a log10 (provirus load) of 2.5 with the genotype *TNF-α*−863A+, *SDF-1* +801AA, *HLA-A∗02*−, *HLA-Cw∗08*+, HTLV-1 subgroup B has a predicted ln odds of HAM/TSP of −1.716 −(0.145 ×60) + (0.003 × 60^2) + (0.46 × 2.5) + (0.487 × 2.5^2) + 3.057 − (4.616 × 2.5) + (1.476 × 2.5^2) − 1.689 − 0.894 −1.587 = -1.86 = exp(-1.86) = 0.15. This equation quantify the contribution of each factor to predcit the odds that an HTLV-I–infected individual of specified genotype has HAM/TSP.
Adapted from Vine et. al 2002 (Vine, 2002).

7.2. Adult T-cell leukaemia/lymphoma (ATL)

ATL is an aggressive malignancy of CD4+ T lymphocytes in which the HTLV-1 provirus is integrated. Based on diverse clinical features, namely lactate dehydrogenase levels, calcium levels and organ involvement, a classification into 4 categories is proposed: acute, lymphomatous, chronic and smouldering ATL (Table 5).

Table 5. Diagnostic criteria for adult T-cell leukaemia

Common features

Histology and cytology

1. Infiltration by malignant activated lymphocytes (i.e. flower cells)
2. Expression of CD2, CD3, CD4, and CD5 antigens
3. Absence of CD7 and CD8 antigens
4. Expression of lymphocyte-activation HLA class II markers (HLA-DR) and interleukin-2 receptor α chain (CD25)
5. Positive serology for human T-cell lymphotropic virus
6. Clonal integration of provirus in tumour cells

Subtype classification

Smouldering

1. Skin lesions and lung infiltrates
2. No lymphadenopathy or other visceral involvement
3. 1–5% leukemic cells on blood smear
4. No hypercalcaemia
5. Normal serum concentrations of lactate dehydrogenase

Chronic

1. Skin, liver, lung, or lymph-node involvement
2. No other visceral involvement
3. Lymphocytosis $>4.10^9$/L with circulating adult-T-cell leukemic cells
4. No hypercalcaemia
5. Serum concentration of lactate dehydrogenase <2N*

Acute

1. Organomegaly
2. Multiple visceral involvement
3. Massive blood infiltration by adult T-cell-leukemic cells
4. Frequent hypercalcaemia
5. High serum concentrations of lactate dehydrogenase

Lymphoma

1. Organomegaly
2. Multiple visceral involvement.
3. Less than 1% leukemic cells on blood smear
4. Possible hypercalcaemia
5. High serum concentrations of lactate dehydrogenase

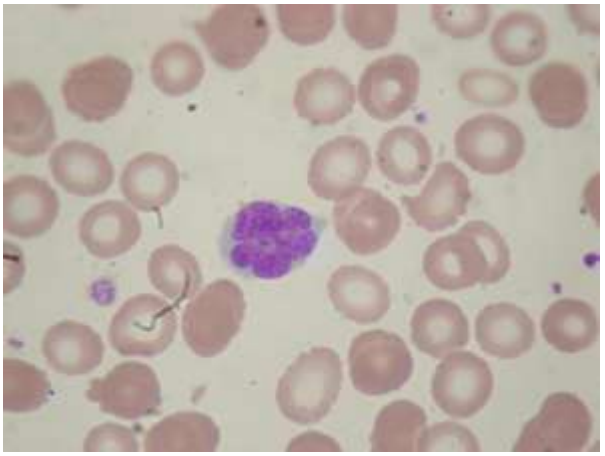
7.2.1. Diagnosis and clinical characteristics

Three diagnostic criteria for ATL have been proposed (Matsuoka M, 2003): 1) the presence of morphologically proven lymphoid malignancy with T-cell surface antigens (typically CD4+, CD25+), 2) the presence of antibodies against HTLV-1 in the serum and 3) the demonstration of monoclonal integration of HTLV-1 provirus. Clinical manifestations of ATL include lymphadenopathy, hepatosplenomegaly, skin lesions and hypercalcaemia. The hypercalcaemia is a characteristic feature of ATL. About 70% of patients with ATL have high serum Ca^{2+} levels during the clinical course of the disease and particularly during the aggressive stage of ATL. This hypercalcaemia may be explained by an increased number of osteoclasts and accelerated bone resorption. (Blayney et al., 1983; Haratake et al., 1985).

The diverse clinical features of ATL have led to its classification in four clinical subtypes: acute, lymphoma, chronic, and smouldering ATL (Shimoyama M, 1991). This classification is based on lymphocyte counts, the frequency of abnormal T cells in peripheral blood, the presence of hypercalcaemia, the level of lactate dehydrogenase and various clinical manifestations such as (histologically-proven) skin or pulmonary lesions, lymphadenopathy, involvement of lung, liver, spleen, central nervous system, bone, gastrointestinal tract, the presence of ascites and pleural effusion (Table 5) (Shimoyama M, 1991).

Acute and lymphoma subtypes are the most aggressive ATL; both are characterized by massive lymphadenopathy (Bazarbachi et al., 2011). In acute ATL, abnormal T lymphocytes with multi-lobulated nuclei and highly condensed chromatin called “flower cells” are common (Figure 8) and 70% of cases will develop hypercalcemia. In smouldering and chronic ATL, the abnormality of the nuclear shape as well as the frequency of ATL cells is generally milder than in acute ATL. In smouldering ATL, skin or lung infiltration is observed and a normal leukocyte counts. Chronic ATL is characterized by high leukocyte count with lymphadenopathy and hepatosplenomegaly without hypercalcemia. (Bazarbachi et al., 2011). Few abnormal cells in the peripheral blood characterize lymphoma-type ATL. However, a small proportion of flower cells can also be found in blood smears of ACs (Shimoyama M, 1991). In fact, a “pre-ATL” state has been described and refers toa state between AC and ATL, it is an asymptomatic phase with abnormal peripheral blood (morphology of ATL cells). This state can progress to one of the four clinical forms of disease or disappear spontaneously (Bazarbachi et al., 2011).

Figure 8.- Flower cell of an ATL patient from the Peruvian HTLV-1 cohort.



Leukemic cells with multilobulated nuclei called "Flower cells" (flower-shaped nuclei) are typically observed in the peripheral blood from patients with adult T-cell leukaemia (ATL). Wright´s eosin methylene blue staining solution, 100x.

7.2.2. Epidemiology

ATL develops in 3% to 5% of individuals infected with HTLV-1. ATL has been related to HTLV-1 infection during infancy, more in particular by infection through breast-feeding (Pawson, 1998). The latency period from the initial infection until onset of ATL is about 60 years in Japan (Takatsuki K, 1994) and 40 years in Jamaica (Hanchard B., 1996).

7.2.3. Prognosis and treatment

The prognosis of ATL is very poor because of the limited effectivity of various anti-cancer drugs, a large tumour burden with multi-organ failure, hypercalcaemia, and frequent infectious complications as a result of a profound T-cell immunodeficiency (Tsukasaki K, 2009). The median survival

time is 6 months for the acute type, 10 months for the lymphoma type and 24 months for the chronic type. The median survival time of smouldering ATL appears to be somewhat longer, but just like chronic ATL, this subtype can convert into one of the more aggressive types.

Patients with acute ATL may have some benefit from combination therapy including zidovudine and interferon alpha. For patients with lymphoma-type ATL, intensive chemotherapy appears to be a better option. The role of therapy in smouldering and chronic ATL remains to be clarified. In addition, allogeneic stem-cell transplantation appears to have improved the survival of some patients who had suitable donors and achieved remission (Utsunomiya A, 2001). Nevertheless, treatment options for ATL remain very limited and do not significantly improve the prognosis of the majority of ATL patients (Dasanu CA, 2011).

7.2.4. Pathogenesis

ATL is characterized by an abnormal growth of HTLV-1-infected cells. Some clones persist for a long time and finally transform into malignant leukemic cells. What exactly determines tumorigenesis is not yet fully understood, but Tax plays an important role in the early stages. Tax inactivates different types of tumour suppressor proteins, activates cellular promoters of several genes such as interleukin 2, interleukin 15 and their cognate receptors and it activates viral promoters, originating the proliferation of T cells. Tax also generates aneuploidy, chromosomal mis-segregation, chromosomal breakage

and fusion events. The accumulation of genetic and epigenetic changes in the host genome, such as mutations in TP53, $P15^{INK4B}$ and $P16^{INK4A}$ and deletion of CDKN2A, finally might lead to Tax-independent proliferation (Yamada Y, 1997).

7.2.5. Host genetics

The genetic background of individuals affected by ATL was investigated. The HLA-A26, HLA-B4002, HLA-B4006, and HLA-B4801 alleles were significantly increased in ATL compared to HAM/TSP and AC in Japan (Yashiki et al., 2001). In Jamaica, HLA-A36, HLA-B18 and HLA–DR53 were more frequent in ATL patients compared to ACs. However, only the HLA-A36 allele remains almost significantly associated with ATL after correction for multiple comparisons (White et al., 1996). In another study Tsukasaki (Tsukasaki et al., 2001) found that the frequency of the TNF-α -857T allele was higher in ATL patients compared to ACs. This allele has been associated with an increased TNF-α production (Tsukasaki et al., 2001).

7.3. Other inflammatory diseases

Uveitis is an inflammatory disorder that affects intraocular tissues (iris, ciliary body and vitreous body). In Japan, a higher seroprevalence of HTLV-1 was found in patients with uveitis of an unknown aetiology than in the general population (Miyanaga M, 2009; Mochizuki M, 1992). Proviral DNA has been detected in the T cell clones derived form the aqueous humor of the patient

(Kamoi and Mochizuki, 2012; Mochizuki M, 1992). The ocular inflammation can be unilateral or bilateral. Usually, uveitis has a good prognosis (normally it disappears after a few weeks), but in some cases, sight-threatening complications can occur.

HTLV-1 also has been associated with other inflammatory diseases such as Sjögren's syndrome, polymyositis, arthritis, synovitis and thyroiditis (Kawai H, 1992; Pinheiro SR, 1995; Sowa, 1992).

7.4. Infectious complications

Several opportunistic infections have been documented in HTLV-1-infected subjects. These include mainly strongyloidiasis, tuberculosis and crusted scabies.

7.4.1. Strongyloidiasis

Individuals infected with *Strongyloides stercoralis* will remain asymptomatic or suffer mild diarrhoea. HTLV-1 infection has been associated with an increased susceptibility and hyperinfection with *S. stercoralis*, as well as with relapse after treatment (Gotuzzo, 1999; Tanaka et al., 2016; Terashima et al., 2002). In individuals with HTLV-1 and *S. stercoralis*, high levels of IFN-γ are observed, which decreases the levels of IL-4, IL-5, IL-13 and IgE, molecules involved in the host defence mechanism against helminths (Carvalho and Da Fonseca Porto, 2004).

In Japan, it was shown that the risk of infection with *S. stercoralis* was twice as high in HTLV-1-infected than in HTLV-1-negative subjects (Hirata T, 2006). Some researchers have suggested that co-infection of HTLV-1 and *Strongyloides* predisposes to ATL development (Plumelle et al., 1997; Sato and Shiroma, 1989). In Japan the infection by *S. stercoralis* is associated with risk to develop cancer and the HTLV-1 prevalence is higher in patients with liver cancer and lymphomas other than ATLL in comparison with other types of cancer (Tanaka et al., 2016).

7.4.2. Tuberculosis

Epidemiological studies showed that active tuberculosis (TB) is more frequent in HTLV-1-positive than in HTLV-1-negative subjects (Matsuzaki T, 1993; Verdonck K, 2008). In addition, in Brazil, the prevalence of HTLV-1 in patients with active tuberculosis was higher (4%) than in controls (1%) (Marinho J, 2005). These findings suggest that HTLV-1 might increase the risk of developing tuberculosis and the severity of this disease (Pedral-Sampaio et al., 1997). It is known that TNF-α plays an important role in the protection against *M. tuberculosis*. TB-HTLV-1 co-infected individuals showed a reduction in TNF-α production. This impairment of the Th1 response in HTLV-1 positive individuals may increase the susceptibility to *M. tuberculosis* (Bastos Mde et al., 2012).

7.4.3. Crusted scabies

Crusted scabies is a rare, severe skin infection caused by massive infestation with *Sarcoptes scabiei* and characterised by hyperkeratotic lesions in pressure areas.

HTLV-1 infection appears to increase the risk of crusted scabies in several countries (Bergman JN, 1999). In Peru, 16 out of 23 patients with crusted scabies were HTLV-1 positive (Blas M, 2005). In Brazil, scabies was found to be associated not only with HIV but also with HTLV-1 infection (Brites, 2002).

7.4.4. Infective dermatitis

Infective dermatitis is a severe and relapsing eczematous disease. A papular rash preferentially affects the scalp, neck, retro auricular region, axillae, and groin. Watery nasal discharge and crusts in the nostrils are common, as is blepharo-conjunctivitis. Positive cultures for *Staphylococcus aureus* and beta-haemolytic *Streptococcus* are frequent. This syndrome usually affects young children but can occur in adults as well. The response to systemic antibiotics and topical steroids is usually good and immediate, but prolonged treatment is recommended due to relapsing (Mahe et al., 2004; Martin et al., 2014).

8. Conclusions

More than a quarter of a century ago, HTLV-1 was first associated with HAM/TSP. Until now, the HTLV-1 PVL remains the only marker of

symptomatic neurological disease in HTLV-1-infected individuals, but with poor discriminatory power. Since the HTLV-1 gene sequences are mostly invariant, intrinsic differences in expression or function of viral genes are unlikely. Clearly, with the possible exception of Tax subgroups, the viral RNA sequence itself does not show relevant differences between HAM/TSP and AC. The role of host cellular immunity in the pathogenesis remains ambiguous. On one hand, HTLV-1 specific CTL may lower PVL and possibly protect against complications. On the other hand, however, both HTLV-1-specific and bystander CD4+ and CD8+ T cells can promote neural damage by infiltrating into the CNS and producing pro-inflammatory IFN-γ, TNF-α and other cytokines/chemokines.

Host genetic factors may account for the difference in efficiency of the CTL response to kill HTLV-1-infected cells and to control the PVL. Genetic factors may influence susceptibility of HTLV-1-infected individuals to HAM/TSP through mechanisms different from PVL as well. Several candidate genes have associated with HAM/TSP but most of these studies did not correct by population stratification or were not replicated in a different population, therefore further validation is needed before to drawn any conclusion about the implication of these genes in HAM/TSP disease. Therefore, we set out to investigate host genetic markers in a well-described cohort of HAM/TSP patients and AC in Lima, Peru.

Chapter 2: Hypothesis, objectives and common procedures

The retrovirus HTLV-1 is genetically stable, because this virus mainly proliferates by cell division and not by reverse transcriptase-based replication. Therefore, HTLV-1-related diseases may not be associated with the genetic variation of the virus, but environmental factors and/or the genetic background of the infected individual host may contribute to the clinical outcome. In this work, we focus on the hypothesis that host genetic constitution is implicated in HTLV-1 disease manifestation.

In Peruvian HTLV-1-infected subjects, several clinical manifestations are observed, including HAM/TSP, ATL and infectious complications. Here we will focus on HAM/TSP because it is the most frequent HTLV-1-associated condition in the cohort of the Instituto de Medicina Tropical Alexander von Humboldt (IMTAvH) in Lima. No previous genetic studies have been performed in this population; only the PVL was found to be increased in HAM/TSP patients compared to AC (Adaui, 2006).

The general aim of this thesis is to identify those human genetic factors that are associated with protection or susceptibility to HAM/TSP development in HTLV-1- infected individuals in the IMTAvH HTLV-1 cohort.

We will address the following specific questions.

1) The validity, in a Peruvian population, of the combined algorithm described in Japan to discriminate HAM/TSP from AC, including the

parameters: *TNF-α*, *SDF-1*, *HLA-02*, *HLA-Cw08*, HTLV-1 subtype, PVL, and age (Vine, 2002).

2) The possibility that, in our population, specific combinations of *KIR* and *HLA* alleles may discriminate HAM/TSP from AC, because of their known role in NK and T cell mediated antiviral responses in general and the proven associations of *KIR* and *HLA* alleles with clinical complications in other viral infections.
3) The possibility that certain markers, including SNPs or tag SNPs, in selected candidate genes may be associated with HAM/TSP in our cohort. The selected genes are related to control of viral load in other diseases, cell adhesion, inflammation, the NF-kB pathway and finally a number of genes that have been associated with HAM/TSP in other populations. In total 45 genes are analysed. For every gene, the SNPs are selected based on previous analyses in other diseases, or, in those cases where there is no previous information; tag SNPs are selected based on the HapMap catalogue.

Setting

The IMTAvH belongs to the Universidad Peruana Cayetano Heredia and is located in northern Lima on the premises of the Hospital Nacional Cayetano Heredia, a 400-bed public referral hospital. HTLV-1 infected subjects are being referred to IMTAvH from inside and outside the hospital. The department of dermatology, infectious and tropical diseases of this public hospital is linked to the IMTAvH. In addition, candidate blood donors in Peru

are screened for HTLV-1 infection, and positive cases detected in this hospital are often referred to IMTAvH.

Since 1989, the IMTAvH has been offering HTLV-1 testing and clinical follow up of HTLV-1-infected people. All diagnosed individuals are invited to participate in the IMTAvH clinical cohort study. In the first years, these were mainly patients with HAM/TSP. Since 1993, the cohort has also recruited relatives of HTLV-1-infected people, candidate blood donors that tested positive for HTLV-1 and patients with other HTLV-1-associated diseases, including ATL, uveitis, infective dermatitis, strongyloidiasis and scabies among others. Demographic, clinical, immunological and virological characteristics of this study population have been published before (Adaui, 2006; Gotuzzo, 2007). At the time when this project started (March 2006), the IMTAvH cohort included more than 2000 HTLV-1-infected individuals.

Study population

For all the studies reported in this thesis, the study participants came from the IMTAvH clinical cohort.

Inclusion criteria for the current host genetics study were:

- Willingness to participate and written informed consent for the IMTAvH clinical cohort as well as for the host genetics studies;
- Confirmed diagnosis of HTLV-1 infection based on enzyme immunoassay and western blot or line immunoassay;

- HTLV-1-infected individual that could be classified either as a patient with HAM/TSP or an AC. The diagnosis of HAM/TSP was based on clinical characteristics and was confirmed by an expert clinician. AC were HTLV-1-infected individuals without signs of HTLV-1-associated diseases.

Exclusion criteria for the host genetics studies were:

- Blood relative of any other participant in the host genetics studies;
- HTLV-2 infection;
- Known HIV infection;
- Probable or confirmed diagnosis of a neurological disorder other than HAM/TSP, such as polyneuropathy, Parkinson's disease, or spastic bladder without gait problems;
- For AC only: probable or confirmed diagnosis of any of the following diseases at any time: ATL, other haematological malignancies, uveitis, dry eye syndrome, scabies, strongyloidiasis, or infective dermatitis.

Study participants were interviewed and sampled when they attended the IMTAvH spontaneously for a follow-up consultation, when they attended the IMTAvH after a phone call by the study team, or during a home visit by the study team.

