Himmelfarb Health Sciences Library, The George Washington University
Health Sciences Research Commons

Microbiology, Immunology, and Tropical Medicine Faculty Publications | Microbiology, Immunology, and Tropical Medicine

2017

A Fashi lymphoproliferative phenotype reveals non-apoptotic Fas signalling in HTLV-1-associated neuroinflammation.

Soraya Maria Menezes

Fabio E Leal
George Washington University

Tim Dierckx

Ricardo Khouri

Daniele Decanine

See next page for additional authors

Follow this and additional works at: http://hsrc.himmelfarb.gwu.edu/smhs_microbio_facpubs

Part of the Medical Immunology Commons, Medical Microbiology Commons, Tropical Medicine Commons, and the Virus Diseases Commons

APA Citation

Menezes, S. M., Leal, F., Dierckx, T., Khouri, R., Decanine, D., Silva-Santos, G., & +several additional authors (2017). A Fashi lymphoproliferative phenotype reveals non-apoptotic Fas signalling in HTLV-1-associated neuroinflammation.. *Frontiers in Immunology,* (). http://dx.doi.org/10.3389/fimmu.2017.00097

This Journal Article is brought to you for free and open access by the Microbiology, Immunology, and Tropical Medicine at Health Sciences Research Commons. It has been accepted for inclusion in Microbiology, Immunology, and Tropical Medicine Faculty Publications by an authorized administrator of Health Sciences Research Commons. For more information, please contact hsrc@gwu.edu.

Authors
Soraya Maria Menezes, Fabio E Leal, Tim Dierckx, Ricardo Khouri, Daniele Decanine, Gilvaneia Silva-Santos, and +several additional authors

This journal article is available at Health Sciences Research Commons: http://hsrc.himmelfarb.gwu.edu/smhs_microbio_facpubs/253

frontiers in Immunology

A Fashi lymphoproliferative phenotype reveals non-apoptotic Fas signalling in HTLV-1-associated neuroinflammation.

Soraya Maria MENEZES[1], Fabio E. LEAL[2], Tim DIERCKX[1], Ricardo KHOURI[1, 3], Daniele DECANINE[3], Gilvaneia SILVA-SANTOS[3], Saul V. SCHNITMAN[3], Ramon KRUSCHEWSKY[3], Giovanni López[4], Carolina ALVAREZ[1, 4], Michael TALLEDO[4], Eduardo GOTUZZO[4, 5], Douglas F. NIXON[2], Jurgen VERCAUTEREN[1], David BRASSAT[7], Roland LIBLAU[7], Anne-Mieke VANDAMME[1, 6], Bernardo Galvão-Castro[3], Johan VAN WEYENBERGH[1*]

[1]Department of Microbiology and Immunology, KU Leuven, Belgium, [2]Department of Microbiology, Immunology & Tropical Medicine,, The George Washington University, USA, [3]Gonçalo Moniz Research Center (CPqGM),, Oswaldo Cruz Foundation (FIOCRUZ), Brazil, [4]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Peru, [5]Departamento de Enfermedades Infecciosas, Tropicales y Dermatológicas, Hospital Cayetano Heredia, Peru, [6]Center for Global Health and Tropical Medicine, Unidade de Microbiologia, Instituto de Higiene e Medicina Tropical,, Universidade Nova de Lisboa, Portugal, [7]INSERM UMR1043 and Pôle des Neurosciences, Hôpital Purpan, Université de Toulouse, France

Provisional

Submitted to Journal:
Frontiers in Immunology

Specialty Section:
Inflammation

ISSN:
1664-3224

Article type:
Original Research Article

Received on:
06 Oct 2016

Accepted on:
19 Jan 2017

Provisional PDF published on:
19 Jan 2017

Frontiers website link:
www.frontiersin.org

Citation:
Menezes S, Leal FE, Dierckx T, Khouri R, Decanine D, Silva-santos G, Schnitman SV, Kruschewsky R, López G, Alvarez C, Talledo M, Gotuzzo E, Nixon DF, Vercauteren J, Brassat D, Liblau R, Vandamme A, Galvão-castro B and Van_weyenbergh J(2017) A Fashi lymphoproliferative phenotype reveals non-apoptotic Fas signalling in HTLV-1-associated neuroinflammation.. *Front. Immunol.* 8:97. doi:10.3389/fimmu.2017.00097

Copyright statement:
© 2017 Menezes, Leal, Dierckx, Khouri, Decanine, Silva-santos, Schnitman, Kruschewsky, López, Alvarez, Talledo, Gotuzzo, Nixon, Vercauteren, Brassat, Liblau, Vandamme, Galvão-castro and Van_weyenbergh. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution and reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

This Provisional PDF corresponds to the article as it appeared upon acceptance, after peer-review. Fully formatted PDF and full text (HTML) versions will be made available soon.

Provisional

1 **A Fas^{hi} lymphoproliferative phenotype reveals non-apoptotic Fas signalling in HTLV-**
2 **1-associated neuroinflammation**

3

4 **Running head:** Fas signalling fuels retroviral neuroinflammation

5

6 Menezes SM[1]*, Leal FE[2]*, Dierckx T[1], Khouri R[1,3], Decanine D[3], Silva-Santos G[3], Schnitman
7 SV[3], Kruschewsky R[4], López G[5], Alvarez C[1,5], Talledo M[5], Gotuzzo E[5,6], Nixon DF[2],
8 Vercauteren J[1], Brassat D[7], Liblau R[7], Vandamme A-M[1,8], Galvão-Castro B[4], Van
9 Weyenbergh J.[1]

10

11 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
12 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, Belgium.

13 [2]Department of Microbiology, Immunology & Tropical Medicine, The George Washington
14 University, Washington DC, USA.

15 [3]LIMI, Gonçalo Moniz Research Center (CPqGM), Oswaldo Cruz Foundation (FIOCRUZ),
16 Salvador-Bahia, Brazil.

17 [4]Bahiana School of Medicine and Public Health, Salvador-Bahia, Brazil.

18 [5]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano
19 Heredia, Lima, Peru.

20 [6]Departamento de Enfermedades Infecciosas, Tropicales y Dermatológicas, Hospital
21 Cayetano Heredia, Lima, Peru.

22 [7] INSERM UMR1043 and Pôle des Neurosciences, Hôpital Purpan, Université de Toulouse,
23 Toulouse, France
24

25 [8]Center for Global Health and Tropical Medicine, Unidade de Microbiologia, Instituto de
26 Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal.

Provisional

27 *SMM and FEL are shared first authors.

28

29 **Corresponding author**: Johan Van Weyenbergh, Rega Institute for Medical Research,

30 Clinical and Epidemiological Virology, Herestraat 49 box 1040, 3000 Leuven, Belgium;

31 j.vw@live.be; johan.vanweyenbergh@kuleuven.be

32

33 **Word count:** Abstract 350, Text: 3584

34 **Figures/Tables:** 8+1

35 **Supplementary Figures:** 1

36 **Supplementary Tables:** 3

37 **References:** 55

38 **Keywords:** Fas/CD95; proliferation; HTLV-1-associated myelopathy/tropical spastic

39 paraparesis; lymphoproliferative disease; apoptosis; interferon, NF-kB, multiple sclerosis

40 **Key Points:**

41 • A two-step increase in cell death receptor Fas occurs upon HTLV-1 infection and
42 disease progression
43 • Unexpectedly, higher Fas level was linked to decreased cell death, increased
44 lymphocyte proliferation/activation and early disease onset
45

46

Provisional

ABSTRACT

Human T-cell lymphotropic virus (HTLV) -1 was the first human retrovirus to be associated to cancer, namely Adult T-cell Leukemia (ATL), but its pathogenesis remains enigmatic, since only a minority of infected individuals develops either ATL or the neuroinflammatory disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). A functional *FAS -670* polymorphism in an interferon (IFN)-regulated STAT1-binding site has been associated to both ATL and HAM/TSP susceptibility. Fas^{hi} T stem cell memory (Tscm) cells have been identified as the hierarchical apex of ATL, but have not been investigated in HAM/TSP. In addition, both *FAS* and *STAT1* have been identified in an IFN-inducible HAM/TSP gene signature, but its pathobiological significance remains unclear. We comprehensively explored Fas expression (protein/mRNA) and function in lymphocyte activation, apoptosis, proliferation and transcriptome, in PBMC from a total of 47 HAM/TSP patients, 40 asymptomatic HTLV-1-infected individuals (AC) and 58 HTLV-1 -uninfected healthy controls.