Study period

Participants were recruited from 2006 to 2008. The third study (candidate gene study) was conducted in two phases. Patients for the first phase were

included between March 2006 and August 2007. Patients for the second (replication) phase were included between September 2007 and August 2008.

Data variables and sources

- Demographic variables that were used in one or more of the human genetic studies were: age, ethnic background, mother tongue, birthplace of study participant, and birthplace of the parents of the study participant. This information was extracted from the IMTAvH cohort database.
- Clinical information from the cohort database was used to select the study participants and to classify them as HAM/TSP patients or AC. In case of doubt, candidate participants were invited for a new physical and neurological examination.
- The HTLV-1 PVL was a key variable in the three studies and was measured as reported by Adaui (Adaui, 2006). The DNA was extracted from PBMC, and the PVL determined by using real-time quantitative PCR, with human endogenous retrovirus 3 as reference gene. The PVL was expressed as the number of HTLV-1 copies per 10^4 PBMC.
- In the first study, the HTLV-1 subtype was determined. The typing methods are explained in chapter 3.
- The motivation for selection of particular human genes, and the specific methods used, is discussed in more detail in chapters 3 to 5.

Motivation of methodological approach

Specific genetic factors have been associated with HAM/TSP before. However, in genetic case-control studies, population stratification can result in spurious association findings due to a non-homogeneous distribution of ethnic groups (subgroup) in cases and controls. When a subgroup is overrepresented, for example in cases, any allele that has a higher frequency in this subgroup may appear falsely associated with the disease. None of the previous association studies in the field of HTLV-1 has taken into account such population stratification effects.

One possible approach to deal with the problem of population stratification is to perform a family-based study, in which the controls are relatives of the cases. However, as HAM/TSP is commonly a late onset disease, it is difficult to collect samples from the parents. For this reason, we decided to use a case-control design, in which the cases are HAM/TSP patients and controls are AC. In practice it is not possible to match cases and controls by genetic ancestry, especially in admixed populations such as the Peruvian. In order to minimize the population stratification effect, Ancestry Informative Markers (AIM) were used. AIM that are distributed across the genome and are unlinked to candidate genes were selected in our analyses.

Another challenge in genetic case-control studies with multiple SNPs analysed, is that if correction for multiple comparisons is performed, these studies are often underpowered, and when correction for multiple comparison

is avoided, spurious associations are likely to be found. Replication of association studies is therefore essential (Hattersley and McCarthy, 2005). In the context of HTLV-1-associated diseases, most of the genetic associations have not been replicated yet and it was our aim to address this shortcoming.

- The first study tried to replicate a previous study form Japan, where several host genes were found to be associated with HAM/TSP disease.
- The second and third study investigated genes and SNPs from various candidate genes. Those studies were performed in two phases by analysing independent individuals; the genes or SNP candidates with main effects in the first phase were evaluated in the second phase to determine the replicability of the results. The two-phase studies were performed according to availability of samples and therefore are not considered strictly a multistage design (van den Oord, 2008).

In ideal case-control-based genetic association studies, several hundreds or thousands of cases and controls are required to achieve sufficient power in order to detect variants with low relative risks. This is, however, difficult to accomplish in HTLV-1 infection, in view of the limited number that can be reached from one study centre. We have collected all possible HAM/TSP and AC cases from the pre-existing IMTAvH cohort during approximately two and a half years and reached a study population of 141 cases and 260 controls. This is the largest study of HAM/TSP patients and AC achieved so far at the IMTAvH and is the largest sample size used compared to most of studies evaluating host genetic factors and HAM/TSP disease.

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Chapter 3: Evaluation of Host Genetic and Viral Factors as Surrogate Markers for HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis in Peruvian HTLV-1-Infected Patients

The aim ofthis study was to evaluate the performance of a prognostic model for HAM/TSP developed in Japan in a Peruvian population.

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Evaluation of Host Genetic and Viral Factors as Surrogate Markers for HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis in Peruvian HTLV-1-Infected Patients

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ABSTRACT

Human T-lymphotropic virus 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a complication that affects up to 5% of HTLV-1-infected individuals. Several host genetic and viral factors have been associated with the risk of HAM/TSP. The aim of this study was to evaluate the performance of a prognostic model for HAM/TSP developed in Japan in a Peruvian population of 71 HAM/TSP patients and 94 AC. This model included age, PVL, the presence of *HLA-A*02* and *HLA-Cw*08* alleles, *SDF-1 +801* and *TNF-*α -863 polymorphisms and viral subgroup. We describe frequencies for the four host genetic markers and demonstrate the presence of the HTLV-1 *tax* B subgroup in Peru. Using cross-validation, we show that the predictive ability of the prognostic model, as characterized by the area under the receiver-operating characteristic curve (Jacobson), does not differ from a model containing PVL only (both AUC=0.74). We found some suggestive evidence of a protective effect of the *HLA-A*02* allele but failed to replicate the associations with the other three genetic markers and with viral subgroup. A logistic model containing PVL, age, gender and *HLA-A*02* provided the best predictive ability in the Peruvian cohort (AUC=0.79).

Key words: human T-lymphotropic virus 1; paraparesis, tropical spastic; genetic predisposition to disease; Peru.

3.1 INTRODUCTION

Human T- lymphotropic virus 1 (HTLV-1) infects an estimated 15 to 20 million people world-wide (de The, 1993). The infection is particularly frequent in Japan, Melanesia, Central Australia, Iran, Central and West Africa, the Caribbean, and South America (Proietti, 2005). In South America, Peru shows one of the highest prevalence rates, with an estimated 1 to 2% of the adult population being infected (Alarcon, 2006; Sanchez-Palacios, 2003).

Although most HTLV-1-infected people remain lifelong asymptomatic, approximately 5-10% will develop severe diseases such as adult T-cell leukaemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Verdonck, 2007). ATL is a neoplastic disease of CD4+T lymphocytes, subclassified in acute, lymphoma, chronic and smoldering types (Tsukasaki K, 2009). HAM/TSP manifests itself as a progressive paraparesis of the legs, often accompanied by back pain, bladder disorders and constipation (Verdonck, 2007).

It is not well understood why some HTLV-1-infected people develop disease and others do not. Disease outcome most likely depends on an interaction between viral, immune and host genetic factors (Bangham CR, 2003). In this regard, high PVL and viral *tax* A subgroup have been associated with the presence of HAM/TSP (Furukawa, 2000; Kira J, 1991; Nagai, 1998) . In a study in Kagoshima, Japan, the host genetic factors *HLA-A*02* and *HLA-Cw*08* were independently associated with both lower PVL and protection against HAM/TSP (Jeffery KJ, 2000; Jeffery KJ, 1999), while the *SDF-1* +801A

allele was linked to HAM/TSP protection and the *TNF-α* -863A allele to increased risk of HAM/TSP (Vine AM, 2002). Using these factors, a logistic regression equation was developed to estimate the odds of HAM/TSP in this Japanese population (Vine AM, 2002). Among the predictors included in this prognostic model, the association of high PVL with HAM/TSP was confirmed in a Peruvian sample set as well as in other settings (Adaui, 2006; Best, 2006). However, PVL shows an extensive overlap between HAM/TSP patients and AC as previously reported (Nagai, 1998) and does not fully explain why some people will develop HAM/TSP (Adaui, 2006).

Besides PVL, no other viral and host genetic factors have been tested for association with HAM/TSP in Peruvian HTLV-1-infected patients. The present study aimed to assess the usefulness of the aforementioned factors as surrogate markers for HAM/TSP in Peru. We determined the frequency of the *HLA-A*02* and *HLA-Cw*08* alleles, *SDF-1* +801 and *TNF-α* -863 polymorphisms and viral *tax* A and B subgroups in a Peruvian study population of 71 HAM/TSP patients and 94 ACs. We subsequently investigated the contribution of these factors to the risk of having HAM/TSP in this cohort and evaluated the predictive ability of the prognostic model developed by Vine et al. (Vine AM, 2002).

3.2 METHODS

Subjects

We included 71 HAM/TSP patients and 94 ACs. They were participants of the HTLV-1 cohort at the “Instituto de Medicina Tropical Alexander von Humboldt” (Lima, Peru). For HAM/TSP diagnosis, internationally accepted clinical criteria were considered (Osame M, 1990). All subjects were genetically unrelated. Origin was defined as Andean (Quechua) if the place of birth of both parents was in the Andean highlands, whereas it was considered Mestizo, if the place of birth of one or both parents was not in the highlands. Patients with known African or Asian ancestry were excluded from the study. Informed consent was obtained from all patients. The study protocol was approved by the Institutional Ethics Committee of the Universidad Peruana Cayetano Heredia.

DNA extraction

For real time quantitation, the DNA was extracted from peripheral blood mononuclear cells (PBMC) using the QIAamp DNA minikit (QIAGEN, Hilden Germany). For genotyping purposes, DNA was extracted from EDTA-treated blood samples using the genomic prep Blood DNA Isolation Kit (Amersham Biosciences UK Limited, England).

Quantitation of HTLV-1 provirus

PVL was measured as reported previously (Adaui, 2006). Briefly, we used a SYBR Green-based real-time quantitative PCR on an iCycler Thermal Cycler

(Bio Rad), with human endogenous retrovirus 3 as reference gene. The PVL was expressed as the number of HTLV-1 copies per 10^4 PBMC.

The performance of our PVL assay was further confirmed by an external validation performed by the group of Prof Anne-Mieke Vandamme (Rega Institute for Medical Research, Katholieke Universiteit Leuven-Belgium). Twenty two blinded samples were analysed using Taqman technology, resulting in a Pearson correlation coefficient of 0.87 ($r^2 = 0.87$).

Genotyping methods

To detect *HLA* types *A*02* and *Cw*08*, we used sequence-specific PCR primers as described elsewhere (Bunce M, 1995). *TNF-α* -863 and *SDF-1* +801 (rs1801157) single nucleotide polymorphism (SNP) genotyping was performed by Kbiosciences (http://www.kbioscience.co.uk).

HTLV-1 tax subgroup determination

The HTLV-1 *tax* gene subgroup (*tax* A or B) was determined by restriction fragment length polymorphism (RFLP) analysis of PCR products (amplifying the *tax* gene) with the enzyme *Acc*II, as previously reported (Furukawa, 2000). The digested PCR products were analysed by electrophoresis on 2% agarose gels.

Admixture proportion

To investigate potential population stratification problems, thirty six Ancestry Informative Markers (AIMs), with large differences in allele frequency between Native American and European populations (Mao et. al.2007), were analysed

in 125 samples (53 HAM/TSP and 72 AC). Admixture proportions for each individual were subsequently estimated using STRUCTURE software (Falush et. al. 2003, Pritchard et, al 2003).

Statistical analysis

Pearson Chi-square or Fisher's exact test (for categorical variables) and Mann-Whitney U test (for continuous variables) were used when appropriate. Logistic regression analysis was performed to test if the host genetic and viral factors analysed were significant after correction for age, gender and PVL. Three predictive logistic regression equations were obtained by using 1) PVL alone, 2) backward elimination on a model containing all variables analysed in this study, and, 3) the factors included in the model developed by Vine et al. (Vine AM, 2002). Assessing the predictive ability of these models by reclassifying the training set to obtain an estimate of the misclassification rate would lead to estimates that are biased upwards due to inevitable overfitting. Therefore, the predictive ability of the different logistic regression models was evaluated by means of ten fold cross-validation. In this procedure, the data is randomly split into ten parts. Each part is omitted in turn from the data and the model is fitted to the other nine parts. Each time, the predictions for the omitted part (test set) are obtained using the model fitted to the other nine parts (training set). We subsequently drew receiver operator characteristic (ROC) curves to compare the performance of the three formulas in discriminating HAM/TSP patients from ACs. The area under the curve (Jacobson, 1990) was used to estimate the accuracy of the prediction. To

reduce fluctuations due to chance divisions of the data, the cross-validation procedure was repeated 20 times and results were averaged. R-software was used for all calculations using the packages MASS, SNPassoc (González JR, 2007) and ROCR (Sing T, 2005).

3.3 RESULTS

Characteristics of HTLV-1-infected patients

The median age of HAM/TSP patients (52 years; range 17-78 years) was significantly higher than that of ACs (44 years; range 13-77 years) (*P*=0.004; Table 1). There were more women among HAM/TSP patients (79%) than among ACs (49%) (*P*<0.001; Table 1). The median PVL, expressed as the copy number of HTLV-1 proviral DNA per 10^4 PBMC was significantly higher for HAM/TSP patients (2602; first quartile (Q1) - third quartile (Q3) = 1586-3943) compared to ACs (1101; Q1-Q3 = 374-2115) (*P*<0.001; Table 1).

Table 1. Descriptive characteristics of HTLV-1-infected AC and HAM/TSP patients

	ACs (n=94)	**HAM/TSP (n=71)**	***P*-value**
Male gender[a]	48(51)	15(21)	<0.001
Age in years, median	44	52	0.004
Andean origin[a]	50(53)	47(66)	0.09
PVL[b], median (Q1-Q3)	1101 (374-2115)	2602 (1586-3943)	<0.001

[a]Data are presented as absolute numbers and percentages (between brackets).
[b]The PVL is expressed as the copy number of HTLV-1 per 10^4 peripheral blood mononuclear cells. ACs: Asymptomatic carriers. Q1-Q3: first quartile-third quartile

Regarding the origin of the participants, 66% of HAM/TSP patients and 53% of ACs were Andean (*P*=0.09). When analysing 36 AIMs using STRUCTURE, we observed no differences in admixture proportions between HAM/TSP patients and ACs (Mann Withney U test, *P*>0.05). In addition, a quantile-quantile plot (data not shown) revealed that the distribution of observed X^2 values obtained from testing the association between AIMs and HAM/TSP status, did not deviate from the expected distribution under the null hypothesis. In contrast, significant differences in admixture proportions were observed between Mestizos and Andeans (Mann Withney U test *P*<0.001) and the distribution of observed X^2 values obtained from testing the association of AIMs and ethnicity deviated from the expected distribution under the null hypothesis. These results indicate that the number of AIMs we selected was appropriate and that confounding due to population stratification was of minor concern in our study.

Comparison of genotypes and allele frequency between HAM/TSP patients and ACs

The presence of *HLA-A*02* and *HLA-Cw*08* genotypes have been associated with lower PVL and with a diminished risk to have HAM/TSP in Japan (Jeffery KJ, 2000; Jeffery KJ, 1999). To examine their link with the presence of HAM/TSP in Peruvian individuals, *HLA-A*02* and *HLA-Cw*08* were genotyped in HTLV-1-infected ACs and HAM/TSP patients. Although *HLA-A*02* and *HLA-Cw*08* were more frequent in ACs than HAM/TSP patients, univariate analysis did not reveal any statistically significant difference in the frequency of these markers between ACs and HAM/TSP patients (*P*>0.05; Table 2).

Table 2. Genotype/allele frequencies in Peruvian HTLV-1-infected AC and HAM/TSP patients

Gene	**Genotype**	**ACs (%) (n= 94)**	**HAM/TSP (%) (n=71)**	***P*-value**	***P*-value corrected[a]**
*HLA-A*02*		79(84)	51(72)	0.06	0.04
*HLA-Cw*08*		17(18)	10(14)	0.5	0.83
TNF-α -863	AA	0(0)	0(0)	0.85	0.98
	AC	11(12)	9(13)		
	CC	83(88)	62(87)		
	A	11(6)	9(6)	0.85	
	C	177(94)	133(94)		
SDF-1 +801	AA	9(10)	5(7)	0.64	0.56
	AG	31(33)	28(39)		
	GG	54(57)	38(54)		
	A	49(26)	38(27)	0.89	
	G	139(74)	104(73)		
HTLV-1 subgroup	B	0(0)	2(3)	0.2	0.99

Data are presented as absolute numbers and percentages (between brackets).
[a]Corrected by age, gender and proviral load.

The effect of *HLA-A*02* and *HLA-Cw*08* on PVL was tested. Median PVL was higher for *HLA-A*02*-positive patients compared to *HLA-A*02*-negative patients but this difference was not significant (1717 copies per 10^4 PBMC compared to 1444 copies per 10^4 PBMC; P=0.19; Table 3). In an analysis stratified according to clinical status, no significant differences in PVL were observed among *HLA-A*02*-positive and *HLA-A*02*-negative patients (P>0.05) (Table 3). No difference in median PVL was observed between *HLA-Cw*08*-positive and *HLA-Cw*08*-negative subjects (1105 copies per 10^4 PBMC

compared to 1776 copies per 10^4 PBMC, *P*=0.07; Table 3). In relation to the clinical status, no significant differences were observed in PVL among *HLA-Cw*08*-positive and *HLA-Cw*08*-negative patients (*P*>0.05; Table 3).

Table 3. Proviral load per *HLA* type for all HTLV-1-infected ACs and HAM/TSP patients and stratified by disease status

	All subjects		**ACs**		**HAM/TSP**	
HLA allele	**median PVL (*N*)**	***P*-value**	**median PVL (*N*)**	***P*-value**	**median PVL (*N*)**	***P*-value**
*HLA-A*02* pos	1717 (130)	0.19	1182 (79)	0.1	2884 (51)	0.06
*HLA-A*02* neg	1444 (35)		611 (15)		1950 (20)	
*HLA-Cw*08* pos	1105 (27)	0.07	907 (17)	0.21	2232 (10)	0.38
*HLA-Cw*08* neg	1776 (138)		1248 (77)		2602 (61)	

Data are presented as median of HTLV-1 copy number per 10^4 PBMCs and number of subjects (between brackets)

TNF-α -863 and *SDF-1* +801 genotypes were determined in ACs and HAM/TSP patients. Both markers were in Hardy-Weinberg equilibrium (*P*=0.4 and *P*=0.3 respectively) and no differences in the distribution of the genotypes or alleles were observed between ACs and HAM/TSP patients for both markers (Table 2). The *TNF-*α -863A allele, previously associated with risk of HAM/TSP, or the *SDF-1* +801AA and *SDF-1* +801GA genotypes, previously associated with protection against HAM/TSP, both in the Japanese cohort (Vine AM, 2002), were not significant in the Peruvian sample set (*P*=0.8, *P*=0.5 and *P*=0.6 respectively). The hypothesis that the *TNF-*α -863A allele increases the risk to develop HAM/TSP in patients with proviral load equal or

higher than 3 copies/100 PBMC (Vine AM, 2002), was not confirmed in our study. No significant results were obtained by univariate and multivariate analysis (*P*>0.05*).*

tax viral subgroup

Furukawa found that *tax* subgroup A increased the risk of developing HAM/TSP (Furukawa, 2000). In our study only two subjects were infected with HTLV-1 *tax* B subgroup; all other subjects (98.8%) were infected with HTLV-1 *tax* A subgroup and no significant differences were established for *tax* viral subgroup between ACs and HAM/TSP (*P*=0.2; Table 2).

Multivariate logistic regression analysis

Logistic regression analysis was performed to determine the effects of *HLA-A*02*, *HLA-Cw*08, SDF-1* +801, *TNF-*α -863 and viral subgroup on HAM/TSP while adjusting for the effects of age, gender and PVL. Only *HLA-A*02* showed an association with a corrected *P*-value <0.05 (*P*=0.04; Table 2). However, this value did not remain significant after correction for multiple testing.

Three different logistic regression models were evaluated using cross-validation (see Methods). The AUC values of the ROC curves were used to compare the performance of these equations in HAM/TSP outcome prediction. No appreciable difference was observed between the equation developed by Vine (AUC=0.739) using Age, PVL, *TNF–*α 863A/C, *SDF-1* +801G/A, *HLA-A*02*, *HLA-Cw*08*, tax viral subgroup (Vine AM, 2002) and the equation using

PVL alone (AUC=0.732). The model obtained using backward elimination, which includes age, gender, PVL and *HLA-A*02* (Table 4) showed better discriminative accuracy (AUC=0.799) compared to either the equation using PVL alone (AUC=0.732) or the model developed by Vine (AUC=0.739) (Vine AM, 2002) (Figure1).

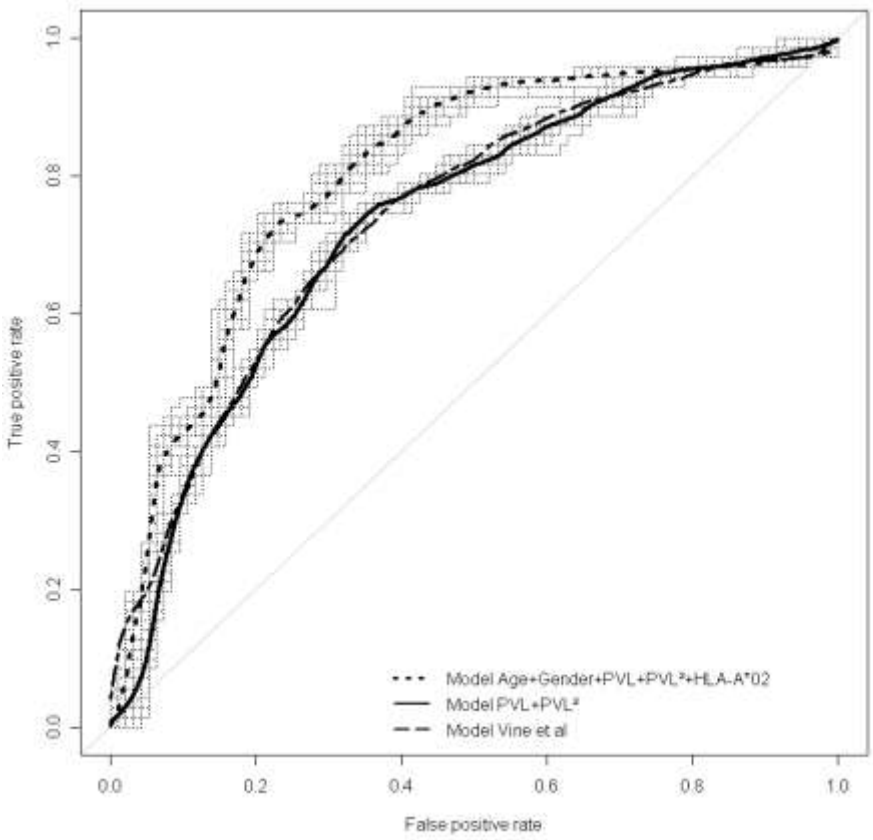
Although *HLA-A*02* remains included in the model obtained by backward elimination (*P*=0.04, uncorrected for multiple testing), its inclusion in the model does not improve the predictive accuracy of the model noticeably (AUC=0.799 compared to AUC = 0.794 for the model without *HLA-A*02*).

Table 4. Odds ratios, confidence intervals and *P*-values for the final logistic regression model obtained by backward elimination

Factor	**OR**	**95% CI**	***P*-value**
Age [a]	1.41	1.12 – 1.7	0.02
Gender (male)	0.31	0.14 – 0.67	0.003
PVL [b]	2.79	1.69 – 4.61	–[c]
*HLA-A*02*	0.37	0.14 – 0.94	0.04

OR = odds ratio, PVL= proviral load, CI= confidence interval
[a] OR associated with a 10 years increase in age
[b] OR associated with an increase of 1000 HTLV-1 copies/ 10^4 PBMC
[c] *P*-values for PVL and PVL^2 were 0.0001 and 0.011 respectively

Figure 1. Receiver operating characteristic (ROC) curve of the model containing proviral load, the model developed by Vine *et al* and the model described in this study.



The model obtained in this study using backward elimination includes Age + Gender + PVL + PVL^2 + HLAA2.
The PVL model includes PVL + PVL^2.
The model by Vine *et. al*. includes Age + Age^2 + PVL + PVL^2 + TNF863.A + TNF863.A*PVL + TNF863.A*PVL^2 + SDF.GA + SDF.AA + HLAA2 + HLACw08 + *tax* viral subgroup.
ROC curves were obtained using 10-fold cross-validation (Martin MP). Bold lines are the averages over 20 CV runs.

3.4 DISCUSSION

In this study, we evaluated the usefulness of *HLA-A*02*, *HLA-Cw*08*, *TNF-α* -863, *SDF-1* +801 and *tax* viral subgroup as surrogate markers for the presence of HAM/TSP in Peruvian HTLV-1-infected subjects. These factors have previously been implicated in susceptibility to HAM/TSP in Kagoshima, Japan and a prognostic model using these factors has been proposed by Vine et al. (Vine AM, 2002). Until now, the association between host genetic or viral factors other than PVL and HAM/TSP in a Peruvian HTLV-1-infected population had not been investigated.

It has been proposed that *HLA-A*02* presents an immunodominant peptide (Tax 11-19) from HTLV-1 to CD8+ cells, thereby improving the efficiency of CD8+ to kill HTLV-1-infected cells (Sakai JA, 2001). The possession of either *HLA-A*02* or *HLA-Cw*08* was associated with a reduction of both HTLV-1 PVL and the risk of developing HAM/TSP in a study from Kagoshima Japan (Jeffery KJ, 2000; Jeffery KJ, 1999). The phenotype frequencies for *HLA-A*02* (78.8%) and *HLA-Cw*08* (16.4%) obtained in this study were similar to the phenotype frequencies reported previously in subjects originating from Arequipa, Peru (*HLA-A*02* = 79.1% and *HLA-Cw*08* = 15.3%) (de Pablo R, 2000). The *HLA-A*02* type is more frequent in Peruvian compared to Iranian (31.1%) or Japanese (37.9%) (Sabouri AH, 2005) HTLV-1-infected subjects. In our Peruvian study population, univariate analysis did not show any significant difference in either PVL or risk of HAM/TSP between *HLA-A*02* or *HLA-Cw*08* positives and negatives. *HLA-A*02*-positive patients showed a

trend towards an increased PVL. The same trend was previously described in a study from Iran (Sabouri AH, 2005). Intriguingly, at the same time we obtained some suggestive evidence from our multivariate analysis that for a fixed proviral load, *HLA-A*02* confers protection to HAM/TSP (OR = 0.37; 95% confidence interval (CI) = [0.14-0.94]; *P*-value = 0.04). The lack of association of the *HLA-A*02* type with low PVL and the only marginal evidence for HAM/TSP protection in the Peruvian cohort is consistent with the hypothesis that *HLA-A*02* is associated with HAM/TSP protection only in *tax* subgroup B-infected subjects (Sabouri AH, 2005). Our results are in concordance with a previous report from Masshad–Iran (Sabouri AH, 2005), except for the suggestive association with HAM/TSP protection shown by *HLA-A*02* after correction for age, gender and PVL.

TNF-α production is of clinical importance because of its inflammatory effects and its possible contribution to tissue damage of the central nervous system (Umehara F, 1994). Several studies reported contradictory results concerning TNF–α production and *TNF-α* -863 alleles (Higuchi, 1998; Skoog and Eriksson, 1999; Udalova IA, 2000). The presence of the *TNF-α* -863A allele has been associated with HTLV-1-associated uveitis (Seki N, 1999) and with increased odds of developing HAM/TSP in HTLV-1-infected subjects with a high PVL (≥3 proviral copies/100 PBMC) (Vine AM, 2002). However, this interaction was not observed in our study. Our result is in agreement with a study from our group (Best, 2006) and a study from Brazil (Santos SB, 2004), in which no significant differences were found in the spontaneous production

of TNF-α between HAM/TSP patients and ACs. The association of *SDF-1* +801 with HAM/TSP risk among HTLV-1-infected subjects in Kagoshima (Vine AM, 2002) was also not replicated in the Peruvian cohort.

The discrepancies between our and previous reports (Furukawa, 2000; Jeffery KJ, 2000; Jeffery KJ, 1999; Nose H, 2006) may be due to the fact that the host genetic factors studied might not be involved in the aetiology of HAM/TSP across all populations. In this aspect, it is worthwhile noting that our study was in agreement with an Iranian study (Sabouri AH, 2005). However, another plausible explanation for the inconsistencies between our study and the study by Vine et al. (Vine AM, 2002) may be that in the latter study no corrections for multiple comparisons were applied and that because of this reason some of the reported associations may in fact be false positives. Indeed, also Yashiki et al. failed to replicate the associations between *HLA-A*02* and *HLA-Cw*08* alleles and HAM/TSP in an independent group of patients from Kagoshima, Japan (Yashiki S, 2001). When association results cannot be replicated, this might indicate that these variants do not influence disease susceptibility (Hattersley AT, 2005).

Concerning the *tax* viral subgroup, almost all patients from the Peruvian cohort were infected with HTLV-1 *tax* subgroup A, corresponding to the cosmopolitan subtype A as reported by Van Dooren et al. (Van Dooren S, 1999). The *tax* subgroup B was observed in only 1.2% of the patients and we did not detect significant differences between the *tax* viral subgroup of HAM/TSP patients

and AC. An identical result was reported previously for Iranian HTLV-1-infected patients (Sabouri AH, 2005).

Although we recognize that our study is limited in sample size, it does not appear to be underpowered when testing for associations between *HLA-A*02*, *HLA-Cw*08, TNF-*α -863A, *SDF-1* +801 polymorphisms, *tax* viral subgroup and HAM/TSP. It is unlikely that our negative results are due to type II errors since the sample size used in our study was larger than the sample size used in the first stage of the discovery of the association between *HLA-A*02* and protection against HAM/TSP in the Japanese setting (50 HAM/TSP and 56 AC) (Jeffery KJ, 1999). In fact, their replication set from London consisted of only 15 HAM/TSP patients and 14 ACs.

We have shown that, in the Peruvian cohort, the prognostic model developed by Vine et al. (Vine AM, 2002) has the same discriminative accuracy to distinguish between HAM/TSP patients and ACs as a model containing only PVL. In addition, we noted that Vine et al. (Vine AM, 2002) evaluated their prognostic model using their training set, a procedure that inevitably results in an overoptimistic estimation of the predictive ability due to overfitting. Furthermore, our data did not provide sufficient evidence for associations between *HLA-A*02*, *HLA-Cw*08*, *TNF-*α -863, *SDF-1* +801 and *tax* viral subgroup to HAM/TSP susceptibility in the Peruvian setting.

In conclusion, the differences observed in HAM/TSP susceptibility between Peruvian and Japanese HTLV-1-infected subjects may be due to possible spurious association in the Japanese population, genetic heterogeneity, gene-

gene and gene-environment interactions and diverging HTLV-1 variants between these populations as important determinants of HAM/TSP. Different factors from the ones evaluated in this study may determine risk of or protection to HAM/TSP disease in Peruvian HTLV-1 infected subjects. As such, efforts towards detecting universal host genetic and viral factors associated with HAM/TSP different from proviral load need to be made. Such efforts may ultimately lead to a better understanding of the pathological mechanisms as well as to predictive testing for HAM/TSP.

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Chapter 4: KIR-HLA distribution between HAM/TSP and asymptomatic carriers

This study aims to analyze if KIR-HLAC combinations are associated with HAM/TSP presence. The frequency distribution of KIR genes, HLA-C1, HLA-C2 groups are determined.

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The role of KIR gene content and HLA-C group in susceptibility to HTLV-1-associated myelopathy / tropical spastic paraparesis in Peru

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KEYWORDS: HTLV-1; HAM/TSP; KIR; HLA-C; Peru

ABBREVIATIONS

HTLV-1	Human T-lymphotropic virus 1
HAM/TSP	HTLV-1-associated myelopathy/tropical spastic paraparesis
PVL	Proviral load
HLA	Human leukocyte antigen
KIR	Killer cell immunoglobulin-like receptors
NK	Natural killers

Abbreviated Title:
"KIR genes and HAM/TSP disease in Peru"

SUMMARY

Human T-lymphotropic virus 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) affects approximately 5% of HTLV-1-infected individuals. It is poorly understood why only some infected subjects develop this disease, but host genetic factors may determine susceptibility. The innate immune system may influence disease outcome in HTLV-1-infected subjects because of its role in early immune responses to viral infections. Variation in genes encoding killer cell immunoglobulin-like receptors (Martin, MP 2007) and their human leukocyte antigen (HLA) molecule ligands may affect the risk of HAM/TSP.

We performed a two-stage case-control study to examine the distribution of KIR genes and HLA-Cw groups in Peruvian HTLV-1-infected HAM/TSP subjects and AC. We also tested for epistatic effects between specific KIR genes and HLA-Cw groups. In the first stage, we found several trends towards association with HAM/TSP or proviral load (PVL). However, these results were not replicated in the second stage. In conclusion, this is the first report on KIR gene frequencies in the Peruvian population and may be of significance in hematopoietic stem-cell transplants. Our study did not reveal significant associations between KIR genes and HLA-Cw groups and HAM/TSP or PVL. However, since our study was only powered to detect larger effects, additional studies using larger cohorts are needed.

4.1 INTRODUCTION

Human T-lymphotropic virus 1 (HTLV-1) affects an estimated 15 to 20 million people around the world, with areas of high prevalence in Africa, the Caribbean, Japan, Melanesia and South America. In South America, the north of Brazil, Colombia, Guyana, and Peru are endemic zones (Proietti et al., 2005; Verdonck et al., 2007). Most HTLV-1-infected individuals remain asymptomatic throughout life. However, between 5 and 10% of infected subjects develop associated diseases such as adult T-cell lymphoma/leukaemia (ATLL) or HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is a progressive inflammatory disease that affects the ability to walk and is considered one of the most severe diseases associated with HTLV-1 (Verdonck et al., 2007).

It is poorly understood why some HTLV-1-infected subjects develop a disease, while others remain asymptomatic throughout life. The pathogenesis of HTLV-1-associated diseases likely depends on interactions between viral and host factors. Several factors have been linked to HAM/TSP, but only the association with high proviral load (PVL) was consistent across all populations analysed (Adaui, 2006; Nagai, 1998; Sabouri AH, 2005). Nevertheless, the PVL alone can not explain the risk of HAM/TSP in all HTLV-1-infected subjects because there is a large overlap in PVL between HAM/TSP patients and AC.

An increased cytotoxic T-lymphocyte (CTL) response has been observed in HAM/TSP patients compared to ACs, which was associated with a high

proviral load of HTLV-1 (Greten, 1998). This increased CTL response suggests that HTLV-1-specific CTL fail to eradicate the virus and might be considered pathogenic rather than protective (Daenke et al., 1996). Additionally, an autoimmune origin of HAM/TSP due to molecular mimicry has been hypothesized, as it has been shown that antibodies that recognize the HTLV-1 tax protein can cross-react with the host heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (Levin MC, 2002).

The ability to kill virus infected cells early in the immune response (the innate immune response) suggests that there may be a role for natural killer (NK) cells in determination of the course of HTLV-1 infections. In fact, it has been reported that HTLV-1-infected HAM/TSP patients have lower numbers of NK cells (Ndhlovu LC, 2009) and a decreased activity of NK cells in comparison with ACs (Fujihara K, 1991; Yu F, 1991).

The activity of NK cells is partially regulated by the balance between inhibitory and activating signals transmitted by a diverse family of receptors. These receptors are located at the surface of the NK cells and are called killer cell immunoglobulin-like receptors (Martin, MP, 2007). KIR are encoded by 14 genes and may transmit either activating or inhibitory signals. Carrying more activating KIR genes has been associated with a stronger immune response and an increased risk for autoimmune diseases such as psoriasis or rheumatoid arthritis (Nelson GW, 2004; Parham P, 2005; Williams F, 2005; Yen, 2001). KIR gene receptors have the capacity to recognize the human leukocyte antigen (HLA) class I molecules on target cells. When the

expression of certain HLA molecules is down-regulated by viral infections or cellular transformation, the KIR and HLA engagement will be altered. Cells that lack expression of self HLA molecules may be destroyed by the cytolytic activity of the NK cells (Raulet DH, 2001).

The HLA-ligands for KIR genes have been divided into 2 major groups based on amino acid differences of HLA-Cw antigens. KIR2DL2, KIR2DL3 and KIR2DS2 bind HLA-Cw group 1 (Cw*01 *03, *07, *08, *12, *13, *14, *1507, *15, and HLA-B*4601 and HLA-B*7301). All have an asparagine at position 80. KIR2DL1 and KIR2DS1 bind HLA-Cw group 2 (HLA-Cw*02, *307, *04, *05 *06, *0707, *0709, *1204, *1205, *15, *1602, *17, *18). Group 2 ligands have a lysine at position 80. The ligands for KIR3DL1 are HLA-Bw4-80I molecules (Gardiner C., 2007; Williams, 2005). Particular combinations of KIR and HLA antigens have been implicated in affecting the course of infectious diseases. *KIR3DS1-HLA-B* delays the progression to AIDS (Martin MP, 2002). *KIR2DL3-HLA-Cw1* favors the resolution of hepatitis C virus infection (Khakoo SI, 2004); and the presence of *KIR2DL2* and *KIR2DS2* predispose to symptomatic herpes simplex virus type 1 infection (Estefanía E, 2007).

Studies on the role of KIR or particular KIR-HLA combinations on HTLV-1 proviral load and HAM/TSP susceptibility are lacking. In the present case-control study, we evaluate the association of KIR genes and HLA-Cw with the susceptibility to HAM/TSP among Peruvian HTLV-1-infected subjects. In addition, we tested for epistatic effects between specific KIR genes and HLA-Cw groups.

4.2 MATERIAL AND METHODS

Subjects

All HTLV-1-infected subjects were recruited from the HTLV-1 cohort of the Institute of Tropical Medicine Alexander von Humboldt in Lima, Peru. The clinical diagnosis of HAM/TSP was made by one or two experts according to international guidelines (De Castro-Costa et al., 2006; Osame M, 1990). Subjects with a sub-normal neurological exam or some other clinical manifestations previously associated with HTLV-1 infection were not included in the current study (Table 1). All selected subjects were genetically unrelated. Their origin was defined as Andean if both parents were born in the Andes, or as Mestizo if at least one parent was not born in the Andes. Patients with known Asian or African ancestry were not included in the study.

We performed a case-control study in two stages in order to avoid false-positive findings. In the first stage we analysed samples from 55 HAM/TSP patients and 109 ACs. In the second stage, we included 85 HAM/TSP patients and 146 ACs. This study was approved by the Research Ethics Committee of the Universidad Peruana Cayetano Heredia and written informed consent was obtained from all participants.