Fas surface expression followed a two-step increase from HC to AC and from AC to HAM/TSP. In HAM/TSP, Fas levels correlated positively to lymphocyte activation markers, but negatively to age of onset, linking Fas^{hi} cells to earlier, more aggressive disease. Surprisingly, increased lymphocyte Fas expression in HAM/TSP was linked to decreased apoptosis and increased lymphoproliferation upon *in vitro* culture, but not to proviral load. This Fas^{hi} phenotype is HAM/TSP-specific, since both *ex vivo* and *in vitro* Fas expression was increased as compared to multiple sclerosis another neuroinflammatory disorder. To elucidate the molecular mechanism underlying non-apoptotic Fas signalling in HAM/TSP, we combined transcriptome analysis with functional assays, i.e. blocking vs. triggering Fas receptor *in vitro* with antagonist and agonist- anti-Fas mAb, respectively. Treatment with agonist anti-Fas mAb restored apoptosis, indicating biased but not defective Fas signalling in HAM/TSP. *In silico* analysis revealed biased Fas signalling towards proliferation and inflammation, driven by RelA/NF-kB. Correlation of Fas transcript levels with proliferation (but not apoptosis) was confirmed in HAM/TSP *ex vivo* transcriptomes. In conclusion, we demonstrated a two-step increase in Fas expression, revealing a unique Fas^{hi} lymphocyte phenotype in HAM/TSP, distinguishable from multiple sclerosis. Non-apoptotic Fas signalling might fuel HAM/TSP pathogenesis, through increased lymphoproliferation, inflammation and early age of onset.

Provisional

78

Provisional

79 **Soraya Maria Menezes**

80 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
81 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, 3000 Belgium.

82 **Fabio E. Leal**

83 [2]Department of Microbiology, Immunology & Tropical Medicine, The George Washington
84 University, Washington DC, USA.

85 **Tim Dierckx**

86 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
87 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, 3000 Belgium.

88 **Ricardo Khouri**

89 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
90 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, 3000 Belgium.

91 [2]LIMI, Gonçalo Moniz Research Center (CPqGM), Oswaldo Cruz Foundation (FIOCRUZ),
92 Salvador-Bahia, 40296-710 Brazil.

93 **Daniele Decanine**

94 [2]LIMI, Gonçalo Moniz Research Center (CPqGM), Oswaldo Cruz Foundation (FIOCRUZ),
95 Salvador-Bahia, 40296-710 Brazil.

96 **Gilvaneia Silva-Santos**

97 [2]LIMI, Gonçalo Moniz Research Center (CPqGM), Oswaldo Cruz Foundation (FIOCRUZ),
98 Salvador-Bahia, 40296-710 Brazil.

99 **Saul V Schnitman**

100 [2]LIMI, Gonçalo Moniz Research Center (CPqGM), Oswaldo Cruz Foundation (FIOCRUZ),
101 Salvador-Bahia, 40296-710 Brazil.

102 **Ramon Kruschewsky**

Provisional

103 [4]Bahiana School of Medicine and Public Health, Salvador-Bahia, Brazil.

104 **Giovanni López**

105 [4]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano
106 Heredia, Lima 31, Perú.

107 **Carolina Alvarez**

108 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
109 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, 3000 Belgium.

110 [4]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano
111 Heredia, Lima 31, Perú.

112 **Michael Talledo**

113 [4]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano
114 Heredia, Lima 31, Perú.

115 **Eduardo Gotuzzo**

116 [4]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano
117 Heredia, Lima 31, Perú.

118 [5]Departamento de Enfermedades Infecciosas, Tropicales y Dermatológicas, Hospital
119 Cayetano Heredia, Lima 31, Perú.

120 **Douglas F. Nixon**

121 [2]Department of Microbiology, Immunology & Tropical Medicine, The George Washington
122 University, Washington DC, USA.

123 **Jurgen Vercauteren**

124 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
125 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, 3000 Belgium.

126 **David Brassat**

Provisional

127 [7] INSERM UMR1043 and Pôle des Neurosciences, Hôpital Purpan, Université de Toulouse,
128 Toulouse, France
129 **Roland Liblau**
130 [7] INSERM UMR1043 and Pôle des Neurosciences, Hôpital Purpan, Université de Toulouse,
131 Toulouse, France
132 **Anne-Mieke Vandamme**

133 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
134 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, 3000 Belgium.

135 [8]Center for Global Health and Tropical Medicine, Unidade de Microbiologia, Instituto de
136 Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal.

137 **Bernardo Galvão-Castro**

138 [4]Bahiana School of Medicine and Public Health, Salvador-Bahia, Brazil.

139 **Johan Van Weyenbergh**

140 [1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Rega
141 Institute for Medical Research, Clinical and Epidemiological Virology, Leuven, 3000 Belgium;

142

Provisional

143 **INTRODUCTION**

144 Human T-lymphotropic virus 1 (HTLV-1) is an exogenous human retrovirus infecting 5-10
145 million people worldwide, mostly in HTLV-1 endemic regions.[1] While a majority of HTLV-1
146 carriers remain asymptomatic (AC) lifelong, a minority (0.25-3%) progresses to either adult
147 T-cell leukemia/lymphoma (ATL) or HTLV-1-associated myelopathy/tropical spastic
148 paraparesis (HAM/TSP)[2,3]. Thirty years after its discovery it is still enigmatic how a single
149 retrovirus causes either fatal hematologic malignancy or neuroinflammation in a small subset
150 of infected individuals. Among factors that allow to discriminate between the three clinical
151 groups (AC, ATL, HAM/TSP), humoral immunity,[4] proteome[5,6] have been described. In
152 agreement with a role for immune activation [4,6-9] in HAM/TSP pathogenesis, promising
153 preclinical results were obtained with Jak kinase and NFkB inhibitors.[10,11] Very few drugs, e.g.
154 valproate, have actually overcome the hurdle in transition from preclinical results[12] to clinical trial in
155 HAM/TSP.[13] Taken together, these studies point at a possible clinical benefit of decreasing
156 lymphoproliferation and/or increasing apoptosis in HAM/TSP patients. HTLV-1-infected cells are
157 driven towards spontaneous lymphoproliferation and oligoclonal expansion.[14,15] On the other
158 hand, apoptosis (programmed cell death) is known to play a role in controlling
159 lymphoproliferation in autoimmune diseases.[16,17] Fas (TNFRSF6/CD95/APO-1) is a death-
160 domain containing receptor of the tumor necrosis factor (TNF) receptor superfamily inducing
161 apoptosis[17], when ligated by Fas ligand (FasL) or agonist antibodies.[18] Fas-FasL signalling is
162 proposed to play a role in both autoimmune and infectious diseases.[17] In multiple sclerosis
163 (MS) patients, increased Fas expression has since long been known,[19] while resistance of T
164 cells to Fas-mediated apoptosis has been linked to MS.[20] In HTLV-1 infection, a wealth of
165 data is available on pro- and anti-apoptotic effects of HTLV-1 infection, mainly its proto-
166 oncogene Tax.[21] In the context of HAM/TSP immunopathogenesis, a role for Fas-FasL in the
167 down-regulation of immune response in the CNS has been suggested.[22] Previous studies on
168 Fas in HAM/TSP have shown increased levels of soluble Fas in serum,[23,24] and CSF,[24] as
169 well as surface expression in CD8 cells.[25] A systems biology approach identified *FAS* (but
170 not *FASL*) as part of an IFN-regulated gene signature in HAM/TSP patients.[7] In addition,
171 immunogenetic data revealed that a functional *FAS* -670 gene polymorphism is associated
172 to both ATL[26] and HAM/TSP[27] disease susceptibility. Therefore, we hypothesized that
173 lymphocyte Fas expression and/or apoptosis may reflect clinical status in HAM/TSP patients.

Provisional

174 **PATIENTS AND METHODS**

175 A flow chart diagram (Figure 1) provides an overview of the study outline, cohorts, as well as
176 *ex vivo*, *in vitro* and *in silico* experimental approach, while patient information and sample
177 use is summarized in Table 1.

178 HAM/TSP patients (n=47, 66.0% female, mean age 50.2±11.5 years, mean disease duration
179 5.6±4.0 y (range 0.8-14 y), EDSS range 3-7 (mean 5.1±1.2)) were recruited from three
180 endemic regions (Sao Paulo and Salvador-Bahia, Brazil and Lima, Peru) following written
181 informed consent. Age- and gender-matched HTLV-1-infected asymptomatic carriers (AC,
182 n=40) and uninfected healthy controls (HC, n=58) from the same endemic regions were
183 included in the study. The study was approved by the Ethics Committees of University of
184 Sao Paulo and FIOCRUZ-Bahia in Brazil and Universidad Peruana Cayetano Heredia in
185 Lima, Peru. Diagnosis of HAM/TSP was according to WHO criteria[29] Antibodies to HTLV-1/2
186 were investigated by diagnostic ELISA (Murex, Abbott, Germany; Bioelisa HTLV-1+2, Biokit
187 Spain) and confirmed by Western blot capable of discriminating between HTLV-1 and HTLV-
188 2 (HTLV Blot 2.4, Genelab, Singapore). All HTLV-1-infected individuals were seronegative
189 for HTLV-2 and HIV. For comparison with another neuroinflammatory disorder, data from MS
190 patients (recruited during our previous study[30]) was used.

Provisional

191 **Isolation of PBMC and *in vitro* cell culture**

192 PBMC isolated from 5-10ml of heparinized venous blood by Ficoll-Hypaque density gradient
193 centrifugation (Sigma-Aldrich) were washed twice with PBS and were plated in 24-well tissue
194 culture plates (Costar, NY) at 4×10^6 cells/ml and incubated at 37°C and 5% CO_2 in
195 RPMI1640 medium supplemented with 2mM L-glutamine, gentamycin (50µg/ml) and 10%
196 heat-inactivated fetal calf serum (Gibco, NY).

197 **HTLV-1 p19 and Proviral load quantification**

198 HTLV-1 matrix protein p19 was quantified in cell-free supernatant of HAM/TSP patients'
199 PBMC and AC and HC using RetroTek HTLV-1/2 p19 Antigen ELISA kit (ZeptoMetrix) after
200 48h of *in vitro* culture. Proviral load (PVL, i.e. viral DNA integrated into the host genome) in
201 HAM/TSP patients and AC was quantified as published.[30,31]

202 **Quantification of cell surface markers by flow cytometry**

203 For phenotypic analysis, PBMC were resuspended at a density of 200,000 cells in 50μL of
204 1% BSA, 0.1% NaN_3 in PBS (+20% human serum to block Fc receptors) and incubated for
205 30min on ice with mAbs specific for CD3, CD4, CD8, , CD80, CD86, CD95/Fas, HLA-DR
206 and corresponding isotype controls (BD Biosciences). For total Fas surface quantification
207 and apoptosis, a minimum of 100,000 events/sample were stained and acquired with
208 FACSort and FACSCanto II flow cytometers (BD Biosciences) and analyzed using CellQuest
209 and Diva software, respectively.

210 **Proliferation and Apoptotic assays**

211 Lymphoproliferation was quantified by [^{3}H]-thymidine incorporation and flow cytometry (as
212 described in[30,32]), the initial stage of apoptosis was analyzed using annexin V staining,
213 whereas cells in the late/final stage of apoptosis were identified as a sub-diploid population
214 by flow cytometry. Nuclear fragmentation was quantified by fluorescence microscopy and
215 ELISA (Cell Death Detection plus, Boehringer-Mannheim, Germany).

216 **Fas triggering and blocking experiments**

217 PBMC were cultured as above for 48h in the presence or absence of agonist or antagonist
218 anti-Fas mAbs (1μg/ml, Alexis Biochemicals) or anti-CD3 mAb (Butantan Institute, Sao
219 Paulo-Brazil) as a positive control for *in vitro* apoptosis.

220 **Microarray analysis**

221 Total RNA was extracted from PBMC according to manufacturer's protocol (QIAgen, Venlo,
222 The Netherlands). Whole genome microarray was performed at VIB Nucleomics (Leuven,
223 Belgium) using GeneChip® Human Gene1.0 ST Array (Affymetrix, Santa Clara, CA),
224 according to manufacturer's specifications. Data was analyzed using Bioconductor limma
225 package (Smyth, GK, 2005), using a moderated t-test, resulting p-values were corrected for
226 genome-wide testing (5% FDR). All microarray raw data are available at Gene Expression
227 Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) series accession number
228 GSE82160.

229 **Statistical analysis**

Provisional

The use of parametric (t-test, Pearson correlation) or non-parametric (Mann-Whitney or Spearman rank correlation) tests was based upon normal distribution as determined by Kolmogorov-Smirnov test (all GraphPad Prism v5.0 or v6.0). A p-value of <0.05 was considered significant for all statistical tests. Transcriptome-wide correlation of FAS mRNA expression levels was calculated using Spearman rank correlation test, with stringent correction for multiple testing (5% FDR).

RESULTS

A two-step increase in *ex vivo* total lymphocyte Fas surface expression, in HTLV-1-infected individuals and HAM/TSP patients, distinguishable from MS patients.

In a first cohort, we quantified surface Fas levels as well as apoptosis by flow cytometry, *ex vivo* in PBMC from HC (HTLV-1-negative, n=14), AC (HTLV-1-positive, n=30) and HAM/TSP patients (n=18). We observed a significant increase in *ex vivo* levels (%) of Fas^{+} lymphocyte in AC (1.8-fold) as well as in HAM/TSP patients (2.1-fold), when compared to HC (Kruskal-Wallis, Dunn's post-test, $p<0.05$, $p<0.001$; respectively, Figure 2A). Moreover, lymphocyte Fas level on a per-cell basis, expressed as mean fluorescence intensity (MFI), revealed an 8-fold increase in AC and a striking 19-fold increase in HAM/TSP (Kruskal-Wallis, Dunn's post-test, $p<0.001$), when compared to HC, but also when compared to AC ($p<0.05$, Figure 2B), indicating that clinical progression to HAM/TSP is characterized by a predominant Fas^{hi} lymphocyte population, possibly primed for apoptosis. To confirm the two-step model of Fas increase, we performed a post-hoc test for linear trend, which was highly significant ($p<0.001$) for both % (slope 18.8) and MFI (slope 64.1).

Next, we proceeded to examine Fas expression in CD4, CD8 and B cell subsets in more detail in an independent second cohort of HC (n=7), AC (n=6) and HAM/TSP patients (n=9). There was no difference in the percentage of cells expressing Fas between the three clinical groups for either cellular subset (Figure 2C.). However, we observed a small but significant linear trend in Fas MFI of $CD4^{+}$ T cells with clinical status (ANOVA p=0.067, post-test for linear trend $p<0.05$, slope=349.2), but not in $CD8^{+}$ T cells or B cells. Thus, the strongest difference between the clinical groups was in total Fas^{+} lymphocytes rather than specific subsets, revealing a Fas^{hi} phenotype in HAM/TSP. To verify if this Fas^{hi} phenotype might be

Provisional

259 shared among neuroinflammatory disorders, we compared Fas expression between
260 HAM/TSP and multiple sclerosis (MS) patients. As shown in Figure 2D, we found a
261 significant 1.6-fold increase in % of *ex vivo* Fas^+ lymphocytes in HAM/TSP (Mann Whitney,
262 p=0.03), as well as a 2.4-fold increase in Fas MFI, which approached statistical significance
263 (Mann Whitney, p=0.08).

264 Finally, *ex vivo* spontaneous apoptosis in HAM/TSP and AC, as measured by DNA
265 degradation, (quantified as sub-diploid cells in flow cytometry) occurred at very low levels
266 (<0.2% of PBMC, data not shown). Therefore, we questioned if the observed *ex vivo*
267 increase in lymphocyte Fas surface expression in HAM/TSP reflected the immunological,
268 virological or clinical status of HAM/TSP patients, rather than an apoptosis-prone status.