Table 1. Description of HTLV-1-positive patients with subnormal manifestations or other manifestations associated to HTLV-1 not included in the study.

Manifestations	**Motive for HTLV-1 testing**		
	Suspicion of an associated disease (n)	**Symptoms, signs or laboratory results suggestive of an HTLV-1-associated disease (n)**	**Total (n)**
Strongyloidiasis	53	14	67
Neurological problem (not HAM)	8	6	14*
Infective dermatitis	18	2	20
Adult T-cell lymphoma/leukaemia	18	0	18
Dry eye syndrome	1	13	14
Uveitis	10	1	11
Scabies	6	1	7
Tuberculosis as motive for HTLV testing	3	0	3
Other diseases	8	6	14
Total	125	43	168[a]

[a]All of these patients can not be classified as HAM/TSP or AC and were not included in the study.

* Patients with subnormal neurological manifestations not included in the study.

Proviral Load

DNA was extracted from peripheral blood mononuclear cells (PBMC) using the genomic prep Blood DNA Isolation Kit (Amersham Biosciences UK Limited, England). To determine the PVL, we used a SYBR Green-based real-time quantitative PCR on an iCycler Thermal Cycler (Bio Rad), with human endogenous retrovirus 3 as reference gene (Adaui, 2006). The PVL was expressed as the number of HTLV-1 copies per 10^4 PBMC.

Genotyping

DNA was extracted from EDTA-treated blood samples using the Genomic Prep Blood DNA Isolation Kit (Amersham Biosciences UK Limited, England).

In the first stage, KIR genotyping was performed by multiplex polymerase chain reaction (PCR) for the KIR genes *2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4, 2DS4s, 2DS5* and *3DS1* (Sun JY, 2004). The frequency of each KIR gene was calculated as the percentage of positive individuals among all individuals evaluated.

The genotyping in the second stage was performed by singleplex reactions, using sequence-specific primers (Vilches C, 2007). The data obtained in the first stage with the multiplex genotyping reactions were also confirmed with the singleplex reactions to avoid differences due to the use of different primer sets and techniques.

In both stages, we used the primers described by Jones to determine HLA-Cw group (Jones DC, 2006). As some of the primers cross-react with specific *HLA-Bw* alleles, reactions to detect *HLA-Bw* alleles *0713, *6702, *5401, *5402, and *5507 were included to increase specificity of the HLA-Cw subgroup typing (Jones DC, 2006).

The genetic substructure of a population and an admixture may lead to spurious associations in genetic association studies. Therefore, we typed 37 unlinked ancestry-informative markers (AIMs), thus enabling us to correct for population stratification. These genetic markers were selected based on their

large differences in allele frequency between Native Americans and European Americans (Mao et al., 2007) and were located in regions not previously associated to HAM/TSP. Based on other studies (Choudhry S, 2006), we anticipated that this number of markers would be sufficient to detect and correct for population stratification. AIMs were genotyped by Kbiosciences (http://www.kbioscience.co.uk).

Statistical Analysis

For univariate analysis, i.e. comparisons of frequencies of KIR genes, HLA-C groups and specific combinations of KIR genes and HLA-C groups between HAM/TSP patients and ACs, we used the Fisher's exact test. The Mann-Whitney U test was used to compare continuous variables.

For multivariate analysis, logistic regression was carried out to evaluate the association of KIR genes, HLA-Cw group and specific combinations of KIR genes and HLA-Cw group with HAM/TSP, including age, gender and logarithmically transformed PVL as covariates. To investigate whether a putative genetic effect acted through the PVL, the analysis was carried out both with and without the PVL term. If a gene solely acts through PVL, the association between gene and disease status will no longer be significant when the PVL term is included in the model. Conversely, if a gene remains significantly associated with the diseases status, the effect of the gene is, at least in part, independent of PVL.

To deal with potential population stratification problems, we conducted a principal component analysis on the AIMs using the EIGENSOFT software (Price AL, 2006) and included the first three principal components (PC1, PC2, PC3) as covariates in the regression model. The full logistic regression model used was: logit(disease status) = gender + age + PC1 + PC2 + PC3 + PVL + genotype. Genotype was included in the equation as a binary variable (i.e., carrying or not carrying the KIR gene or KIR-HLA combination coded as 1 and 0, respectively).

Linear regression was used to evaluate the association of KIR genes, HLA-Cw group and specific combinations of KIR genes and HLA-Cw group with $\log_{10}$(PVL). Disease status, age, gender and the three first principal components were included as covariates. To test whether a putative genetic effect on PVL was independent of disease status, this analysis was also carried out omitting disease status from the model. We used the R statistical software package for all analyses.

To avoid false positive results, we took a pragmatic approach to the multiple testing issue. We considered replication in an independent data set more important than surviving an overly stringent significance level. As the first stage of this study probably has low power to detect any genetic associations, we used a liberal significance threshold of 0.10 for selecting genes or gene combinations for genotyping in stage 2.

4.3 RESULTS

The characteristics of the participants according to disease status are summarized in Table 1. In both stages, the majority of the HAM/TSP patients were women (*P*<0.01; Table 1). The age of HAM/TSP patients was significantly higher than that of ACs (*P*<0.001 in both stages; Table 1). The PVL, expressed as the copy number of HTLV-1 proviral DNA per 10^4 PBMC was significantly higher in HAM/TSP patients when compared to ACs (*P*<0.001 in both stages; Table 2).

Table 2. Descriptive characteristics of HTLV-1-infected HAM/TSP patients and asymptomatic carriers

	Stage 1			Stage 2		
	HAM/TSP (*N*=55)	ACs (*N*=109)	*P*-value	HAM/TSP (*N*=85)	ACs (*N*=146)	*P*-value
	n (%)	*n* (%)		*n* (%)	*n* (%)	
Male gender[a]	10(18)	62(57)	<0.001	15(18)	54(37)	<0.002
Age in years, median	52	44	<0.001	55	45	<0.001
Andean origin[a]	41(75)	62(57)	0.03	52(61)	79(54)	0.29
PVL[b], median	2994	1290	<0.001	2650	871	<0.001
(Q1-Q3)	(2090-4438)	(537-2574)		(1596-4709)	(226-1971)	

[a] Data are presented as absolute numbers and percentages (between brackets).
[b] The proviral load is expressed as the copy number of HTLV-1 per 10^4 peripheral blood mononuclear cells.
Q1-Q3: first quartile-third quartile

The KIR gene frequencies are shown in Table 3. The “framework” KIR genes *KIR2DL4, KIR3DL2* and *KIR3DL3* were present in all individuals tested. In the univariate analysis, comparing HAM/TSP patients with ACs, there were no significant differences in the frequency of any of the KIR genes and no differences in the number of activating KIR genes ($P > 0.05$).

A multiple logistic regression analysis was used to evaluate the effect of KIR genes alone or KIR-HLA-C combinations on HAM/TSP status. Age, gender and the three first principal components obtained using EIGENSOFT on the AIMs were included as covariates. Since it is not well known whether the elevated PVL is a cause or consequence of the disease, multivariate analysis was carried out both including and omitting PVL as a covariate in the logistic regression model. For none of the KIR genes alone, or KIR gene–HLA-C combinations tested, significant associations with HAM/TSP status or logarithmically transformed PVL were observed. However, a trend (defined as P-value < 0.10) was observed for *KIR2DL3*/*KIR2DL3-HLA-C1C2* when $\log_{10}$(PVL) was included as a covariate in the logistic model (P-value = 0.07; OR = 0.38; 95% CI = 0.14-1.06). Similarly, a trend was observed for *KIR3DL1* (P-value = 0.09, OR 0.23, 95% CI = .04-1.23). When we omitted $\log_{10}$(PVL) from the model, there was a trend for HLA-C1C1 (P-value = 0.06; OR = 2.09; 95% CI = 0.97-4.50).

Multiple linear regression analysis was performed to evaluate the effect of KIR genes alone or KIR-HLA-C combinations on $\log_{10}$(PVL) using age, gender and the three first principal components calculated from the AIMs as covariates. To

investigate whether the effect was independent of disease status, associations were also tested by including the HAM/TSP status in the model. We observed a trend for 2DS4 that was independent of HAM/TSP status and in which absence of this gene was associated with low PVL (P-value = 0.05 and 0.09 when disease status was included and excluded from the model, respectively). In addition, a trend was observed between absence of *HLA-C1C1* and low PVL (P-value = 0.09).

No other trends with HAM/TSP status or PVL were observed in the first stage. All KIR and KIR-HLA-C combinations associated with HAM/TSP status and/or PVL with a P-value < 0.10 were genotyped in a second independent group of samples to investigate whether the associations observed in the first stage could be replicated.

The *KIR2DL3/KIR2DL3-C1C2* combination was near-significantly associated with HAM/TSP outcome (P-value = 0.09) in the second stage. However, the association was in the opposite direction when compared to the first stage. PVL and *HLA-C1C1* were associated with a P-value of 0.03, however, again the association was in the opposite direction. None of the other putative associations could be replicated in the second sample set. Therefore, based on these results, we do not find evidence implicating these genes in the risk of HAM/TSP or the control of PVL.

Table 3. Frequency of KIR genes and HLA-C group in HTLV-1-infected HAM/TSP patients and asymptomatic carriers

Gene	Stage 1		Stage 2	
	HAM/TSP (*N*=55)	ACs (*N*=109)	HAM/TSP (*N*=85)	ACs (*N*=146)
	n (%)	*n* (%)	*n* (%)	*n* (%)
2DL4, 3DL2, 3DL3	55 (100)	109 (100)		
2DS3	5 (9.1)	7 (6.4)		
2DL1	54 (98.2)	108 (99.1)		
3DS1	20 (36.4)	38 (34.9)		
2DS2	20 (36.4)	34 (31.2)		
3DL1[a, b]	50 (90.9)	104 (95.4)	81/84[c] (96.4)	140/145[c] (96.5)
2DL5	24 (43.6)	40 (36.7)		
2DS1	22 (40)	39 (35.8)		
2DS4s	18 (32.7)	36 (33.0)		
2DS4[b]	45 (81.8)	95 (87.2)	71/82[c] (86.6)	122/145[c] (84.1)
2DS5	21 (38.2)	38 (34.9)		
2DL2	23 (41.8)	40 (36.7)		
2DL3	54 (98.2)	108 (99.1)		
C1C1[a,b]	25 (45.5)	37(33.9)	35 (41.2)	58 (39.7)
C1C2	24 (43.6)	50 (45.9)		
C2C2	6 (10.9)	21 (19.3)		
2DL2/2DL3 + C1/C1	8(15)	12(11)		
2DL2/2DL3 + C1/C2	11(20)	17(16)		
2DL2/2DL3 + C2/C2	3(5)	9(8)		3(5)
2DL3/2DL3 + C1/C1	17(31)	24(22)		
2DL3/2DL3 + C1/C2[a]	12(22)	32(30)	7 (8.2)	25 (17.1)
2DL3/2DL3 + C2/C2	3(5)	12(11)		

[a] KIR genes or KIR-HLA C combinations displaying a trend of association (P-value < 0.10) in the logistic regression analysis with disease outcome in stage 1. These associations were followed up in stage 2. See text for association *P*-values.

[b] KIR genes or KIR-HLA C combinations displaying a trend of association (P-value < 0.10) in the multiple linear regression analysis with proviral load in stage 1. These associations were followed up in stage 2.

[c] Total number of typed samples (some samples could not be genotyped).

4.4 DISCUSSION

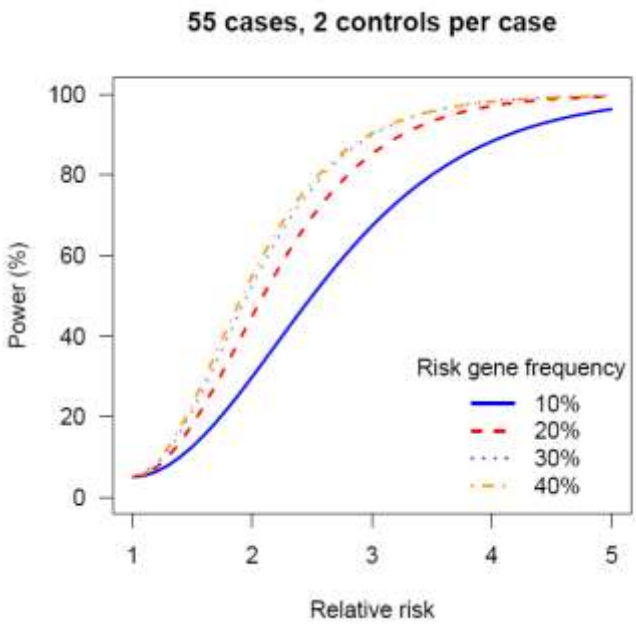
To investigate the possibility that KIR genes may contribute to the genetic susceptibility to HAM/TSP, we analysed whether the distribution of KIR genes differs between HTLV-1-infected HAM/TSP patients and ACs in Peru. In addition, we tested for epistatic effects between specific KIR genes and HLA-C.

The involvement of KIR and HLA class-I molecules in antiviral immune responses has been reported previously. For example, the *KIR2DL3* genotype in the presence of the *HLA-C1C1* genotype influences the resolution of hepatitis C virus infection (Khakoo SI, 2004), *KIR2DL2/KIR2DL3* in the absence of *HLA-C1* is more frequent in HIV-1-exposed but seronegative female sex workers (Jennes W, 2006), and the presence of *KIR2DL2* and *KIR2DS2* increases the risk of symptomatic herpes simplex virus type 1 infection (Estefanía E, 2007).

Despite the fact that KIR-HLA combinations influence the outcome of other viral infections, we did not detect significant differences in the frequency of KIR genes or *KIR2DL2/KIR2DL3* - HLA-C combinations between HTLV-1-infected HAM/TSP patients and ACs in a Peruvian cohort. Even though we found no differences in KIR gene frequency and *KIR2DL2*/*KIR2DL3-HLA-C* combinations in this study, we cannot rule out a role of KIR genes in HAM/TSP development. A limitation of our study is the modest sample size of both stages which only allows the detection of genes with large effect sizes. We calculated the power of our study as a function of effect size for different

risk gene frequencies. Supplementary Figure 1 shows that our study has low power to detect small genetic effect sizes that are typical for common multifactorial diseases such as metabolic, auto-immune and psychiatric disorders (relative risks in the range of 1.1 to 1.5). However, genetic determinants for susceptibility to infectious diseases generally display much higher effect sizes with relative risks greater than 2.5 not being an exception (Kaslow RA, 2008; Martin MP, 2007). Figure 1 shows that we have good power to detect these larger effect sizes. E.g., for a relative risk of 2.5 and assuming a risk gene frequency of 20% we have 70% power to detect an association in stage 1 of our study.

Figure 1. Power as a function of genetic effect size for different risk gene frequencies.



In the calculations we assumed a HAM/TSP prevalence of 5% among HTLV-1 infected subjects and used a significance level of 0.05. Calculations were performed in Quanto (Gauderman and Morrison, 2006).

Furthermore, specific combinations of *KIR3DL1* variants, with different levels of surface expression on NK cells, and HLA-B alleles have been shown to be involved in the control of plasma HIV RNA levels and progression to AIDS in HIV-1-infected individuals (Martin MP, 2007). In the present study only KIR gene content was investigated. The impact of the allelic variation, variation in gene expression and epistatic interactions between *KIR* and *HLA-B* alleles, will be the subject of future research. In addition, other genes expressed in NK cells display a different expression level between HAM/TSP patients and ACs, e.g., expression of CD94/NKG2A is lower in HAM/TSP patients, while an over-expression of NKG2D and NKG7 receptors has been found in HTLV-1-positive patients with low proviral load. Therefore, the participation of NK cells in disease outcome of HTLV-1-infected subjects remains a possibility (Saito M, 2003; Vine et al., 2004).

Universal risk factors for HAM/TSP other than high PVL and female gender have not been reported. Given that HAM/TSP appears to be a complex disease, multi-centre studies using large sample sizes are likely to be necessary to unravel its pathogenic basis. The discovery of host genetic factors involved in HAM/TSP development across all populations will lead to a better understanding of the mechanisms of disease progression.

Our study constitutes the first study evaluating associations between KIR genes and HAM/TSP disease and the first report on KIR gene content in the Peruvian population. As KIR genotype may affect the outcome of tissue

transplantation, pregnancy and autoimmune diseases, our reported population frequencies are of practical relevance.

In conclusion, our results did not reveal significant associations between the presence or absence of certain KIR genes and HLA-C groups and HAM/TSP or PVL. However, since our study was only powered to detect larger effects, further studies are needed to completely discard the role of KIR genes in HAM/TSP disease.

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Chapter 5: Candidate gene analysis for susceptibility to HTLV-1-associated myelopathy / tropical spastic paraparesis in Peruvian HTLV-1 infected patients

This study aims to analyze host genetic factors by evaluating several SNPs from 45 candidates and six HLA alleles between HAM/TSP and AC. In addition ancestry informative markers are sued to minimize the possibility of false positive results due to population stratification.

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Candidate gene analysis for susceptibility to HTLV-1-associated myelopathy / tropical spastic paraparesis in Peruvian HTLV-1 infected patients

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ABSTRACT

The human T-cell lymphotropic virus type 1 (HTLV-1) is the etiological agent of HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), a progressive disease causing paraparesis of the lower limbs. Only a minority of HTLV-1-infected subjects develop HAM/TSP. Universal susceptibility factors for HAM/TSP are unknown. Only a high PVL has been associated with HAM/TSP, but this factor does not fully explain the presence of disease in HTLV-1-infected subjects. Most likely, host genetic factors play an important role in HAM/TSP development. We performed a two-stage case-control study to evaluate the association between HAM/TSP and candidate single nucleotide polymorphisms (SNPs) from 45 genes in addition to six Human leukocyte antigen alleles. Several SNPs belonging to *NF-KB1A* and *NKG2D* showed a trend of association in the same direction in both stages, suggesting that *NFKB1A* and *NKG2D* might be implicated in HAM/TSP development. Further replication studies in independent HTLV-1-infected groups should validate these observed associations.

KEYWORDS: Human T-lymphotropic virus 1, Paraparesis, Tropical Spastic, Peru, Genetic Association Studies

5.1 INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) was the first human retrovirus discovered. HTLV-1 infects approximately 20 million people around the world. Although this virus has a worldwide distribution, some areas are considered endemic: Japan, Africa, the Caribbean basin and South America. HTLV-1 is transmitted via three main routes a) mother-to-child transmission b) sharing of infected blood (drug users, transfusions) and c) sexual intercourse.

HTLV-1 preferentially infects CD4+ T cells and to a lesser extent CD8+ T cells (Nagai M, 2001; Ruscetti et al., 1983). Most of the infected individuals remain asymptomatic but between 5 and 10% develop adult T- cell Leukemia (ATL), HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), infective dermatitis, or other infectious (e.g. strongyloidiasis, scabies) or inflammatory (e.g. uveitis, alveolitis) complications. HAM/TSP is a chronic disease characterized clinically by spastic weakness of the legs, back pain, sensory signs and symptoms, constipation and bladder problems.

The causative factors that predispose to HAM/TSP development are not well known. Many viral and genetic factors have been analysed in several populations to evaluate their association with HAM/TSP. So far, only high PVL remains consistently associated with HAM/TSP across populations (Adaui, 2006; Nagai M, 1998; Sabouri AH, 2005), although some AC have a high PVL and some HAM/TSP patients have a low PVL.

The fact that overlapping values of PVL exist between HAM/TSP patients and AC, and the fact that HTLV-1 strains with identical genomic sequences are associated with two clinically different disease outcomes such as ATL and HAM/TSP suggest that HTLV-1 infection alone is not sufficient to cause HAM/TSP. A multifactorial etiology is proposed for HAM/TSP, with host genetic, viral and environmental factors as contributors to disease susceptibility. As such, the identification of genetic determinants of HAM/TSP susceptibility constitutes a major challenge.

The purpose of this study was to ascertain whether certain human genetic factors are associated with HAM/TSP in Peruvian HTLV-1-infected subjects, using a two-stage candidate gene association study. We selected genes related to immune functions including genes involved in innate immunity, genes encoding cytokines, chemokines and human leukocyte antigens (HLA), and genes involved in cell adhesion.

5.2 METHODS

Study participants

All subjects were recruited from the HTLV-1 cohort of the Institute of Tropical Medicine Alexander von Humboldt in Lima, Peru. All HTLV-1-infected subjects underwent clinical examination. HAM/TSP status was determined by one or two experts according to international guidelines (De Castro-Costa et al., 2006; Osame et al., 1990). Patients with subnormal neurological

manifestations, with other disease manifestations associated with HTLV-1 and HTLV-II positive subjects were excluded from the study. A subject's origin was defined as Andean if both parents or all grandparents were born in the Andes or as Mestizo if at least one parent was not born in the Andes. Patients with known Asian or African ancestry were excluded from the study. All subjects were unrelated.

We performed a case-control study in two stages in order to avoid false-positive findings. In the first stage we analysed samples from 56 HAM/TSP patients and 114 AC. With this number of samples we have 80% power to detect susceptibility variants with relative risks of 2.1 or higher. In the second stage, we evaluated 85 HAM/TSP patients and 146 AC. The study protocol was approved by the Institutional Research Ethics Committee of the Universidad Peruana Cayetano Heredia and a written informed consent was obtained from each participant.

Candidate genes

The candidate genes for the analysis were selected based on the literature according to the following criteria:

1. Genes previously associated with HAM/TSP protection or risk in other populations (e.g., *TNF-α*, *IFN-γ*, *SDF-1*, *HLA-A02*, *HLA-Cw08*).
2. Genes associated with control of viral load or disease progression in other viral diseases (*CX3CR1*, *DC-SIGN* and *PD-1*).

3. Genes with different expression levels between HAM/TSP patients and AC (e.g., *Foxp3*, *Granzyme A*, *Granzyme H*, *Granulysin*, *IL6*, *NKG2D*, *NKG7* and *Perforin*).
4. Genes involved in the NF-kB pathway (e.g., *NEMO* and *NFκBIA*).
5. Genes involved in self-reactive immune responses (e.g., *PD-1*) or autoimmune diseases (e.g., *CXCL10*).
6. Genes encoding cytokines (e.g., *IL2* and *IL10*) and their receptors (*IL2R*).
7. Genes encoding cellular adhesion molecules (e.g., *ICAM-1* and *VCAM*).

Relevant references underscoring the selection of each gene are given in Supplementary Table S1.

SNP selection

Within the selected genes and based on literature, SNPs previously associated with other viral or inflammatory diseases or to HAM/TSP in other populations were selected for analysis in this study. Some selected SNPs were located within the gene, whereas others were located in the promoter region. For instance, for some genes differentially expressed between HAM/TSP and AC, functional SNPs located in the promotor region were selected (e.g., *Metalloproteinase2* -1306).

At least one SNP was selected for each gene. When no candidate SNPs were available for a candidate gene, the HapMap catalogue was used to select

tagSNPs (Consortium 2005), using the Tagger option of the Haploview software (Barrett et al., 2005) and the European-derived HapMap CEU panel as the reference panel. Service *et al.* and González-Neira *et al.* (González-Neira A., 2006; Service S., 2007) showed that tagSNPs defined in the CEU reference panel are also efficient for populations from the Americas.

For SNPs showing a trend of association in both stages of the present study, fine mapping was performed by analyzing additional tagSNPs in the regions adjacent to both sides of the associated SNPs. The 6 HLA alleles, the 45 candidate genes, and the rs number of the 94 selected SNPs are listed in Supplementary Table S1.

Ancestry-Informative Markers

Population stratification can lead to spurious associations between a phenotype and a marker locus, or indeed may mask true associations. To reduce this possibility, we used 37 ancestry-informative markers (AIMs) to correct for population stratification. We selected AIMs with large differences in allele frequencies (Δ>0.67) between Native Americans and European Americans (Mao and and Parra, 2007). The AIMs were distributed across the genome and unlinked to the selected candidate genes. The rs number, the chromosome number and chromosome position of the analysed AIMs are given in Supplementary Table S2.

Genotyping

DNA was extracted from EDTA-treated blood samples using the genomic prep Blood DNA Isolation Kit (Amersham Biosciences UK Limited, England). For HLA typing, sequence-specific PCR primers were used as described previously (Bunce M, 1995). SNP genotyping was performed by Kbiosciences (http://www.kbioscience.co.uk).

Proviral Load

The DNA was extracted from peripheral blood mononuclear cells (PBMC) using the QIAamp DNA minikit (Qiagen, Hilden, Germany). The was determined using a SYBR Green-based real-time quantitative PCR on an iCycler Thermal Cycler (Bio Rad) as described elsewhere (Adaui, 2006). Human endogenous retrovirus 3 was used as reference gene. The PVL was expressed as the number of HTLV-1 copies per 10^4 PBMC.

Statistical Analysis

Quality control. Samples and SNPs with more than 10% missing genotypes and non-polymorphic SNPs, were excluded from the analysis. A test for deviation of Hardy-Weinberg equilibrium was performed and SNPs were excluded if $P<0.001$.

Detecting and correcting for population stratification. To evaluate whether the AIMs show differences in allele frequency between cases and controls, a χ^2-test was performed and Quantile-Quantile plots (Q-Q plots) were

constructed. Under the null hypothesis, no differences are expected between the distribution of observed and expected χ^2 values and the points should fall approximately along the reference line. A deviation from the reference line might be indicative of the presence of population stratification between cases and controls. An identical approach was used for a comparison between Andeans and Mestizos. Principal component analysis (PCA) was conducted on the AIMs using the EIGENSOFT software (Price AL, 2006) and the first three principal components were used to correct for population stratification.

Association analysis. We first carried out a univariate analysis. A Chi square (χ^2) or Fisher exact test for categorical variables and a Mann-Whitney U-test for continuous variables were used when appropriate. Logistic regression analysis was performed to test for association between disease status and genotype under the assumption of an additive genetic model. Age, gender, PVL and the first three principal components from the PCA on the AIMs were used as covariates to adjust each association. Replication analysis was performed in an independent data set. Due to the low power because of the moderate sample size and the exploratory nature of the study, replication in an independent data set was considered more important than correcting for multiple testing. Therefore, we used a liberal threshold in the first stage. Those SNPs displaying a trend of association in the first stage (P-value ≤0.1), were evaluated in the second stage. The statistical software package R was used for all analyses.

5.3 RESULTS

Patient characteristics

The characteristics of the participants are summarized in Table 1. In both stages there were more women among HAM/TSP patients than among AC ($P<0.01$; Table 1). HAM/TSP patients were older than AC ($P<0.001$; both stages; Table 1). Overall, the proviral load (expressed as the copy number of HTLV-1 proviral DNA per 10^4 PBMC) was significantly higher in HAM/TSP patients than in AC ($P<0.001$; Table 1). Regarding ethnicity, a marginal difference was observed in the first stage ($P=0.044$; Table 1), while no statistical difference was observed in the second stage ($P=0.29$; Table 1), although in both stages, a slight excess of Andean origin was noted among HAM/TSP patients (Table 1).

Table 1. Descriptive characteristics of HTLV-1-infected HAM/TSP patients and asymptomatic carriers

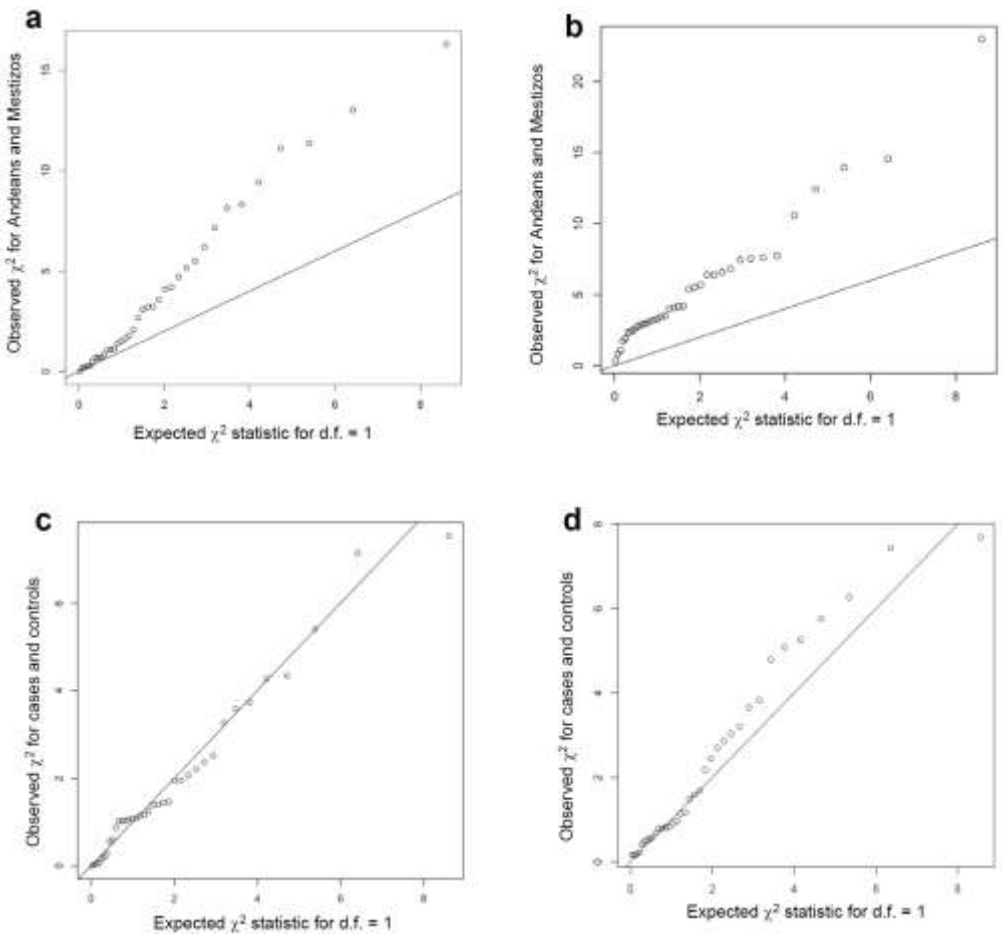
	Stage 1			Stage 2		
	HAM/TSP (*N*=56)	AC	*P*-value	HAM/TSP (*N*=85)	AC (*N*=146)	*P*-value
	n (%)	(*N*=114)		*n* (%)	*n* (%)	
		n (%)				
Male gender[a]	10(18)	63(55)	<0.001	15(18)	54(37)	0.003
Age in years, median	52.5	44	0.001	55	45	<0.001
(Q1-Q3)	(44.8 – 58.3)	(37 – 54.5)		(43 – 65)	(38 – 53)	
Andean origin[a]	42(75)	66(58)	0.044	52(61)	79(54)	0.29
PVL[b], median	2943	1273	<0.001	2650	871	<0.001
(Q1-Q3)	(2057–4418)	(535-2567)		(1596-4709)	(226-1971)	

[a] Data are presented as absolute numbers and percentages (between brackets).
[b] The proviral load is expressed as the copy number of HTLV-1 per 10^4 peripheral blood mononuclear cells.

Population structure

The Q-Q plots for Andeans and Mestizos show a higher deviation from the reference line in both stages (Fig.1a, 1b). This fact suggests differences in admixture proportion between Andean and Mestizos. The differences observed in the Q-Q plot evaluating ethnic origin suggest that the number of AIMs analysed in this study is sufficient to detect differences in admixture proportion. For cases and controls, the Q-Q plots show a closer match with the reference line in both stages, suggesting that no significant differences in admixture proportion exist between cases and controls (Fig 1c, 1d).

Figure 1. Q-Q plots of expected χ^2 statistic for one degree of freedom (x-axis) versus observed χ^2 (y-axis) for the comparison of AIM frequencies between Andeans and Mestizos from the first stage (a), and the second stage (b), and for the comparison between HAM/TSP and AC from the first stage (c) and the second stage (d).



First stage

After genotyping, quality control was performed resulting in the exclusion of 12 samples with more than 10% missing genotypes, 10 non-polymorphic SNPs

(Supplementary Table S3) and 2 SNPs with more than 10% missing genotypes. None of the SNPs deviated from Hardy-Weinberg equilibrium.

Initially, univariate analysis was performed to investigate the relationship between each genotype and disease. The complete list of analysed candidate SNPs and their *P-values* resulting from the univariate analysis are listed in Supplementary Table S1.

To correct for possible confounding variables, a multivariate analysis was also performed. Age, gender and the three first principal components obtained using EIGENSOFT based on the AIMs, were included as covariates in a logistic regression model. After analysis, two HLA alleles and ten SNPs belonging to seven genes showed a trend of association ($P<0.1$, Supplementary Table 1). When PVL was included as covariate in the logistic regression model, two HLA alleles and twelve SNPs belonging to nine genes showed a trend of association ($P<0.1$, Table 2 and Supplementary Table 1). The trends initially observed for *CTLA4*_49 rs231775 and for *TLR9*_1486 rs187084 (Supplementary Table 1), disappeared when PVL was included into the model, indicating that the effect of these SNPs may be acting through PVL. Four additional SNPs showed a trend of association when PVL was included as a covariate, *NKG7*_2 rs3009, *MMP2*_1306, *TLR2*_16934 rs4696480 and *PD1*_19 rs2227982 ($P<0.1$, Table 2 and Supplementary Table 1). The two HLA alleles and the twelve SNPs found when PVL was included in the logistic regression model were evaluated in the second stage, as the

objective of the study was to identify markers associated with HAM/TSP but not acting through PVL.

Second stage

To determine whether the trends of association observed in the first stage were consistent, the relevant SNPs were analysed on the samples from the second stage. Three of the twelve evaluated SNPs showed a trend of association after logistic regression analysis including age, gender, PVL and the three first principal components as covariates ($P \leq 0.1$; Table 2), *NF-KBIA* (rs3138053 $P=0.1$, rs2233406 $P=0.074$) and *NKG2D* (rs1049174 $P=0.0823$). *P*-values for the complete data set including both stages were also calculated (Table 2). *P*-values < 0.05 were observed for SNPs belonging to *NF-KBIA* (rs3138053 $P=0.0279$, rs2233406 $P=0.0167$) and *NKG2D* (rs1049174 $P=0.0038$, rs12821887 $P=0.0248$, rs12819494 $P=0.0235$), indicating that the association was in the same direction in the two stages.

Table 2. HLA alleles and SNPs showing a trend of association in the first stage, and their *P*-values observed in the first stage, the second stage and when using the complete data set

HLA / SNP	rs number	*P*-value*	*P*-value*	*P*-value*
		First stage	Second stage	Complete data set
HLA.B07		0.0886	0.4548	0.6712
HLA.Cw07		0.0107	0.4068	0.2771
*IFNg*_874	rs2430561	0.0433	0.1333	0.8281
*MMP2*_1306		0.0173	0.3768	0.1954
*NF-KBIA*_2	rs3138053	0.036	0.1096	0.0279
*NF-KBIA*_3	rs2233406	0.0316	0.074	0.0167
*NKG2D*_2	rs1049174	0.0225	0.0823	0.0038
*NKG2D*_3	rs12821887	0.0692	0.2728	0.0248
*NKG2D*_6	rs12819494	0.0536	0.3553	0.0235
*NKG7*_2	rs3009	0.0804	0.9873	0.2758
*PD1*_19	rs2227982	0.0473	0.818	0.3103
*RANTES*_403	rs2107538	0.0895	0.1216	0.8923
*TGFbeta*_509	rs1800469	0.0214	0.9867	0.1591
*TLR2*_16934	rs4696480	0.0503	0.9134	0.3949

* *P*-values obtained after logistic regression analysis including age, gender, PVL and three principal components as covariates. Genes or SNPs displaying a trend of association (*P*-value < 0.10) in the first stage were analysed in the second stage. *P*-values for the complete data set are also given. The *P*-values are not corrected for multiple testing.

Fine mapping

To finemap the associated region, we evaluated additional tag SNPs adjacent to the initially associated SNPs. One SNP for *NF-KBIA* and four SNPs for *NKG2D* were analysed. *P*-values resulting from the analysis of these extra SNPs are given in Table 3. This experiment did not result in the detection of

stronger association signals or in a further delimitation of the region possibly associated with HAM/TSP. When both sample sets were combined, we found a strong association signal for two SNPs; *NKG2D* (rs11053781, *P*=0.0042) and *NF-KBIA* (rs3138045, *P*=0.0085) (Table 3). However, these associations did not survive a correction for multiple comparisons.

Table 3. Fine mapping using additional tagSNPs within *NF-KBIA* and *NKG2D*

Gen / SNP	rs number	*P*-value*	*P*-value*	*P*-value*
		First stage	Second stage	Complete data set
*NF-KBIA*_6	rs3138045	0.0078	0.1078	0.0085
*NKG2D*_8	rs10772271	0.2755	0.0699	0.0299
*NKG2D*_9	rs10845123	0.1148	0.8732	0.4702
*NKG2D*_10	rs12231827	0.0108	0.3992	0.2756
*NKG2D*_11	rs11053781	0.0318	0.0803	0.0042

* *P*-values obtained after logistic regression analysis including age, gender, PVL and three principal components as covariates. *P*-values for the complete data set are also given. The *P*-values are not corrected for multiple testing.

5.4 DISCUSSION

A consensus exists on the fact that host genetic factors are important for the development of HAM/TSP in HTLV-1-infected subjects. However, so far, no host genetic factors that are consistent across populations have been established in association with HAM/TSP. We previously evaluated a limited set of genetic factors associated with HAM/TSP in other populations, but we could not replicate these associations in our samples of Peruvian HAM/TSP patients and AC (Talledo M, 2010). This fact and the possibility that other host genetic factors might be involved in the development of HAM/TSP in HTLV-1-infected subjects, prompted us to analyse other gene polymorphisms as candidates for susceptibility to HAM/TSP.