269 ***Ex vivo* lymphocyte Fas surface expression correlates to immune activation markers**
270 **in HAM/TSP**

271 To explore possible clinical relevance of this increased lymphocyte Fas in HAM/TSP
272 patients, we correlated *ex vivo* Fas surface expression to patient demographic and clinical
273 data. We observed that, in HAM/TSP, *ex vivo* lymphocyte Fas (% or MFI) was not correlated
274 to age, gender, disease duration or severity. In addition, *ex vivo* lymphocyte Fas was not
275 significantly correlated to PVL in AC or HAM/TSP (p>0.05). However, *ex vivo* Fas levels (%)
276 correlated significantly to lymphocyte activation markers HLA-DR and CD86 (Figure 3A-B),
277 implying that increased Fas expression may be coupled to immune activation and/or
278 inflammation in HAM/TSP.

279 ***In vitro* Fas^+ lymphocyte levels correlate negatively to both age of onset and *in* vitro**
280 **apoptosis: a selective defect in HAM/TSP patients?**

281 Upon quantification of *in vitro* Fas^+ lymphocyte expression in HC, AC and HAM/TSP patients
282 by flow cytometry, we again observed a two-step increase in % Fas^+ lymphocytes: 2-fold in
283 AC and 3.4-fold in HAM/TSP vs. HC (Post-test for linear trend, p=0.0001, slope=27.0)
284 (Figure 4A). In HAM/TSP, *in vitro* Fas levels per-cell (MFI) were even more pronounced, with
285 an 8-fold increase over HC. Hence, clinical status impacts both *ex vivo* (Figure 2A-B) and *in*
286 *vitro* (Figure 4A) Fas expression. In addition, Fas *in vitro* levels showed a significant negative
287 correlation to age of disease onset in HAM/TSP patients (p=0.019, Pearson's r =-0.69, n=11)

Provisional

288 (Figure 4B), but not to age, disease duration and gender, suggesting Fas^{hi} phenotype
289 predisposes to earlier, aggressive disease manifestation. Further, *in vitro* Fas expression
290 neither correlated to viral p19 protein level (p=0.41), nor to PVL (p=0.14) in HTLV-1-infected
291 individuals (data not shown).

292 In agreement with its role as a death receptor in immune homeostasis, Fas surface
293 expression positively correlates with spontaneous *in vitro* apoptosis in HC, while this
294 correlation was lost in AC (data not shown). Surprisingly, *ex vivo* Fas expression correlated
295 negatively (Supplementary Figure 1) to spontaneous *in vitro* apoptosis in HAM/TSP.
296 Furthermore, *in vitro* Fas level (MFI) also correlates negatively to lymphocyte apoptosis in
297 HAM/TSP (Figure 5A). This negative correlation was confirmed by fluorescence microscopy.
298 As shown in Figure 5B, Fas^{hi} cells are negative for annexin V staining and display normal
299 nuclear morphology, whereas Fas^{lo} cells were seen to undergo apoptosis by both annexin V
300 staining and nuclear condensation/fragmentation, occasionally triggering phagocytosis by
301 macrophages, emphasizing their apoptotic nature. Since resistance to Fas induced
302 apoptosis has been observed *in vitro* in lymphocytes from MS patients,[34] we compared *in*
303 *vitro* lymphocyte Fas expression and apoptosis between HAM/TSP and MS patients. As
304 shown in Figure 5C, there was a significant increase (2.4-fold, Mann-Whitney test, p=0.019)
305 in Fas MFI in HAM/TSP as compared to MS patients, but not apoptosis (as measured by
306 annexin V staining, Mann-Whitney test, p=0.84). In contrast to HAM/TSP, no correlation was
307 observed between Fas MFI and apoptotic cells in MS patients (p=0.35, data not shown).
308 Taken together, the significant negative correlations between *ex vivo* and *in vitro* Fas
309 lymphocyte expression and *in vitro* apoptosis observed only in HAM/TSP, suggest a possible
310 selective defect in Fas-mediated apoptosis. Hence, we next aimed to comprehensively
311 explore non-apoptotic Fas signalling in HAM/TSP.

312 **Fas expression positively correlates to lymphoproliferation *in vitro* and *ex vivo* in**
313 **HAM/TSP**

314 We quantified *in vitro* spontaneous lymphoproliferation by [^{3}H]-thymidine incorporation in
315 HAM/TSP patients. Surprisingly, we found that Fas expression positively correlates to
316 spontaneous lymphoproliferation *in vitro* (Figure 6A), which might imply that the observed
317 defect in Fas-mediated pro-apoptotic signalling in HAM/TSP might be explained as a bias in

Provisional

318 Fas signalling towards proliferation rather than apoptosis. Therefore, we hypothesized that
319 Fas^{hi} cells might be already proliferating *in vivo* in HAM/TSP although at very low level. We
320 thus extended our previously described[27] sensitive flow cytometry assay to quantify Fas^{+}
321 diploid vs. tetraploid (proliferating) lymphocytes *ex vivo* in HAM/TSP patients, stained
322 immediately after PBMC isolation, without *in vitro* culture. As shown in Figure 6B, virtually all
323 of the proliferating cells were Fas^{hi} (99.2±0.8%), as compared to non-proliferating
324 lymphocytes (69.4±5.9%, Paired t test, p=0.0082).

325 **Stimulation with agonist Fas mAb *in vitro* can trigger apoptotic signalling in HAM/TSP**

326 We then examined if this apparent defect in Fas-mediated apoptosis might be reversible by
327 stimulating with agonist anti-Fas mAb, and if blocking with antagonist anti-Fas mAb could
328 reveal ongoing Fas-FasL signalling in HAM/TSP. Hence, we treated PBMC *in vitro* with anti-
329 Fas mAb (agonist or antagonist) or anti-CD3 mAb as a positive control. No decrease in
330 spontaneous apoptosis was observed upon treatment with antagonist anti-Fas mAb,
331 confirming our hypothesis of inactive Fas-FasL signalling *in vitro* in HAM/TSP. Interestingly,
332 treatment with agonist anti-Fas mAb resulted in significantly increased apoptosis (1.7-fold,
333 $p<0.05$), similar to treatment with anti-CD3 mAb (positive control, 1.8-fold, $p<0.01$) (Figure
334 7A). These results imply that agonist anti-Fas mAb treatment can restore the apparent
335 defect in apoptosis in HAM/TSP, at least *in vitro*.

Provisional

336 **Systems analysis of gene expression profiles upon Fas triggering vs. Fas blocking in**
337 **HAM/TSP**

338 Considering the significant correlation between *in vitro* Fas expression to age of onset in
339 HAM/TSP, we resorted to genome-wide transcriptional analysis of PBMC treated *in vitro* with
340 agonist or antagonist Fas mAb, to explore the broad pro/anti-apoptotic, inflammatory,
341 proliferative and immunoregulatory Fas signalling pathways specifically triggered in
342 HAM/TSP. Microarray analysis revealed that *in vitro* treatment with agonist anti-Fas mAb,
343 significantly down-regulated 190 genes and up-regulated 59 genes (Supplementary Table
344 1A and B), while treatment with antagonist anti-Fas mAb down-regulated 38 genes and up-
345 regulated 18 genes (Supplementary Table 1C and D). Thus, triggering Fas signalling effects
346 a broader gene spectrum than inhibiting it. This was also evident from Ingenuity® pathway
347 analysis (IPA), since no biological functions were significantly associated with antagonist

anti-Fas mAb treatment, whereas treatment with agonist anti-Fas mAb resulted in 22 significantly associated biological functions (5% FDR-adjusted and a stringent cut-off of at least five enriched molecules per pathway) (Supplementary Table 2). The top 10 biological functions activated by agonist anti-Fas mAb (Supplementary Table 2), highlight cellular migration, especially of myeloid cells. In addition, IPA network analysis (Figure 7B) of Fas-triggered gene expression reveals a central role for NFkB pro-survival signalling, connecting several up-regulated proliferative and inflammatory molecules (TNF, JNK, RNA Polymerase II, POLR2D, HIST1H3A, HIST1H2AB) as well as down-regulated anti-proliferative genes (L3MBTL2, CARD6). This central role for NFkB signalling was confirmed by Ingenuity upstream regulator analysis, identifying RelA as the top upstream regulatory molecule upon triggering Fas signalling (target genes: BCL2A1, CASR, CXCL3, ICAM1, L3MBTL2, PTGES, TGM2, TNF and TPMT; p= 0.000032). Again, blocking Fas signalling did not yield any significantly enriched upstream regulators (using the same stringent cut-off of five enriched molecules/pathway, data not shown).