Genes previously associated with HAM/TSP in other populations such as *IL6*-634 in Japan and Brazil (Gadelha et al., 2008; Nishimura M, 2002) or *Perforine* 418*C/T in Iran (Rafatpanah H, 2004) were not replicated in our population despite the similar sample sizes. Additionally, no significant differences were found for *IL10*-592, which is in agreement with the results obtained in Brazil (Gadelha et al., 2008) and in contrast to the association found by Sabouri in Japan (Sabouri AH, 2004).

Similar to a previous report by our group, no trends of association between HAM/TSP and *HLA-A*02* or *HLA-Cw*08* were observed in the first stage of our study (Talledo M, 2010). In addition, *HLA-A*24* and *HLA-DRB1*01* were not associated with HAM/TSP. *HLA-B*07* and *HLA-Cw*07* showed a trend for

association with HAM/TSP in the first stage, but were not replicated in the second stage.

Population stratification can lead to false positive and false negative findings. Therefore, we used AIMs to correct for population stratification. The minor deviation from the reference line observed between cases and controls in the Q-Q plots in both stages suggest that population stratification might not be a problem in our study. However, to completely exclude a possible influence of population stratification we used the first three principal components of an EIGENSOFT analysis as covariates in the logistic regression model.

Although no correction for multiple testing was performed due to the exploratory nature of the study, the *P*-values observed for some SNPs of *NF-KBIA* and *NKG2D* suggest that these genes may influence susceptibility to HAM/TSP. These findings need to be confirmed in larger populations of HTLV-1- infected subjects and the importance of these genes in HAM/TSP development across populations should also be evaluated in populations different from the Peruvian.

NKG2D is a C-type lectin-like receptor constitutively expressed on all human natural killer (NK) cells, CD8+ T cells, and $\gamma\delta$ T cells. NKG2D engagement stimulates the secretion of cytokines and release of cytolytic granules. The expression of *NKG2D* in CD8+ cells from AC or HAM/TSP patients with low PVL was high compared to the expression observed in AC or HAM/TSP patients with high PVL (Vine et al., 2004), suggesting an inverse correlation

between *NKG2D* expression and PVL. Our findings are in agreement with those observed by Vine *et al*. (2004). We conclude that NK cells might play an important role in the host defence against HTLV-1 infection.

Tax protein is a potent transcriptional activator of HTLV-1 genes as well as specific cellular genes. It is known, for example, that tax stimulates the NF-kB pathway (Sun and Ballard, 1999). NF-kB is tightly sequestered in the cytoplasmic compartment due to its interaction with members of the IkB family of inhibitory proteins such as NF-KBIA. The SNPs of *NF-KBIA* that showed a trend of association with HAM/TSP susceptibility in this study are localized in the promoter region of the gene. Possibly, these SNPs are involved in the modulation of *NF-KBIA* expression. A plausible hypothesis may be that the susceptibility alleles lead to a decreased expression of *NF-KBIA*, resulting in a decreased inhibition of NF-kB. This may in turn lead to NF-kB activation and an enhanced transcription of a large number of immunorelevant genes as a consequence. As such, *NF-KBIA* may play a crucial role in the pathogenesis of HTLV-1 infection. However, functional studies need to be performed to confirm this hypothesis.

The primary objective of the present study was to identify markers associated with HAM/TSP that act independently of PVL, as these are the most valuable for prognosis. However, PVL itself has been shown to be under (partial) host genetic control (Jeffery, 1999).

For several alleles belonging to different genes we could not detect an association with HAM/TSP. Possibly, the effect sizes of these factors are very small and our sample size may not have sufficient power to detect the effect of these alleles on HAM/TSP development. Given that no large cohorts, comparable with those used in association studies of malaria, tuberculosis or HIV, are available to attain sufficient statistical power, it is imperative to perform multicentric studies on HTLV-1-infected subjects to find genes that are significantly associated with HAM/TSP susceptibility across diverse populations.

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Supplementary Data

Supplementary Table 1. Genes and SNPs analysed and *P*-values obtained after univariate and multivariate analysis.

					Multivariate Analysis					
Variant name	**Official gene symbol**	**Gene name**	**rs number**	**Univariate analysis[a]**	**PVL term omitted[b]**	**Corrected for PVL[c]**	**Chrom.**	**Contig accesion**	**Position of RefSNP in chromosome coordinates**	**Refer.**
CCR2_64	*CCR2*	chemokine (C-C motif) receptor 2	rs1799864	0.8116	0.8795	0.9409	3	NT_022517	46399208[d]	19 , 44
CTLA4_318	*Ctla4*	cytotoxic T-lymphocyte-associated protein	rs5742909	0.6649	0.4378	0.653	2	NT_005403	204732347[d]	27
CTLA4_49			rs231775	0.04898	0.0459	0.1615	2	NT_005403	204732347[d]	27
CX3CR1_249	*CX3CR1*	chemokine (C-X3-C motif) receptor	rs3732379	0.9624	0.1261	0.2253	3	NT_022517	39307256[d]	45, 53, 57
CX3CR1_280			rs3732378	0.2282	0.1831	0.2835	3	NT_022517	39307162[d]	45, 53, 57
CXCL10_1	*Cxcl10*	chemokine (C-X-C motif) ligand 10	rs3921	0.5831	0.3735	0.202	4	NT_016354	76942943[d]	16, 53
CXCL10_2			rs8878	0.5831	0.3735	0.202	4	NT_016354	76942300[d]	16, 53
DC_SIGN_336	*CD209*	CD209 molecule	rs4804803	0.7604	0.6039	0.6758	19	NT_077812	7812733[d]	3, 10, 33,
DC_SIGNR	*CLEC4M*	C-type lectin domain family 4, member	rs2277998	0.5877	0.2951	0.6147	19	NT_077812	7831628[d]	17
Eotaxin_384	*CCL26*	chemokine (C-C motif) ligand 26	rs17809012	0.8237	0.5729	0.6627	17	NT_010799	32612444[d]	10
Foxp3_1	*FOXP3*	forkhead box P3	rs3761549	0.3314	0.1465	0.2134	X	NT_079573	49117345[d]	37, 57
Foxp3_2			rs2232365	0.154	0.7715	0.879	X	NT_079573	49115886[d]	37, 57
Foxp3_3			rs2280883	0.06841	0.5832	0.6027	X	NT_079573	49109128[d]	37, 57
Foxp3_4			rs2294021	0.1139	0.4739	0.5313	X	NT_079573	49105610[d]	37, 57
Foxp3_5			rs6609857	0.09798	0.5322	0.5022	X	NT_079573	49101623[d]	37, 57
Granulysin_1	*GNLY*	granulysin	rs1561285	0.975	0.8881	0.7824	2	NT_022184	85924384[d]	53, 57
Granulysin_2			rs11887686	0.5921	0.1535	0.1537	2	NT_022184	85924131[d]	53, 57

Granulysin_3			rs2043760	0.8833	0.7128	0.9813	2	NT_022184	85921992[d]	53, 57
Granulysin_4			rs1866138	0.7272	0.5588	0.4654	2	NT_022184	85921761[d]	53, 57
granzymeA_1	*GZMA*	granzyme A	rs2069186	0.3153	0.9238	0.8998	5	NT_006713	54398065[d]	53
granzymeA_2			rs3136556	0.8095	0.4661	0.4371	5	NT_006713	54400528[d]	53
GranzymeB245	*GZMB*	granzyme B		0.689	0.9728	0.701	14			31, 53
GranzymeB48				1	0.7457	0.5956	14			31, 53
GranzymeB88				0.838	0.9252	0.6926	14			31, 53
granzymeH_1	*GZMH*	granzyme H	rs2332406	1	0.9842	0.8808	14	NT_026437	25079343[d]	53, 57
granzymeH_2			rs12896130	1	0.9746	0.8521	14	NW_001838110	5189995[e]	53, 57
granzymeH_3			rs17257083	0.412	0.9529	0.783	14	NT_026437	25074711[d]	53, 57
granzymeH_4			rs17200187	0.4487	0.9617	0.8021	14	NT_026437	25075057[d]	53, 57
ICAM1_R241G	*Icam1*	intercellular adhesion molecule 1		0.8417	0.4579	0.6556	19			28, 47
ICAM2_1	*Icam2*	intercellular adhesion molecule 2	rs11655833	0.2199	0.5381	0.3888	17	NT_010783	62083212[d]	28, 47
ICAM2_2			rs3764868	0.9173	0.5156	0.3911	17	NW_001838450	57448366[e]	28, 47
ICOS_1624	*ICOS*	inducible T-cell co-stimulator	rs10932037	0.2154	0.2343	0.2062	2	NT_005403	204801768[d]	3, 4
ICOS_173			rs10932029	0.7463	0.7625	0.9155	2	NT_005403	204801768[d]	3, 4
ICOS_2373			rs4675379	0.4137	0.9334	0.8917	2	NT_005403	204826095[d]	3, 4
ICOS_602			rs10183087	0.4869	0.671	0.6447	2	NT_005403	204824324[d]	3, 4
IFNg_874	*IFNG*	interferon, gamma	rs2430561	0.2144	0.0708	0.0433	12	NT_029419	68552522[d]	13
IL10_1082	*IL10*	interleukin 10	rs1800896	0.2093	0.6978	0.552	1	NT_167186	206946897[d]	8
IL10_592			rs1800872	0.4376	0.7432	0.7982	1	NT_167186	206946407[d]	8
IL12_277	*IL12A*	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic	rs568408	0.2199	0.2832	0.1255	3	NT_005612	159713467[d]	58

		lymphocyte maturation factor 1, p35								
IL2RA_1	*IL12RA*	interleukin 2 receptor, alpha	rs706778	0.1019	0.289	0.4179	10	NT_008705	6098949[d]	12, 60
IL2RA_2			rs3118470	0.5462	0.2859	0.3316	10	NT_008705	6101713[d]	12, 60
IL2_330	*IL2*	interleukin 2	rs2069762	0.945	0.7997	0.9664	4	NT_016354	123377980[d]	23
IL6_174	*IL6*	interleukin 6	rs1800795	0.6055	0.418	0.189	7	NT_007819	22766645[d]	7, 15, 35,
IL6_634			rs1800796	0.4625	0.4357	0.6638	7	NT_007819	22766246[d]	7, 15, 35,
IL_4_1098	*IL4*	interleukin 4	rs2243248	0.626	0.4991	0.4635	5	NT_034772	132008644[d]	8
MCP1_2518	*CCL2*	chemokine (C-C motif) ligand 2	rs1024611	0.5188	0.3619	0.6896	17	NT_010799	32579788[d]	2
MIP1a	*CCL3*	chemokine (C-C motif) ligand 3	rs1130371	1	0.4272	0.5205	17	NT_010799	34416537[d]	34
MIP3a_2	*CCL20*	chemokine (C-C motif) ligand 20	rs6749704	0.4816	0.9048	0.8979	2	NT_005403	228677842[d]	3
MMP2_1306	*MMP2*	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)		0.1403	0.1395	0.0173	16			20, 41, 59, 61, 62
MMP9_279	*MMP9*	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	rs17576	0.6893	0.1214	0.3251	20	NT_011362	44640225[d]	20, 24, 36
NF-KB1_1	*NF-KB1*	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	rs1020759	0.5074	0.7655	0.7647	4	NT_016354	103510511[d]	3, 26, 46, 57,

NF-KB1_2	*NF-KB1A*	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	rs3138053	0.06683	0.0399	0.036	14	NT_026437	35874854[d]	26, 46, 57
NF-KB1_3			rs2233406	0.06592	0.0389	0.0316	14	NT_026437	35874799[d]	26, 46, 57
NF-KB1_4			rs2233409	0.5348	0.4352	0.6546	14	NT_026437	35874270[d]	26, 46, 57
NF-KB1_5			rs696	0.4208	0.2017	0.5487	14	NT_026437	35871093[d]	26, 46, 57
NKG2D_1	*KLRK1*	killer cell lectin-like receptor subfamily K, member 1	rs2617156	0.5827	0.2499	0.2173	12	NT_009714	10536879[d]	43, 53, 57
NKG2D_2			rs1049174	0.106	0.0358	0.0225	12	NT_009714	10525365[d]	43, 53, 57
NKG2D_3			rs12821887	0.1993	0.0951	0.0692	12	NT_009714	10554679[d]	43, 53, 57
NKG2D_5			rs2617165	1	0.8173	0.9498	12	NT_009714	10553930[d]	43, 53, 57
NKG2D_6			rs12819494	0.2696	0.1022	0.0536	12	NT_009714	10551541[d]	43, 53, 57
NKG7_1	*NKG7*	natural killer cell group 7 sequence	rs5026035	0.6686	0.4438	0.2717	19	NT_011109	51874741[d]	53, 57
NKG7_2			rs3009	0.05785	0.1273	0.0804	19	NT_011109	51874950[d]	53, 57
PD1_13	*PDCD1*	programmed cell death 1	rs11568821	0.1618	0.134	0.1731	2	NT_005416	242793912[d]	6, 25, 40, 50
PD1_15			rs2227981	0.1569	0.6664	0.7532	2	NT_005416	242793273[d]	6, 25, 40, 50
PD1_19			rs2227982	0.1826	0.2711	0.0473	2	NT_005416	242793433[d]	6, 25, 40, 50
Perforine_418	*PRF1*	perforin 1 (pore forming protein)		0.369	0.2231	0.1925	10			41
RANTES_28	*CCL5*	chemokine (C-C motif) ligand 5	rs2280788	1	0.9865	0.9916	17	NT_010799	34207405[d]	34, 51, 55,
RANTES_403			rs2107538	0.1617	0.0809	0.0895	17	NT_010799	34207780[d]	34, 51, 55,
SDF1_801	*CXCL12*	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) [	rs1801157	0.06202	0.5315	0.5902	10	NT_033985	44868257[d]	54

TGFbeta_509	*TGFB1*	transforming growth factor, beta 1	rs1800469	0.0121	0.025	0.0214	19	NT_011109	41860296[d]	5
TIMP2_596AC	*TIMP2*	TIMP metallopeptidase inhibitor 2	rs8179093	0.858	0.6417	0.7883	17	NT_010783	76921764[d]	1
TLR2_16934	*TLR2*	toll-like receptor 2	rs4696480	0.4336	0.858	0.0503	4	NT_016354	154607126[d]	3
TLR4_299	*TLR4*	toll-like receptor 4	rs4986790	0.1067	0.1226	0.3578	9	NT_008470	120475302[d]	3, 29
TLR9_1486	*TLR9*	toll-like receptor 9	rs187084	0.2253	0.0504	0.9413	3	NT_022517	52261031[d]	3, 48,
TLR9_1174			rs352139	0.532	0.3743	0.8958	3	NT_022517	52258372[d]	3, 48,
TLR9_1237			rs5743836	0.03323	0.4343	0.2974	3	NT_022517	52260782 [d]	3, 48,
TNF_a_863	*TNF*	tumour necrosis factor (TNF superfamily, member 2)		1	0.6003	0.809	6			13
VCAM1_1	*VCAM-1*	vascular cell adhesion molecule 1	rs3181092	0.6255	0.2508	0.3684	1	NT_032977	101204644[d]	49
VCAM1_2			rs3176878	0.5578	0.3342	0.2699	1	NT_032977	101203698[d]	49
HLA.Cw08	*HLA*	Human Leukocyte Antigen		0.5228	0.5463	0.5839	6			54
HLA.A2				0.3055	0.7778	0.4107	6			22, 54
HLA.Cw07				0.004491	0.0118	0.0107	6			30
HLA.A24				0.08189	0.1465	0.2014	6			This study
HLA.B07				0.06005	0.0653	0.0886	6			11, 22
HLA DRB.01				0.76	0.3148	0.13	6			22, 54

[a]*P*-values obtained after univariate analysis, χ^2 or Fisher's exact tests were used when applicable.
[b]*P*-values obtained after logistic regression analysis including age, gender and three principal components as covariates.
[c]*P*-values obtained after logistic regression analysis including age, gender, PVL and three principal components as covariates.
[d]Mapped to the human GRCh37 reference assembly
[e]Mapped to the alternative human reference assembly

Supplementary Table 2. Rs numbers, allele frequency difference (Δ between Native Americans and European Americans and genomic position of the ancestry informative markers (AIMs) selected to correct for population stratification.

Denomination	rs number	Delta Δ	Chromosome	contig accesion	Position of RefSNP in chromosome coordinates[a]	Reference
AIM1	rs4908736	0.88	1	NT_021937	8187211	32
AIM2	rs3017626	0.81	1	NT_032977	112558031	32
AIM3	rs17140601	0.86	3	NT_022459	76311299	32
AIM5	rs2642483	0.8	7	NT_007914	146389259	32
AIM6	rs7012587	0.8	8	NT_008046	130346325	32
AIM7	rs12675813	0.78	8	NT_167187	13366751	32
AIM8	rs1329724	0.73	9	NT_008413	18346065	32
AIM9	rs621348	0.74	11	NT_167190	65586509	32
AIM10	rs2248020	0.73	11	NT_167190	93259965	32
AIM12	rs2148430	0.76	13	NT_009952	89356442	32
AIM14	rs17649077	0.87	15	NT_010274	93586296	32
AIM15	rs2219403	0.73	17	NT_010783	72059031	32
AIM16	rs8097069	0.71	18	NT_010966	51065247	32
AIM17	rs4493170	0.68	18	NT_010966	20663834	32
AIM18	rs487656	0.71	20	NT_011362	58790814	32
AIM19	rs2838101	0.75	21	NT_011515	43139598	32
AIM20	rs132628	0.71	22	NT_011520	36541825	32
AIM21	rs11038170	0.837	11	NT_009237	44852738	32
AIM22	rs2467864	0.802	15	NT_010194	45641098	32

AIM23	rs868767	0.8	3	NT_005612	141377330	32
AIM24	rs2317212	0.793	3	NT_005612	97328284	32
AIM25	rs17066390	0.783	4	NT_016354	179500231	32
AIM26	rs1366363	0.78	5	NT_006576	29949620	32
AIM27	rs1415841	0.771	13	NT_024524	34867290	32
AIM28	rs9320598	0.768	6	NT_025741	98019080	32
AIM29	rs1651007	0.762	16	NT_010393	12774649	32
AIM30	rs6730157	0.754	2	NT_022135	135907088	32
AIM31	rs6715552	0.752	2	NT_005403	163044355	32
AIM32	rs7172	0.738	1	NT_004487	151372138	32
AIM33	rs1429433	0.735	16	NT_010498	72694352	32
AIM34	rs4727586	0.732	7	NT_007933	103706936	32
AIM35	rs169748	0.73	14	NT_026437	54186083	32
AIM36	rs2089222	0.729	12	NT_009775	117002658	32
AIM37	rs4822015	0.729	22	NT_011520	22780596	32
AIM38	rs2219939	0.727	15	NT_010194	79029723	32
AIM39	rs6823628	0.725	4	NT_016354	127464807	32
AIM40	rs4081585	0.724	7	NT_007819	4321255	32

The AIMs selected showed large differences in allele frequency ($\Delta > 0.67$) between Native Americans and European Americans, were distributed across the genome and were unlinked to the selected candidate SNPs.
[a]Mapped to the human GRCh37 reference assembly.