Genome-wide correlation of *ex vivo* Fas RNA levels in HAM/TSP confirms a significant association to proliferation but not apoptosis

Provisional

Finally, we used a pathway-based data mining approach, to test our hypothesis of biased Fas signalling, and to possibly extend our findings by including additional pro- and anti-apoptotic genes (e.g. TRAIL, cFlip, etc.). For this purpose, we explored possible interactions of Fas mRNA within the *ex vivo* global gene expression profile in PBMC of HAM/TSP patients (n=6). Using transcriptome-wide correlation, 4554 genes significantly correlated to Fas transcript levels (Supplementary Table 3), after stringent FDR-correction for multiple testing. Using annotated Ingenuity pathways, we found a significant enrichment for proliferation-related genes (159 of 4554 genes, p=0.023). However, apoptosis, as defined by IPA, was not enriched amongst the *ex vivo* Fas-correlating genes (71 genes out of 4554 genes, p=0.10).

DISCUSSION

In this study, we combined *ex vivo*, *in vitro* and systems analysis of Fas expression with functional apoptosis and proliferation assays, thereby providing an all-inclusive approach of the biological and clinical relevance of Fas signalling in HAM/TSP. We observed a two-step

increase in *ex vivo* Fas expression: first, a greater percentage of Fas^{+} lymphocytes upon HTLV-1 infection and second, a strong increase in expression of the death receptor at the single-cell level upon HAM/TSP disease progression. In addition, for the first time, we demonstrate that Fas expression correlates negatively to apoptosis and age of onset, but positively to immune activation and lymphoproliferation.

The most surprising finding of this study is a selective defect in Fas-mediated apoptosis in HAM/TSP patients. First, both *ex vivo* and *in vitro* Fas levels negatively correlated to *in vitro* apoptosis (Figure 5A and Supplementary Figure 1). Second, by fluorescence microscopy (Figure 5B), we document that Fas^{lo} but not Fas^{hi} cells preferentially undergo apoptosis *in vitro*. Third, *in vitro* treatment of PBMC with agonist anti-Fas mAb, but not antagonist anti-Fas mAb, was able to trigger apoptosis and restore the selective defect in HAM/TSP patients. Fourth, *in silico* analysis of the HAM/TSP transcriptome revealed a large number of transcripts (>4500) significantly correlating to Fas mRNA level, but are not enriched for apoptotic pathways. Taking together, our data indicate that the death receptor is fully functional in HAM/TSP, and not in a dormant state but skewed towards other biological pathways. Similar to our observation in HAM/TSP, increased Fas[35] and resistance to Fas-triggered apoptosis[36] has been reported in MS, which was also supported by gene expression profiling.[37] Nevertheless, our data reveal that the Fas^{hi} phenotype is HAM/TSP-specific, since Fas expression was increased both ex vivo and in vitro, as compared to MS patients. Strikingly, the increase in non-apoptotic Fas receptor is also negatively correlated to age of disease onset in HAM/TSP (Figure 4B), rendering Fas as a clinically relevant molecule. It should be stated, however, that formal demonstration of the possible clinical utility of Fas expression or Fas downstream signalling targets as biomarker(s) in HAM/TSP will require confirmation of our findings in prospective cohort studies with a long-term clinical follow-up. In addition, agonist anti-Fas mAb, although restoring the defect in apoptosis in HAM/TSP, would not be a therapeutic option given that anti-Fas mAb therapy caused liver injury and lethality in mice.[38] In the absence of clinical benefit of antiretrovirals in HAM/TSP, immunomodulatory options include IFN-α/β, glucocorticoids, cyclosporine and ascorbic acid.[32,39,40] We previously demonstrated IFN-β can restore defective B cell CD86 up-regulation in HAM/TSP.[30] As in MS, defective Fas-mediated apoptosis in HAM/TSP patients may be overcome by IFN-β therapy.[41,42] In addition to IFN therapy, our *in silico* analysis

Provisional

409 might reveal novel treatment options. As shown in Figure 7B, a molecular network elegantly
410 describes the interplay between the molecular players of apoptosis (CARD6, caspases),
411 proliferation (POLR2D, L3MBTL2) and inflammation (TNF, JNK), with a central role for
412 NFkB. Therefore, our data confirm and extend the findings of Oh et al.[11] and Talledo et al.,[9]
413 who pointed at the importance of NFkB signalling in HAM/TSP from a pharmacological and
414 immunogenetic perspective. Furthermore, our Fas-triggered gene expression in HAM/TSP
415 reveals the same upstream regulator (Rel A), which is associated to active disease in MS.[37]
416 Thus, transcriptomics can reveal neuroinflammatory disorders sharing analogous biological
417 pathways, indicating approved MS drugs to be considered in HAM/TSP, but also allow the
418 identification of possible novel therapeutic targets, e.g. TGM2 or L3MBTL2 (Figure 7B).

419 Regarding HAM/TSP pathogenesis, both genetic and environmental triggers have been
420 suggested.[43] Interestingly, in a large cohort in the same endemic area (Salvador-Bahia), a
421 city with Afro-descendent demography, probable (but not definite) HAM/TSP occurred in
422 31% of AC during 8-year follow-up,[44] which suggests lifetime risk in this population is 10-fold
423 higher than previously reported.[43] As for environmental factors, co-infection with Gram-
424 positive bacteria, as in infective dermatitis, has been shown to trigger early HAM/TSP in
425 children from the same endemic area.[45,46] Concerning genetics, a single *FAS* -670
426 polymorphism has been associated to both ATL[26] and HAM/TSP[27] susceptibility. Since this
427 polymorphism also determined CD4 Tscm levels in a genome-wide twin study (Khouri et al,
428 submitted), the proliferative, non-apoptotic Fas^{hi} cells in HAM/TSP are reminiscent of a Tscm
429 phenotype,[47] as outlined in Figure 8. However, since CD4 or CD8 Tscm represent only a
430 minor subset of Fas^{+} lymphocytes[28], a Tscm origin of Fas^{hi} cells is not likely, considering the
431 two-step increase we observed both *ex vivo* and *in vitro* (Figures 2A-B and 4A), first in AC
432 and second in HAM/TSP.

433 Non-apoptotic Fas signalling towards proliferation has been previously demonstrated,[48,49]
434 while Tax gene expression and cell cycling but not cell death are selected during HTLV-1
435 infection *in vivo*.[50] Tax mediates its anti-apoptotic activity by activating the NFkB pathway,[51]
436 associating NFkB to cell survival and inflammation, similar to our *in silico* findings. In
437 addition, Tax-deregulated autophagy and cFLIP expression are responsible for resistance to
438 apoptosis *in vitro*,[52] in agreement with our *ex vivo* and *in vitro* results. In contrast, many viral
439 infections are associated with heightened apoptosis. The most striking example is HIV,[53]

Provisional

which manipulates apoptotic pathways to enable efficient viral replication.[54] In the case of HTLV-1, *in vitro* culture triggers viral protein synthesis and subsequent cytokine-driven lymphoproliferation.[14] However, Fas did not correlate to PVL, similar to[25] and two other published cohorts (p>0.5 for test and training sets).[7] Interestingly, PVL also did not correlate to apoptosis or age of disease onset, in contrast to Fas. A previous larger study with sufficient statistical power also demonstrated PVL does not correlate to age of onset in HAM/TSP.[55] Furthermore, viral p19 protein levels did not correlate to Fas in our cohort. Taken together, increased Fas levels in HAM/TSP appear to be driven by a IFN/STAT1 axis, either genetically[27] or environmentally[45] linked, rather than by the virus itself, suggesting the role of Fas in HAM/TSP pathogenesis is independent of PVL. Therefore, it is tempting to speculate that a similar IFN/STAT1 signalling pathway might underlie the suggested deleterious role of $CD80^+$ B cells, correlating positively to disease severity, also independent of PVL.[30]

In conclusion, our results suggest defective Fas-mediated apoptosis is linked to early disease onset and might be an additional factor in HAM/TSP pathogenesis, independent of PVL. Triggering Fas signalling, rather than inhibiting it, induces a specific gene set with a central role for NFkB pro-survival signalling. Thus, our integrated *ex vivo*, *in vitro*, *in silico* approach identifies biased pro-inflammatory and proliferative Fas signalling in HAM/TSP, revealing possible novel therapeutic targets.

Supplementary data

Funding. This research was supported by Brazilian National Research Council (CNPq/Science Without Borders, PVE), Fonds voor Wetenschappelijk Onderzoek (FWO, grant G.0778.10N and G0D6817N), VLIR-UOS project ZEIN2010PR376 and 'Vaast Leysen Leerstoel voor Infectieziekten in Ontwikkelingslanden' (KU Leuven), Belgium.

Potential conflicts of interest. All authors: no reported conflicts.

Provisional

466 **References**

467 1. Gessain A, Cassar O. Epidemiological Aspects and World Distribution of HTLV-1
468 Infection. *Front Microbiol.* 2012;3:388.
469 2. Bangham CR, Araujo A, Yamano Y, Taylor GP. HTLV-1-associated myelopathy/tropical
470 spastic paraparesis. *Nat Rev Dis Primers.* 2015;1:15012.
471 3. Verdonck K, González E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. Human
472 T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect Dis.*
473 2007;7:266-81.
474 4. Enose-Akahata Y, Abrams A, Johnson KR, Maloney EM, Jacobson S. Quantitative
475 differences in HTLV-I antibody responses: classification and relative risk assessment for
476 asymptomatic carriers and ATL and HAM/TSP patients from Jamaica. *Blood.*
477 2012;119:2829-36.
478 5. Ishihara M, Araya N, Sato T, Tatsuguchi A, Saichi N, Utsunomiya A, et al. Preapoptotic
479 protease calpain-2 is frequently suppressed in adult T-cell leukemia. *Blood* 2013;121:4340-
480 7.
481 6. Oliere S, Hernandez E, Lezin A, Arguello M, Douville R, Nguyen TL, et al. HTLV-1 evades
482 type I interferon antiviral signaling by inducing the suppressor of cytokine signaling 1
483 (SOCS1). *PLoS Pathog.* 2010;6:e1001177.
484 7. Tattermusch S, Skinner JA, Chaussabel D, Banchereau J, Berry MP, McNab FW, et al.
485 Systems Biology Approaches Reveal a Specific Interferon-Inducible Signature in HTLV-1
486 Associated Myelopathy. *PLoS Pathog.* 2012;8:e1002480.
487 8. Swaims AY, Khani F, Zhang Y, Roberts AI, Devadas S, Shi Y, et al. Immune activation
488 induces immortalization of HTLV-1 LTR-Tax transgenic CD4+ T cells. *Blood.* 2010;116:2994-
489 3003.
490 9. Talledo M, Lopez G, Huyghe JR, Verdonck K, Gonzalez E, Clark D, et al. Possible
491 implication of NFKB1A and NKG2D genes in susceptibility to HTLV-1-associated
492 myelopathy/tropical spastic paraparesis in Peruvian patients infected with HTLV-1. *J Med*
493 *Virol.* 2012;84:319-26.
494 10. Ju W, Zhang M, Jiang JK, Thomas CJ, Oh U, Bryant BR, et al. CP-690,550, a
495 therapeutic agent, inhibits cytokine-mediated Jak3 activation and proliferation of T cells from
496 patients with ATL and HAM/TSP. *Blood.* 2011;117:1938-46.
497 11. Oh U, McCormick MJ, Datta D, Turner RV, Bobb K, Monie DD, et al. Inhibition of immune
498 activation by a novel nuclear factor-kappa B inhibitor in HTLV-I-associated neurologic
499 disease. *Blood.* 2011;117:3363-9.
500 12. Lezin A, Gillet N, Olindo S, Signate A, Grandvaux N, Verlaeten O, et al. Histone
501 deacetylase mediated transcriptional activation reduces proviral loads in HTLV-1 associated
502 myelopathy/tropical spastic paraparesis patients. *Blood.* 2007;110:3722-8.
503 13. Olindo S, Belrose G, Gillet N, Rodriguez S, Boxus M, Verlaeten O, et al. Safety of long-
504 term treatment of HAM/TSP patients with valproic acid. *Blood.* 2011;118(24):6306-9.
505 14. Itoyama Y, Minato S, Kira J, Goto I, Sato H, Okochi K, et al. Spontaneous proliferation of
506 peripheral blood lymphocytes increased in patients with HTLV-I-associated myelopathy.
507 *Neurology.* 1988;38:1302-7.
508 15. Bangham CR, Osame M. Cellular immune response to HTLV-1. *Oncogene.*
509 2005;24:6035-46.
510 16. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science.*
511 1998;281:1305-8.
512 17. Krammer PH. CD95's deadly mission in the immune system. *Nature.* 2000;407:789-95.
513 18. Suda T, Nagata S. Purification and characterization of the Fas-ligand that induces
514 apoptosis. *J Exp Med.* 1994;179:873-9.
515 19. Ichikawa H, Ota K, Iwata M. Increased Fas antigen on T cells in multiple sclerosis. *J*
516 *Neuroimmunol.* 1996;71:125-9
517 20. Okuda Y, Apatoff BR, Posnett DN. Aoptosis of T cells in peripheral blood and
518 cerebrospinal fluid is associated with disease activity of multiple sclerosis. *J Neuroimmunol.*
519 2006;17:163-70.
520 21. Saggioro D. Anti-apoptotic effect of Tax: an NF-kappaB path or a CREB way? *Viruses.*
521 2011;3:1001-14.
522 22. Osame M. Pathological mechanisms of human T-cell lymphotropic virus type I-
523 associated myelopathy (HAM/TSP). *J Neurovirol.* 2002;8:359-64.
524 23. Kamihira S, Yamada Y, Hiragata Y, Yamaguchi T, Izumikawa K, Matsuo Y, et al. Serum
525 levels of soluble Fas/APO-1 receptor in human retroviral infection and associated diseases.
526 *Intern Med.* 1997;36:166-70.