Supplementary Table 3. Non-polymorphic SNPs, excluded from the analysis

Official gene symbol	Gene name	Variant name	rs number	Chrom.	contig accesion	Position of RefSNP in chromosome coordinates[a]	Mapped to a reference or alternate assembly	Refer.
IKBKG	NF-kB essential modulator	Nemo1		X				14, 38
	NF-kB essential modulator	Nemo2						14, 38
CX3CR1	chemokine (C-X3-C motif) receptor 1	CX3CR1_666	rs4986872	3	NT_022517	39307344	GRCh37	45, 53
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	GCrecep_1	rs6189	5	NT_029289	142780339	GRCh37	52
CD274	CD274 molecule	PDL1_1072GC	rs12551333	9	NT_008413	5457171	GRCh37	56
	CD274 molecule	PDL1_1113GA	rs 4278201	9	NT_008413	5457212	GRCh37	56
	CD274 molecule	PD-L1_6777CG	rs 17718883	9	NT_008413	5462876	GRCh37	56
*PDCD1*LG2	programmed cell death 1 ligand 2	PDL2_24293GC	rs 12339171	9	NT_008413	5534862	GRCh37	56
	programmed cell death 1 ligand 2	PDL2_47103CT	rs 7854303	9	NT_008413	5557672	GRCh37	56
	programmed cell death 1 ligand 2	PDL2_47139TC	rs 7854413	9	NT_008413	5557708	GRCh37	56

[a]Mapped to the human GRCh37 reference assembly

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Chapter 6. Differential mRNA expression of NF-kB pathways genes between HAM/TSP and Asymptomatic HTLV-1 carriers

The objective of this study was to compare the mRNA expression of 84 genes implicated in the NF-kB pathways between HAM/TSP and asymptomatic carriers (unpublished results).

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ABSTRACT

HTLV-1 infection is endemic in Peru. Its infection has been associated with two main diseases: HTLV associated myelopathy / tropical spastic paraparesis (HAM/TSP) and Adult T-cell leukaemia/lymphoma. HAM/TSP is a neuroinflammatory, non-remitting and disabling disease; it is one of the most prevalent diseases among HTLV-1 infected individuals in Peru. Viral factors per se do not explain fully the appearing of HAM/TSP disease; therefore host factors are investigated to determine the HAM/TSP presence in HTLV-1 infected individuals.

Previous results of our group showed an association between SNPs located in the promotor region of the *NFKBIA* gene. Knowing that the NF-κB pathway plays a role in the pathogenesis of this condition, mRNA expression of 84 genes involved in the NF-κB pathway regulation, were compared between asymptomatic HTLV-1 carriers (AC) and HAM/TSP patients. These analyses were performed in two phases: a first exploratory phase and a second confirmatory phase: both using independent patients. Two genes: *NFKBIA* and *EGR1* were dysregulated in HAM/TSP compared to AC ($p<0.05$ after multiple testing correction). *NFKBIA* was higher in AC compared to HAM/TSP patients, while *EGR1* showed a lower expression in AC compared to HAM/TSP patients.

This lower expression is expected to generate lower amounts of inhibitor IκBα and to allow a higher activation of the NF-kB pathway, which is characteristic in HAM/TSP patients. For *EGR1* gene, involved in growth and cell

differentiation of diverse cell types, the higher expression in HAM/TSP might contribute to cell proliferation in those patients.

6.1 INTRODUCTION

Human T-lymphotropic virus 1 (HTLV-1) is a retrovirus infecting between 5-10 million people around the world. In Peru this virus may have infected up to 450,000 individuals (Gessain and Cassar, 2012). HTLV-1 is the causative agent of several diseases such as Adult T-Cell Leukaemia/Lymphoma (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 is also associated with a high rate of opportunistic co-infections such as strongyloidiasis and tuberculosis. Although several strains have been described, no specific subtype has been associated to a particular outcome of disease (Ehrlich et al., 1992).

The focus of this study is HAM/TSP disease, a chronic progressive myelopathy characterised by spastic paraparesis, affecting the central nervous system. The only consistent factor associated with HAM/TSP, when compared with AC, is the high PVL (Adaui, 2006; Sabouri AH, 2005), although many patients with a high load will remain lifelong asymptomatic carriers (Iwanaga et al., 2010; Nagai and Osame, 2003). Therefore, the PVL does not fully explain the presence of disease in those individuals. In this regard, it is necessary to search for host factors associated with HAM/TSP in HTLV-1 infected individuals.

HAM/TSP patients showed an increased NF-kB pathway activity compared to healthy donors (Oh et al., 2011). This pathway is responsible for the regulation of more than 200 genes involved in several processes such as cell survival, adhesion, inflammation, differentiation and proliferation (Hayden and Ghosh,

2012). Therefore, the genes involved in the NF-kB signalling pathway are good candidates to understand the pathogenesis of HAM/TSP disease and to discover targets that may be used in future therapeutic approaches.

In a previous study, we observed a trend for association between HAM/TSP and three SNPs belonging to the *NFKBIA* gene on chromosome 14q13: *NFKB1A*_2 (rs3138053), *NFKB1A*_3 (rs2233406), *NFKB1A*_6 (rs3138045) (Talledo M, 2012). All SNPs are located in the promoter region of the *NF-KBIA* gene. The first two SNPs, rs3138053, rs2233406, are located between 900-1000 bp from the initiation codon, while rs3138045 is located 3700-3800 bp away from the initiation codon. The three SNPs were in linkage disequilibrium. Therefore we hypothesize that these SNPs may influence the expression level of the *NFKBIA* gene. In order to test this hypothesis, we compared the mRNA expression of 84 genes involved in the NF-KB pathway in PBMC from HAM/TSP and AC.

6.2 METHODS

Subjects

Peripheral blood mononuclear cells (PBMC) from HAM/TSP and AC were obtained from 39 patients selected from the HTLV-1 cohort of the Institute of Tropical Medicine Alexander von Humboldt in Lima, Peru. Selection criteria are shown in Table 1. Clinical examinations were performed in all patients by one or two experts according to international guidelines (De Castro-Costa et al., 2006). Patients with non-well defined neurological manifestations and with

other disease manifestations associated with HTLV-1 as well as HTLV-2 positive subjects were excluded from the study. All subjects were unrelated.

This study was performed in two independent phases: 8 HAM/TSP patients and 4 ACs were evaluated in the first exploratory phase. The second confirmatory phase included 18 HAM/TSP and 9 AC. The Institutional Research Ethics Committee of the Universidad Peruana Cayetano Heredia approved the study protocol and a written informed consent was obtained from each participant.

cDNA conversion and Superarray PCR amplification

The purpose of the study was to find common factors that might be associated with HAM/TSP in HTLV-1 infected individuals. Therefore the samples belonging to each outcome were evaluated as pools: all the analyses were performed in triplicate to minimize the technical variability of the assay (Figure 1).

In the first “exploratory” phase, two independent pools of cDNA equivalent to 500ng of RNA were constituted: one with HAM/TSP patients (8 samples) and another with AC (4 samples). Briefly, 150 ng of mRNA (quantified using Nanodrop, Thermo Scientific, Grand Island, NY, USA) was extracted from PBMC and reverse-transcribed into cDNA from each patient using the Superarray RT^2 First Strand Kit (QIAGEN GmbH, Hilden-Germany). Equivalent amounts of cDNA obtained from each patient were used to constitute every pool. To verify the results obtained, a second confirmatory

phase was performed. A pool of cDNA from HAM/TSP patients 18 (samples) and a pool of AC (9 samples) was composed. The pools of both the exploratory and confirmatory phase were encrypted in order to hide the identity of the pool to the researchers performing the experiments in the laboratory.

The cDNA of every pool was evaluated in triplicate using RT^2 SYBR Green Mastermix (QIAGEN GmbH, Hilden-Alemania) using the RT^2 Profiler ^{TM} PCR Array for the NF-kB Signaling Pathway (PAHS-025A, QIAGEN GmbH, Hilden-Germany). Besides the 84 candidate genes, these plates include five housekeeping genes, 1 well for genomic control, 3 wells for reverse transcription control and 3 wells for positive PCR control. Equal amounts of master mix including cDNA were added to each well of the PCR array containing specific primers for the 84-candidate gene, the 5 housekeeping genes and controls. The amplification process was performed according to the RT^2 SuperArray protocol.

Table 1.- Inclusion and exclusion criteria used for HAM/TSP and AC HTLV-1 positive selection.

Inclusion Criteria for Asymptomatic carriers
Subjects between 18 and 60 years old with signed informed consent to use their samples in future studies
HTLV-1 positive with clinical diagnosis of asymptomatic infection.
Inclusion Criteria for HAM/TSP patients
Subjects between 18 and 60 years old with signed informed consent to use their samples in future studies
HTLV-1 positive with complete clinical diagnosis of HAM/TSP
Subjects with more than 2 years of HAM/TSP diagnosis.
Exclusion Criteria for Asymptomatic carriers and HAM/TSP patients
Subjects with undefined clinical diagnosis
Subjects with other inflammatory diseases such as Sjögren syndrome, rheumatoid arthritis, Systemic lupus erythematosus, dry eye syndrome, Hashimoto's thyroiditis
Subjects with neoplastic diseases
Subjects with diagnostic of neurological disease other than HAM/TSP
Subjects with HTLV-2
Patients with known HIV infection

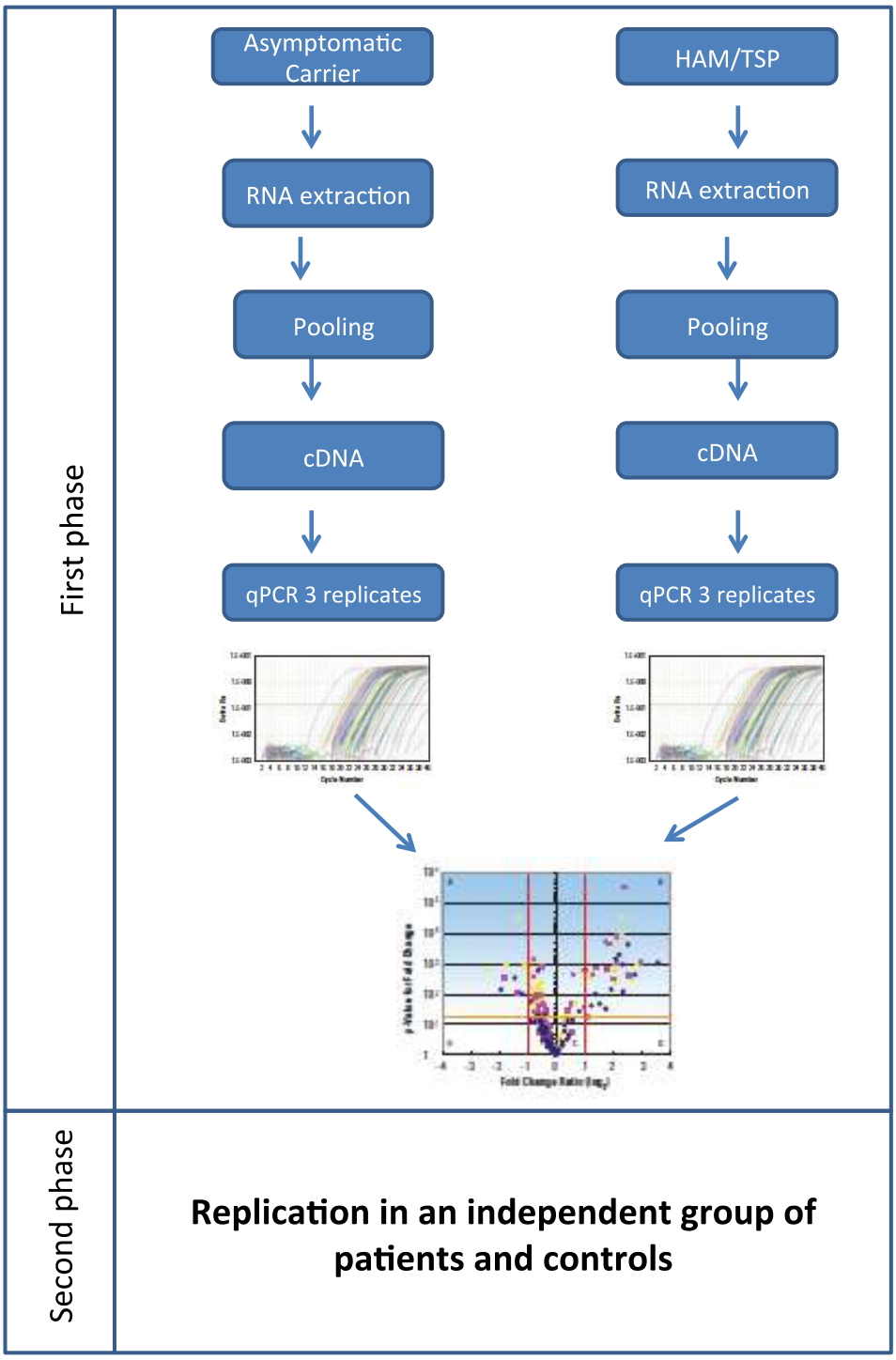

Figure 3: Process of the mRNA expression analysis of genes from the NF-KB pathway between HAM/TSP and AC. The first phase was exploratory and the second phase was confirmatory.

Data analysis

Every amplification was verified according to the conditions suggested by the manufacturer, taking into account the Ct of the genomic control (Ct >cycle 35), Ct of the reverse transcription control (Ct of 20 ± 2), and the Ct of the housekeeping genes. For the candidate genes, only those genes with Ct <cycle 35 in both groups were considered for the analysis. The global mean was used as reference to calculate the normalized expression level for further statistical analysis. Only those genes with differential expression of Ct ≥ 1.5 between HAM/TSP and AC were taken into account. A $P<0.05$, as calculated with an unpaired t-test, was considered as statistically significant. False discovery rate (FDR) was used to correct for multiple comparisons. Qbase software (http://www.biogazelle.com/) was used for all these analysis.

6.3 RESULTS

First exploratory phase

The reproducibility of the triplicate assay was within the requirements suggested by the manufacturer of the array (positive control, Ct 20±2). Contamination of the samples with genomic DNA was not observed (genomic control well with Ct >= 35). Of the 84 genes, 58 showed Ct values below 35 in both AC and HAM/TSP groups. As a Ct below 35 is a quality criterion suggested by the manufacturer, only these 58 genes were taken into account.

After correction for multiple comparisons, three genes *NFKBIA*, *IL8* and *EGR1* showed significant differences in mRNA expression between HAM/TSP and AC (P_{adj}<0.05): *NFKBIA* gene showed higher expression in asymptomatic ("fold change" = 1.77) compared to HAM/TSP patients, whereas *EGR1* and *IL8* genes showed higher expression in HAM/TSP compare to AC (Table 2).

Table2.- Genes with differential expression between HAM/TSP and AC.

Gene Symbol	**Exploratory Phase**		**Confirmatory Phase**	
	Fold-change AC vs. HAM/TSP	P_{adj}	**Fold-change AC vs. HAM/TSP**	P_{adj}
NFKBIA	1.77	0.0157	2.611	0.00038
EGR1	0.213	0.0462	0.092	0.0004
IL8	0.195	0.0479	0.792	0.103

P_{adj} P value corrected for multiple comparisons by FDR.

Second confirmatory phase

Samples independent from the first phase were evaluated in a second confirmatory phase, using exactly the same approach. The *NFKBIA* gene expression was confirmed to be significantly higher in AC compared to HAM/TSP (P_{adj}<0.05). *EGR1* expression was significantly lower in AC

(P_{adj}<0.05). *IL8* expression was lower in AC compared to HAM/TSP patients but was not statistical significant (P_{adj}>0.05) (Table 2).

6.4 DISCUSSION

The activation of the NF-kB pathway leads to the translocation of the NF-kB to the nucleus, modulating the transcription of genes involved in inflammation, cell adhesion, differentiation growing, apoptosis and various immune functions (Sun and Andersson, 2002). It is known that the NF-kB pathway is implicated in HAM/TSP disease in HTLV-1 infected individuals (Oh et al., 2011). Since HAM/TSP is an inflammatory disease with proliferation of infected cells, the processes influenced by the activity of the NF-kB pathway can be good candidates to contribute to HAM/TSP pathogenesis.

In a previous study, we analysed polymorphisms in a number of genes involved in the regulation of this inflammatory pathway. Three SNPs (rs3138053, rs2233406 and rs3138045), located in the promoter region of the *NFKBIA* gene on chromosome 14q13, were associated with HAM/TSP disease in Peruvian individuals (Talledo M, 2012). rs3138053 and rs2233406 SNPs are located in the binding regions for CCAAT/enhancer binding protein of the *NFKBIA* gene (Han et al., 2015). Since the *NFKBIA* gene encodes the protein IκBα, which is an inhibitor of the NF-kB pathway, we investigated the mRNA expression levels of 84 genes implicated in the regulation of the NF-kB pathway, using superarray plates from the NF-kB Signaling Pathway (QIAGEN GmbH, Hilden-Germany). To reduce the workload, we used a pooling

strategy, and analysed a pool of HAM/TSP samples and a pool of asymptomatic carrier samples. The analysis was done in triplicate. In addition, in a second confirmatory phase of the study independent patients were analysed to verify the results of the first phase. The main finding was that mRNA of the NFKBIA gene itself showed a lower expression in HAM/TSP compared to AC, in both phases of the study (P_{adj} <0.05). In addition, *EGR1* and *IL8* genes showed a significant lower expression in AC compared to HAM/TSP patients in the first phase but only *EGR1* gene remains significant in the second phase of the analysis.

The increased activation of NF-kB is one of the consequences of the Tax expression in infected cells. NF-kB controls the expression of different genes implicated in several processes such as inflammatory immune response, apoptosis and cellular proliferation. The activation of the NF-kB pathway depends of the IKK complex and inhibitory proteins called IkB, which includes IκBα, IκBβ, and IκBε, of which IκBα is the most abundant. Tax binds IKK-γ and in turns phosphorylates IκBα. This step is followed by ubiquitination and degradation of IκBα. The absence of IκBα leads to NF-kB activation and translocation of the NF-kB to the nucleus.

This study showed a lower expression of *NFKBIA* gene, encoding IκBα, in HAM/TSP compared to AC. This diminished expression is expected to lead to a lower amount of inhibitor IκBα and therefore a higher activation of the NF-kB pathway, which is characteristic in HAM/TSP patients.

The lower expression of *NFKBIA* gene and therefore its protein IκBα, the main inhibitor of the NF-kB pathway, suggest that IκBα might a good candidate as a therapeutic target to evaluate candidate drugs for HAM/TSP treatments. There are several compounds such Pyrrolidine dithiocarbamate (PDTC) (Hayakawa et al., 2003) and metiltioadenosina (MTA) (Moreno et al., 2006), which inhibit the proteasomal degradation of IκBα. These compounds could perhaps be evaluated in cells of patients to test the effect on the IκBα levels and the NF-kB activity.

Blocking the activity of the NF-kB pathway *in vitro* has been shown to reduce the aberrant cytokine production, which plays a role in the immunopathogenesis of HAM/TSP disease (Waldmann, 2002). In addition, blocking the NF-kB pathway inhibits the spontaneous proliferation of PBMCs causing a reduction of proviral load and increases the susceptibility to apoptosis (Oh et al., 2011).