Provisional

24. Inoue A, Koh CS, Sakai T, Yamazaki M, Yanagisawa N, Usuku K, et al. Detection of the soluble form of the Fas molecule in patients with multiple sclerosis and human T-lymphotropic virus type I-associated myelopathy. *J Neuroimmunol.* 1997;75:141-6.
25. Furukawa Y, Bangham CR, Taylor GP, Weber JN, Osame M. Frequent reversible membrane damage in peripheral blood B cells in human T cell lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP). *Clin Exp Immunol.* 2000;120:307-16.
26. Farre L, Bittencourt AL, Silva-Santos G, Almeida A, Silva AC, Decanine D, et al. Fas 670 promoter polymorphism is associated to susceptibility, clinical presentation, and survival in adult T cell leukemia. *J Leukoc Biol.* 2008;83:220-2.
27. Vallinoto AC, Santana BB, dos Santos EL, Santo RR, Hermes RB, Sousa RC, et al. FAS-670A/G single nucleotide polymorphism may be associated with human T lymphotropic virus-1 infection and clinical evolution to TSP/HAM. *Virus Res.* 2012;163:178-82.
28. Nagai Y, Kawahara M, Hishizawa M, Shimazu Y, Sugino N, Fujii S, et al. T memory stem cells are the hierarchical apex of adult T-cell leukemia. *Blood.* 2015;125:3527-35.
29. Osame M. Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP. In: Blattner WA, editor. Human retrovirology: HTLV. New York: Raven Press; 1990. pp. 191–7.
30. Menezes SM, Decanine D, Brassat D, Khouri R, Schnitman SV, Kruschewsky R, et al. 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;11:18.
31. Grassi MF, Olavarria VN, Kruschewsky Rde A, Mascarenhas RE, Dourado I, Correia LC, et al. Human T cell lymphotropic virus type 1 (HTLV-1) proviral load of HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients according to new diagnostic criteria of HAM/TSP. *J Med Virol.* 2011;83:1269-74.
32. Moens B, Decanine D, Menezes SM, Khouri R, Silva-Santos G, Lopez G, et al. Ascorbic Acid Has Superior Ex Vivo Antiproliferative, Cell Death-Inducing and Immunomodulatory Effects over IFN-alpha in HTLV-1-Associated Myelopathy. *PLoS Negl Trop Dis.* 2012;6:e1729.
33. Adaui V, Verdonck K, Best I, Gonzalez E, Tipismana M, Arevalo J, et al. SYBR Green-based quantitation of human T-lymphotropic virus type 1 proviral load in Peruvian patients with neurological disease and asymptomatic carriers: influence of clinical status, sex, and familial relatedness. *J Neurovirol.* 2006;12:456-65.
34. Comi C, Leone M, Bonissoni S, DeFranco S, Bottarel F, Mezzatesta C, et al. Defective T cell fas function in patients with multiple sclerosis. *Neurology.* 2000;55:921-7.
35. Ichikawa H, Ota K, Iwata M. Increased Fas antigen on T cells in multiple sclerosis. *J Neuroimmunol.* 1996;71:125-9.
36. Comi C, Fleetwood T, Dianzani U. The role of T cell apoptosis in nervous system autoimmunity. *Autoimmun Rev.* 2012;12:150-6.
37. Achiron A, Feldman A, Mandel M, Gurevich M. Impaired expression of peripheral blood apoptotic-related gene transcripts in acute multiple sclerosis relapse. *Ann N Y Acad Sci.* 2007;1107:155-67.
38. Timmer T, de Vries EG, de Jong S. Fas receptor-mediated apoptosis: a clinical application? *J Pathol.* 2002;196:125-34.
39. Nakagawa M, Nakahara K, Maruyama Y, Kawabata M, Higuchi I, Kubota H, et al. Therapeutic trials in 200 patients with HTLV-I-associated myelopathy/ tropical spastic paraparesis. *J Neurovirol.* 1996;2:345-55.
40. Martin F, Castro H, Gabriel C, Adonis A, Fedina A, Harrison L, et al. Ciclosporin A proof of concept study in patients with active, progressive HTLV-1 associated myelopathy/tropical spastic paraparesis. *PLoS Negl Trop Dis.* 2012;6:e1675.
41. Van Weyenbergh J, Wietzerbin J, Rouillard D, Barral-Netto M, Liblau R. Treatment of multiple sclerosis patients with interferon-beta primes monocyte-derived macrophages for apoptotic cell death. *J Leukoc Biol.* 2001;70:745-8.
42. Kaser A, Deisenhammer F, Berger T, Tilg H. Interferon-beta 1b augments activation-induced T-cell death in multiple sclerosis patients. *Lancet.* 1999;353:1413-4.
43. Taylor GP. Editorial Commentary: Human T-Cell Lymphotropic Virus Type 1 (HTLV-1) and HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis. *Clin Infect Dis.* 2015;61:57-8.
44. Tanajura D, Castro N, Oliveira P, Neto A, Muniz A, Carvalho NB, et al. Neurological Manifestations in Human T-Cell Lymphotropic Virus Type 1 (HTLV-1)-Infected Individuals Without HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis: A Longitudinal Cohort Study. *Clin Infect Dis.* 2015;61:49-56.

Provisional

590 45. Primo JR, Brites C, Oliveira Mde F, Moreno-Carvalho O, Machado M, Bittencourt AL.
591 Infective dermatitis and human T cell lymphotropic virus type 1-associated
592 myelopathy/tropical spastic paraparesis in childhood and adolescence. *Clin Infect Dis.*
593 2005;41:535-41.
594 46. Farre L, de Oliveira Mde F, Primo J, Vandamme AM, Van Weyenbergh J, Bittencourt AL.
595 Early sequential development of infective dermatitis, human T cell lymphotropic virus type 1-
596 associated myelopathy, and adult T cell leukemia/lymphoma. *Clin Infect Dis.* 2008;46:440-2.
597 47. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell
598 subset with stem cell-like properties. *Nat Med.* 2011;17:1290-7.
599 48. Siegel RM, Chan FK, Chun HJ, Lenardo MJ. The multifaceted role of Fas signaling in
600 immune cell homeostasis and autoimmunity. *Nat Immunol.* 2000;1:469-74.
601 49. Barca O, Seoane M, Senaris RM, Arce VM. Fas/CD95 Ligation Induces Proliferation of
602 Primary Fetal Astrocytes Through a Mechanism Involving Caspase 8-Mediated ERK
603 Activation. *Cell Physiol Biochem.* 2013;32:111-20.
604 50. Zane L, Sibon D, Jeannin L, Zandecki M, Delfau-Larue MH, Gessain A, et al. Tax gene
605 expression and cell cycling but not cell death are selected during HTLV-1 infection in vivo.
606 *Retrovirology.* 2010;7:17.
607 51. Saggioro D, Silic-Benussi M, Biasiotto R, D'Agostino DM, Ciminale V. Control of cell
608 death pathways by HTLV-1 proteins. *Front Biosci (Landmark Ed).* 2009;14:3338-51.
609 52. Wang W, Zhou J, Shi J, Zhang Y, Liu S, Liu Y, et al. Human T-cell leukemia virus type 1
610 Tax-deregulated autophagy pathway and c-FLIP expression contribute to resistance against
611 death receptor-mediated apoptosis. *J Virol.* 2014;88:2786-98.
612 53. Wood KL, Twigg HL, 3rd, Doseff AI. Dysregulation of CD8+ lymphocyte apoptosis,
613 chronic disease, and immune regulation. *Front Biosci (Landmark Ed).* 2009;14:3771-81.
614 54. Lima RG, Van Weyenbergh J, Saraiva EM, Barral-Netto M, Galvao-Castro B, Bou-Habib
615 DC. The replication of human immunodeficiency virus type 1 in macrophages is enhanced
616 after phagocytosis of apoptotic cells. *J Infect Dis.* 2002;185:1561-6.
617 55. Nagai M, Usuku K, Matsumoto W, Kodama D, Takenouchi N, Moritoyo T, et al. Analysis
618 of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers:
619 high proviral load strongly predisposes to HAM/TSP. *J Neurovirol.* 1998;4:586-93.

620

Provisional

Figure legends

Figure 1. Schematic representation of the methodology (ex vivo, in vitro and in silico approaches).

Figure 2. *Ex vivo* lymphocyte Fas surface expression in HTLV-1-infected individuals, HAM/TSP and MS patients. Using flow cytometry, Fas levels as % (A) and MFI (mean fluorescence intensity on a per cell basis) (B) were quantified in HC, AC and HAM/TSP patients. (*p<0.05, **p<0.01, ***p<0.001; Kruskal-Wallis, with Dunn's multiple comparison post-test). (C) Fas expression in CD4, CD8 and B cells was quantified in *ex vivo* PBMCs in HC, AC and HAM/TSP patients (ANOVA, p=0.067, post-test for linear trend p<0.05). (D) *Ex vivo* Fas levels (% and MFI) are compared between neuroinflammatory diseases HAM/TSP and MS (Mann Whitney test, *p<0.05).

Figure 3. Increased *ex vivo* lymphocyte Fas surface expression in HAM/TSP patients correlates with activation markers. Positive correlation between the percentage of Fas^+ lymphocytes and (A) $HLA\text{-}DR^+$ (*p=0.039, Spearman's r= 0.56, n=14) and (B) $CD86^+$ (*p=0.031, Spearman's r=0.60, n=13) lymphocytes in HAM/TSP patients.

Provisional

Figure 4. Significant linear trend in Fas^+ lymphocyte levels in PBMCs of HC, AC and HAM/TSP patients upon *in vitro* culture, and negative correlation with age of onset of HAM/TSP. (A) Fas levels were quantified by flow cytometry after 48h of *in vitro* culture. Fas^+ lymphocytes (%) gradually increase (HC n=12 AC n=4 HAM n=12) upon infection (AC) and further upon disease progression to HAM/TSP (ANOVA, p=0.0005; post-test for linear trend, p<0.0001). (B) Lymphocyte Fas levels (after 48h of *in vitro* culture) quantified by flow cytometry (MFI) correlate negatively to age of onset in HAM/TSP patients (*p=0.019, Pearson's r= - 0.69, n=11).