EGR1, another gene that showed differential mRNA expression in both phases is a transcription factor involved in growth and cell differentiation of diverse cell types. EGR1 is constitutively expressed in HTLV-1 infected cells (Sakamoto et al., 1992). This gene showed higher expression in HAM/STP compared to AC. This result is in agreement with previous findings (Arainga et al., 2012; Trejo et al., 1997) showing that the expression of this gene is upregulated by Tax. This upregulation might contribute to cell proliferation in HAM/TSP patients, with higher levels of Tax compared to AC.

In conclusion, the finding of a difference in gene expression of the *NFKBIA* gene between HAM/TSP and AC is in line with our earlier findings of disease associated SNPs in the promotor of this gene. This finding is promising, and highlights this gene as a potential target for therapeutic intervention. In addition, the upregulation of *EGR1* is in agreement with previous findings reported in other populations. Together, our observations might help to understand the pathogenesis of HAM/TSP disease.

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Chapter 7 General Discussion

7.1. Pathogenesis of HAM/TSP: implication of viral and host genetics

It is currently not clear why HAM/TSP develops in only a minority of HTLV-1 infected persons. With regard to viral factors, PVL is strongly associated with HAM/TSP in several populations but there is an important overlap in PVL levels between AC and HAM/TSP patients (Adaui, 2006; Sabouri AH, 2005). There is little evidence that viral genetics influence disease outcome (Ehrlich et al., 1992; Major et al., 1993; Sambor et al., 1999). HTLV-1 is a genetically very stable virus because its replication strategy is largely based on proliferation of cells harbouring the provirus. Several genotypes have been described, but there is no evidence that they have different pathogenic effects. Furukawa reported the association of an HTLV-1 variant (*tax* subgroup A) with HAM/TSP disease (Furukawa, 2000) but other studies have not confirmed such association (Sabouri AH, 2005; Talledo M, 2010). Therefore, it is likely that HTLV-1 related diseases such as HAM/TSP have a multifactorial pathogenesis: the presence of the virus is necessary, but not sufficient. Host genetic and possibly also environmental factors may be needed for disease expression. Evidence in Brazil and Japan has suggested family aggregation of HAM/TSP, which supports a possible role of human genetic factors with this disease (Nozuma et al., 2014; Primo et al., 2005).

In the present work, we started from the hypothesis that host genetic factors are implicated in the HAM/TSP susceptibility in HTLV-1 infected individuals. Although HAM/TSP also occurs in children, it is mainly a late onset disease.

Therefore it is difficult to perform family studies and we preferred a case control association study, to identify the host genetic factors associated with HAM/TSP disease in Peruvian HTLV-1 infected individuals.

7.2. Summary of our findings on human genetics in Peruvian HTLV-1 infected subjects

Because no previous genetic studies have been done in HTLV-1 infected subjects in Peru, our first study asked the question whether a published logistic regression equation based on host genetic factors, viral subtype, age and gender, showing a 88% discriminative accuracy between HAM/TSP and AC in a Japanese cohort (Vine, 2002), was also appropriate in Peru. In our cohort age, gender and PVL were associated with HAM/TSP disease, while *HLA-A*02* showed only a marginal association (P=0.04, uncorrected for multiple testing). None of the other viral (tax subgroup) or host genetic factors (*SDF-1*, *TNF-α, HLA-A*02*, *HLA-Cw*08*) were useful in the equation. An adjusted equation using PVL, age, gender, and *HLA-A*02* provided the best predictive ability in the Peruvian cohort (AUC=0.799). When *HLA-A*02* was excluded from the equation the AUC decreases very slightly (AUC=0.794), showing the very small contribution of *HLA-A*02* to discriminate HAM/TSP form AC in our population (Talledo M, 2010).

In a second study, we investigated a set of genes encoding killer cell immunoglobuline-like receptors (KIR). The rationale for this study was that these genes influence disease outcome in several viral infections**:** certain KIR haplotypes delay the progression to AIDS in HIV-1 infected individuals (Martin

et al., 2002), help to resolve hepatitis C virus infections (Khakoo et al., 2004) or predispose to symptomatic herpes simplex virus type 1 (Estefania et al., 2007). We compared the frequency distribution of KIR genes between HAM/TSP and AC, but found no significant difference (Talledo et al., 2010). Our study is in concordance with the subsequent findings of O'Connor where KIR3DS1 failed to influence the control of PVL or disease outcome in three different study population of HTLV-1 infected individuals from Japan, Jamaica and Brazil (O'Connor et al., 2012).

In a third study, a candidate gene analysis was performed in 401 HTLV-1 infected individuals in a two-stage case-control study using genes associated with other viral diseases or genes associated with similar diseases to HAM/TSP without viral origin (Talledo M, 2012). Overall, we have analysed 2 SNPs and 2 HLA alleles in the first study, 14 KIR genes in the second study and 6 HLA alleles, 94 SNPs belonging to 45 candidate genes in the third study, 37 AIMs were included in the second and third study.

In this context, AIMs were preferred to correct for population stratification, because they provide more information on genetic ancestry than random genetic markers and therefore fewer numbers are required (Risch et al., 2002; Rosenberg et al., 2003). Although no differences were observed in the AIMs frequency distribution between HAM/TSP and AC, significant differences were observed between Andean and mestizos analysed in the second and third study, suggesting the presence of subpopulations and the adequate numbers of AIMs used. The AIMs inclusion is important in admixed populations such as

the Peruvian one, in which it is difficult to define ethnicity by questionnaire or by self-description. This correction by using AIM reduces the possibility of false-positive findings. In fact, our study is the first one that uses AIMs to control for the population stratification effect in the setting of HTLV-1 associated diseases.

In our two-stage candidate gene approach, we found a trend of association for two genes: *NKG2D* and *NFKBIA* (Talledo M, 2012). Clearly, although these trends need to be confirmed in larger studies and/or other population, they might be of pathogenic significance. On the other hand, several previous findings reported in other HTLV-1 infected populations were not replicated in the Peruvian cohort. Associations of IL-6-634 C/C genotype with HAM/TSP protection in Brazil and Japan, and IL-10 -592 A with HAM/TSP in Japan (Gadelha et al., 2008; Nishimura M, 2002 ; Sabouri AH, 2004), were not confirmed in our, despite the higher sample size of our study. These differences may be due to several reasons. As no analysis for population stratification was performed in previous studies, the finding may have been falsely positive. Genetic heterogeneity between the populations used in the different studies is another possible explanation.

The trend of association of HAM/TSP with SNPs of *NFKBIA* found in the third study led us to evaluate genes involved in the NF-kB signaling pathway (QIAGEN GmbH, Hilden-Alemania). A differential mRNA expression of the *NFKBIA* gene was detected between HAM/TSP patients and AC in a two-stage study (manuscript in preparation –see Chapter 6). HAM/TSP patients

had a lower mRNA expression of the *NFKBIA* gene than AC. In normal cells the NF-kB pathway is tightly regulated, but in HTLV-1 infected cells Tax binds IKK-γ, which in turn phosphorylates IkBα (encoded by the *NFKBIA* gene) that is subsequently ubiquitinated and degraded by the proteasome. Because IkBα is the most abundant inhibitor of the NF-KB pathway, the finding of a decreased expression of the *NFKBIA* in HAM/TSP patients fits with the current disease model, which emphasises the pathogenic role of NF-KB. In addition, the result is in line with the previous finding that SNPs located in the promotor of the *NFKBIA* gene are associated with HAM/TSP disease.

The tight regulation of the NF-kB pathway control mechanisms is lost in HTLV-1 infected cells, resulting in a constitutive activation of this pathway, inducing the overexpression of NF-kB proteins, stimulatory cytokines and preventing the termination of the pathway. Tax promotes the constant degradation of the IkBα. Clearly, the finding of NFKBIA SNPs associated with HAM/TSP and the lower expression of mRNA NFKBIA gene from this study are consistent with a constitutive activation of the NF-kB pathway, might be one of the causes of the neuropathogenesis and hyperstimulation of the immune system in HAM/TSP patients.

NKG2D is a C-type lectin-like receptor normally absent on CD4+ T-cells Tax stimulates the expression of IL15, which has antiapoptotic activity, the production of IL15 by HTLV-1 infected cells generate the survival and spontaneous proliferation of Tax-specific CTL. IL15 also induces the expression of NKG2D on CD4+ T cells. NKG2D engagement with its receptors

MICA and MICB stimulates the secretion of cytokines, release of cytolytic granules and may promote CTL activities against HTLV-1 infected cells (Azimi et al., 2006).

7.3. Putting our study into historical and future perspective

A number of studies have been published in several countries in order to identify host genes that might be involved in HAM/TSP susceptibility, but the progress is very slow, and not a single host genetic factor has been consistently associated in the different populations evaluated. One of the main reasons is the fact that HTLV-1 is understudied. In fact, until today most studies been performed with a limited number of cases and controls and therefore have insufficient power to detect genetic factors with small effects on HAM/TSP disease susceptibility. The associations found in these studies could often not be replicated in other populations, i.e. HLA-A*02 and HLA-Cw*08 showed no effect on either the risk of developing HAM/TSP or HTLV-1 provirus load in Iran in contrast to previous findings in Japan and, similarly, no effect of HLA-A*02 was found in Martinique (Deschamps et al., 2010; Sabouri AH, 2005; Taghaddosi et al., 2013). Reasons for non-replication could be either the fact that the findings were false positives, artefacts due to population stratification or population specific factors because of genetic heterogeneity.

This thesis started in 2005 with the sample collection. At that time, it was common to start smaller exploratory studies, which have the potential to reveal only factors with large effect size. In addition, candidate gene studies were

often done, under the assumption that genes that were known to be involved in the pathophysiology, would harbour variants with a moderate to large effect size. Under this assumption, candidate genes would be successful with a limited number of samples. For our candidate gene study, the gene selection was based on physiological functions of genes or results from studies in other infectious diseases such as HIV. Given the limited budget, we opted to use selected functional SNPs (SNPs that influence the expression, constitution or activity of the protein) and tag SNPs (representative SNP in a region of the genome with high linkage disequilibrium that represents a group of SNPs called a haplotype) so that we could analyse a reasonable list of candidate genes partially, rather than just a couple of them in depth, in an effort to increase our chances of success.

However, in the meanwhile the insights in complex diseases have greatly increased. A number of recent studies have shown that most complex traits are indeed very complex, with a very small effect size for most associated variants (Lango Allen et al., 2010). In such cases, high sample numbers are needed for sufficient power.

Looking back from today's perspective towards all "historical" genetic association studies (including our own); we can see some limitations and formulate some recommendations for future host genetic HTLV-1 studies.

It is evident that novel approaches such as genome-wide associations studies (GWAS) has promises (7.3.1), but also comes with power requirements with

regard to sample size of thousands of patients, implying the need for multicentre studies. As a consequence, a strict standardization of the phenotypes (7.3.2) and correction by population stratification effect (7.3.3) are necessary. The problems on multiple testing in GWAS needs to be addressed and focusing on extreme phenotypes can be a solution (7.3.4). The potentials and limitations of replication studies and posterior meta-analysis have to be considered (7.3.5). Alternative approaches, such as exome sequencing, might be a future alternative to GWAS (7.3.6).

7.3.1. The potential of GWAS studies

Because the genetic determinants of HAM/TSP are not really known, using a genome wide approach rather than conducting candidate gene studies may give more results about the relationship between genetic markers and HAM/TSP. The availability of genome wide approaches nowadays represents a great contribution to identify genetic variants in complex trait genetic studies. Contrary to HIV infection, where several GWAS have been done until date, this type of studies has not been done to identify genetic factors associated to HAM/TSP disease. Unlike a candidate gene study, a GWAS makes no a priori assumptions on the nature of the genes involved; therefore, a GWAS may discover unexpected genetic risk factors that were impossible to predict based upon functional data. GWAS hold the potential to give us a comprehensive view of the impact of human genetics in HTLV-1 infection, but it requires large sample sizes, not available in a single centre.

7.3.2. Standardized approach for phenotypical and genotypical analysis

In a multicentre GWAS study, it is critical to avoid site-based effects by using standardized phenotype criteria to ensure that phenotype is consistent across multiple sites. This will minimize the variability among clinicians and allow a correct assignment of the affection status. It is very important that standardized protocols for clinical phenotype definition, SNP genotyping, and statistical analysis are used across different sites of the study. In addition, factors known to influence the disease such sex, age, and known clinical covariates should be accounted for.

7.3.3. Population stratification

None of the previous studies into HTLV-1 took population stratification into account, which might lead to false positive results. Since the proposed multicentric approach will pool patients from diverse ethnicities, with possible difference in phenotype prevalence and allele frequencies across population, corrections for population stratification should be carried out to avoid spurious association between a SNP and disease status. Population stratification is best accounted for by unbiased principal component analysis: Principal component scores are indicative of an individual's ethnic origin, and by including the scores for the most important principal components into the association analysis, effects of population stratification are adjusted for (Price AL, 2006).

7.3.4. Multiple testing and sample size: are extreme phenotypes the solution?

The estimation of sample size depends: 1) the effect size of the variants involved in the disease, which is expected to be extremely low, 2) on the frequency of the DNA polymorphisms in the ethnic group to be evaluated, and 3) the cutoff for the p-values that is required to declare a SNP significantly associated with the phenotype. Due to the multiple testing burden, this p-value cutoff is set much lower than the conventional $p<0.05$ that is traditionally used in statistical testing. To have enough power to detect genetic factors with low relative risk and considering the effect of many hypotheses tested, huge samples sizes are required, which might be difficult for a relatively uncommon and heterogeneous disease complication, such as HAM/TSP.

A possible way around this difficulty is to perform GWAS in patients with rapid progression (patients showing a drop in their CD4+ T cell count <300/mm^3 in <3 years after the last seronegative test) and slow progression (individuals with CD4+ T cell count >500/mm^3 for 8 or more years after seroconversion). The idea behind the use of extreme phenotypes is to retain a large fraction of the power of a study, with a limited number of samples. Vasilescu has showed the power of this design with extreme phenotypes for HIV (Vasilescu et al., 2007). Individuals with extreme phenotypes are more probably to be informative. Patients with rapid and slow progression of AIDS were evaluated, and genetic evidence of a protective role of CXCR1 in disease progression was found. By using extreme phenotypes it is expected that rare variants

influencing the risk or protection of the disease might be enriched, improving the power of the study (Barnett et al., 2013; Guey et al., 2011).

However, special care must be taken because it can increase the effect of population stratification (Guey et al., 2011). Another drawback in extreme phenotypes design is that there may be little gain with respect to the cost of sample selection. The effort needed to recruit samples from patients with an extreme phenotype, in this case rapidly progressive HAM/TSP, would be much higher compared to just collecting an even much larger number of "regular" patients, because rapidly progressive HAM/TSP is an infrequent clinical manifestation of HTLV-1 infection and patients are difficult to find.

7.3.5. The need for replication and the limitations of posterior meta-analysis

A major challenge in a GWAS approach is to confirm that a SNP, statistically associated with the phenotype, is truly involved in the pathophysiology. First of all, analysing thousands of associations leads to an inflated probability of false positive associations. The generally accepted threshold to declare an association statistically significant in a GWAS, has been put to 5.0E-8 (Panagiotou and Ioannidis, 2012). However, a significant association in one study is not considered sufficient to confirm the association. A replication study in independent individuals is necessary to confirm the initial results. After this step a generalization effect will be evaluated by analysing those SNPs in different populations, to determine the effect size of this marker in

multiple human populations. It is very important to demonstrate that the association occurs in the same direction.

An interesting option of GWAS, similar to other genetic studies is the possibility to perform posterior meta-analyses of different studies. Nevertheless, we have to remain realistic. Despite the fact that some of the frequent complex diseases have been studied by a number of GWAS, and that meta-analysis has been carried out, they have not explained the genetics of many of these diseases in great detail. One of the much-debated themes in complex genetics is the “missing heritability”. The major part of the heritability is difficult to detect with the current genomic models and the variants most commonly identified have small effect sizes and only explain a small proportion of heritability in complex traits (Maher, 2008; Manolio et al., 2009). The best-known example is the human height where over 250,000 individuals were analysed, and about 700 variants reached genome-wide significance, explaining about one fifth of the phenotype (Wood et al., 2014). In general, the reason of missing heritability will be due to several factors such as variants with small effect sizes not reaching significance, low allele frequencies (rare variants), epistasis, epigenetics, or inflated heritability.

7.3.6. Beyond GWAS: exome sequencing?

A next step that is currently being taken in the study of complex diseases is whole genome sequencing or exome sequencing. Especially for the analysis of rare genetic variants, such an approach will be needed, because rare

variants are not present on the microarrays that are used for GWAs. At this moment, exome sequencing techniques have already led to many breakthroughs in monogenic diseases, but they are not yet used on a large scale for complex diseases. Because the price of the analysis remains relatively elevated, the high sample numbers needed to analyse complex diseases currently prevent their widespread use. Nevertheless, it is to be expected that prices of genome sequencing will drop, and that over time this tool will become affordable.

7.4. Possible clinical relevance of host genetics?

Given that little is known about the host genetic influence in HAM/TSP development, the identification of associated genetic variants that influence disease risk, prognosis or the response to treatment could be useful to define interventional strategies as has been performed for other diseases such as HIV or HCV. For these infections the study of genetic factors has become a routine part of clinical care. In HIV the genetic screening for *HLA-B*5701* is necessary before the prescription of abacavir, a nucleoside reverse-transcriptase inhibitor. Individuals with HLA-B*5701 show a hypersensitivity reaction to abacavir that includes fever, rash, gastrointestinal tract symptoms, and respiratory symptoms, and this requires the medication to be discontinued (Hetherington et al., 2001; Mallal et al., 2002). In patients with chronic genotype 1 HCV infection, a SNP located in *IL28-B* (rs8099917/rs12979860 genotype TT/CC) has been associated with rapid virological response and better treatment response to Pegylate-Interferon (Boglione et al., 2014; Ge et

al., 2009).

In a similar way, virus genotyping can offer tools to treat viral diseases. In HIV for example, the sequencing of the V3 loop allows to determine if HIV uses CCR5 as co-receptor for entry. Those patients infected by virus with CCR5 tropism can be treated with a CCR5 antagonist such as maraviroc (Wyatt et al., 2015).

7.5. Conclusion

Our search to discover genetic differences between HAM/TSP and AC showed two genes with trend of association with HAM/TSP disease, and one of them with differential expression between both groups. For a more complete understanding of HAM/TSP disease, we believe that further genetic studies are needed, and that they can be successful when the correct study design is used. GWAS and exome sequencing approaches can facilitate some progress to identify genes that appear to influence HAM/TSP susceptibility in HTLV-1 infected individuals. Understanding the genetics of this disease may lead us to do further exploration by other technologies (like –omics) to define the functional consequences of these genetic variants. Unexpected connections of cellular processes between HTLV-1 and human can be elucidated inclusive and may emerge possibilities to develop new diagnostic or therapeutic insights.

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