Figure 5. Fas^{hi} cells are apoptosis-resistant in HAM/TSP patients. (A) Fas MFI (mean fluorescence intensity on a per-cell basis) negatively correlates to apoptosis (quantified as % annexin V^+ cells) in lymphocytes of HAM/TSP patients (*p=0.012, Spearman's r= - 0.63, n=15). (B) In the middle panel is a representative image of a non-apoptotic Fas^{hi} cell

652 (indicated by a red horizontal arrow). This Fas^{hi} cell is annexin V negative as visualized in
653 the first panel and displays a normal nuclear morphology seen in the third panel. On the
654 contrary, a Fas^{lo} cell in panel 1 (black vertical arrow), displays pronounced annexin V
655 staining (panel 1) and is undergoing apoptosis, as evidenced by nuclear condensation, and
656 is being engulfed by a macrophage. (C) *In vitro* Fas levels (MFI) and apoptosis (% of
657 Annexin V$^+$ cells) are compared between neuroinflammatory diseases HAM/TSP and MS
658 (Mann Whitney test, *$p<0.05$).
659
660 Figure 6. Fas surface expression correlates positively with *in vitro* and *ex vivo*
661 lymphoproliferation in HAM/TSP patients. (A) *In vitro* Fas expression as measured by flow
662 cytometry (MFI) correlates positively to lymphoproliferation quantified by [3H]-thymidine
663 incorporation (*$p=0.018$, Pearson's $r=0.62$, $n=14$). (B) *Ex vivo* Fas surface expression
664 measured by flow cytometry (% and MFI) is significantly higher in proliferating (tetraploid, 4n)
665 cells vs. diploid (2n) cells in HAM/TSP patients (Paired t test, $p=0.0082$ and $p=0.0023$
666 respectively, $n=5$)
667
668 Figure 7. *In vitro* Fas triggering with agonist anti-Fas mAb induces apoptosis in HAM/TSP
669 and activates a molecular network linking apoptosis, proliferation and inflammation. (A)
670 Agonist (ago) anti-Fas mAb but not antagonist (ant) anti-Fas mAb increased apoptosis
671 (quantified by CellDeathPlus ELISA) in PBMCs upon *in vitro* treatment for 24h when
672 compared to control (untreated) PBMCs. Treatment with anti-CD3 mAb was used as a
673 positive control. (ANOVA, with Bonferroni's post test *$p<0.05$, **$p<0.01$). (B) Top molecular
674 network (score=34, linking cell-to-cell signalling, interaction, and cellular growth and
675 proliferation) identified by Ingenuity pathway analysis (IPA) among 249 genes significantly
676 up- and down-regulated (red and green, respectively) in PBMCs of HAM/TSP patients by *in*
677 *vitro* treatment with agonist anti-Fas mAb.
678
679 Figure 8. Model indicating the two-step increase in *ex vivo* lymphocyte Fas surface
680 expression. First, following HTLV-1 infection, there is an increase in lymphocyte Fas
681 expression (%) in AC. Second, upon progression to HAM/TSP, Fas expression is increased
682 on a per-cell basis as Mean Fluorescence Intensity (MFI), (Figure 2A-B). In agreement with
683 its role as a death receptor, Fas$^+$ cells in HC are primed to follow the apoptotic pathway,

Provisional

684 depicting nuclear condensation and cell blebbing, which is lost upon HTLV-1 infection (AC).
685 In contrast, in HAM/TSP patients, Fas^{hi} cells are driven towards proliferation (Figure 7A-B).
686 We recently discovered a genotype/phenotype interaction for the *FAS* -670 polymorphism
687 with both apoptosis and proliferation in ATL patients and healthy controls (Khouri et al,
688 submitted). This Fas^{hi} proliferating and chemotherapy-resistant leukemic phenotype is in
689 agreement with the recently discovered CD4 Tscm hierarchical apex of ATL. The same *FAS*
690 -670 polymorphism also determined CD4 Tscm levels in a genome-wide twin study,
691 confirming our hypothesis (Khouri et al, submitted). Therefore, a genetically determined
692 IFN/STAT1/FAS axis might help explain the proliferative, non-apoptotic phenotype in
693 HAM/TSP suggesting CD4 Tscm as a pivotal factor not only in ATL but also in HAM/TSP
694 pathogenesis. Considering STAT1 and FAS are in the HAM/TSP gene signature, our data
695 further refine the data of Tattermusch et al.[7] It is not unexpected that a Tscm phenotype is
696 absent from the disease signature, since Tscm are rare (2-3%)[47] and their genome-wide
697 expression profile is intermediate between naïve and central memory T cells. However,
698 Tscm cells have a Fas^{hi}, apoptosis-resistant and drug-resistant, proliferative phenotype, in
699 agreement with their stem cell-like nature. Interestingly, the proliferating cells in HAM/TSP
700 patients were almost exclusively Fas^{hi}, (Figure 6B), compatible with a Tscm phenotype.
701
702
703 Table 1.
704 Patient information and sample use

Provisional

Table 1. Patient information and sample use

Patient	Age	Gender	Cohort	Analysis
1	NA	F	BA	Ex vivo flow cytometry
2	NA	M	BA	Ex vivo flow cytometry
3	NA	F	BA	Ex vivo flow cytometry
4	NA	F	BA	Ex vivo flow cytometry
6	NA	F	BA	Ex vivo flow cytometry
7	51	M	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
8	40	M	BA	Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
9	40	F	BA	Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
10	63	F	BA	Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
11	51	F	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
12	36	M	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
13	40	F	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
14	60	F	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
15	44	M	BA	Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
16	NA	F	BA	Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
17	53	M	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray
18	45	F	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray
20	59	M	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
21	60	F	BA	Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
22	38	M	BA	In vitro lymphoproliferation
23	59	F	BA	In vitro lymphoproliferation
24	56	F	BA	In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray
25	49	F	BA	In vitro apoptosis
26	57	M	BA	In vitro apoptosis
27	49	F	BA	In vitro flow cytometry, In vitro apoptosis In vitro lymphoproliferation
28	60	M	BA	In vitro flow cytometry, In vitro apoptosis In vitro lymphoproliferation, Microarray
29	46	M	BA	In vitro apoptosis, In vitro lymphoproliferation, Microarray
31	50	M	BA	In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray
32	50	F	BA	In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray
33	62	F	BA	In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation
2569	27	F	LI	In vitro apoptosis
2570	50	F	LI	In vitro apoptosis
2574	35	F	LI	In vitro apoptosis
2817	64	F	LI	Ex vivo flow cytometry
2819	32	F	LI	Ex vivo flow cytometry
2821	63	F	LI	Ex vivo flow cytometry
2822	50	F	LI	Ex vivo flow cytometry
2823	64	M	LI	Ex vivo flow cytometry
SP5	32	F	SP	Ex vivo flow cytometry
SP6	65	F	SP	Ex vivo flow cytometry
SP7	62	F	SP	Ex vivo flow cytometry
SP8	47	F	SP	Ex vivo flow cytometry
SP26	35	M	SP	Ex vivo flow cytometry
SP30	72	M	SP	Ex vivo flow cytometry
SP32	27	M	SP	Ex vivo flow cytometry
SP36	52	F	SP	Ex vivo flow cytometry
SP46	61	F	SP	Ex vivo flow cytometry

Cohorts: BA Bahia, LI Lima, SP Sao Paulo

705 NA: Not available

706
707
708

Provisional

709 **Footnote page:**

710 ***Funding.*** This research was supported by Brazilian National Research Council
711 (CNPq/Science Without Borders), Fonds voor Wetenschappelijk Onderzoek (FWO,grant
712 G077810N and G0D6817N), VLIR-UOS project ZEIN2010PR376 and 'Vaast Leysen
713 Leerstoel voor Wetenschappelijk onderzoek over infectieziekten in ontwikkelingslanden' (KU
714 Leuven), Belgium.

715 ***Potential conflicts of interest.*** All authors: no reported conflicts.

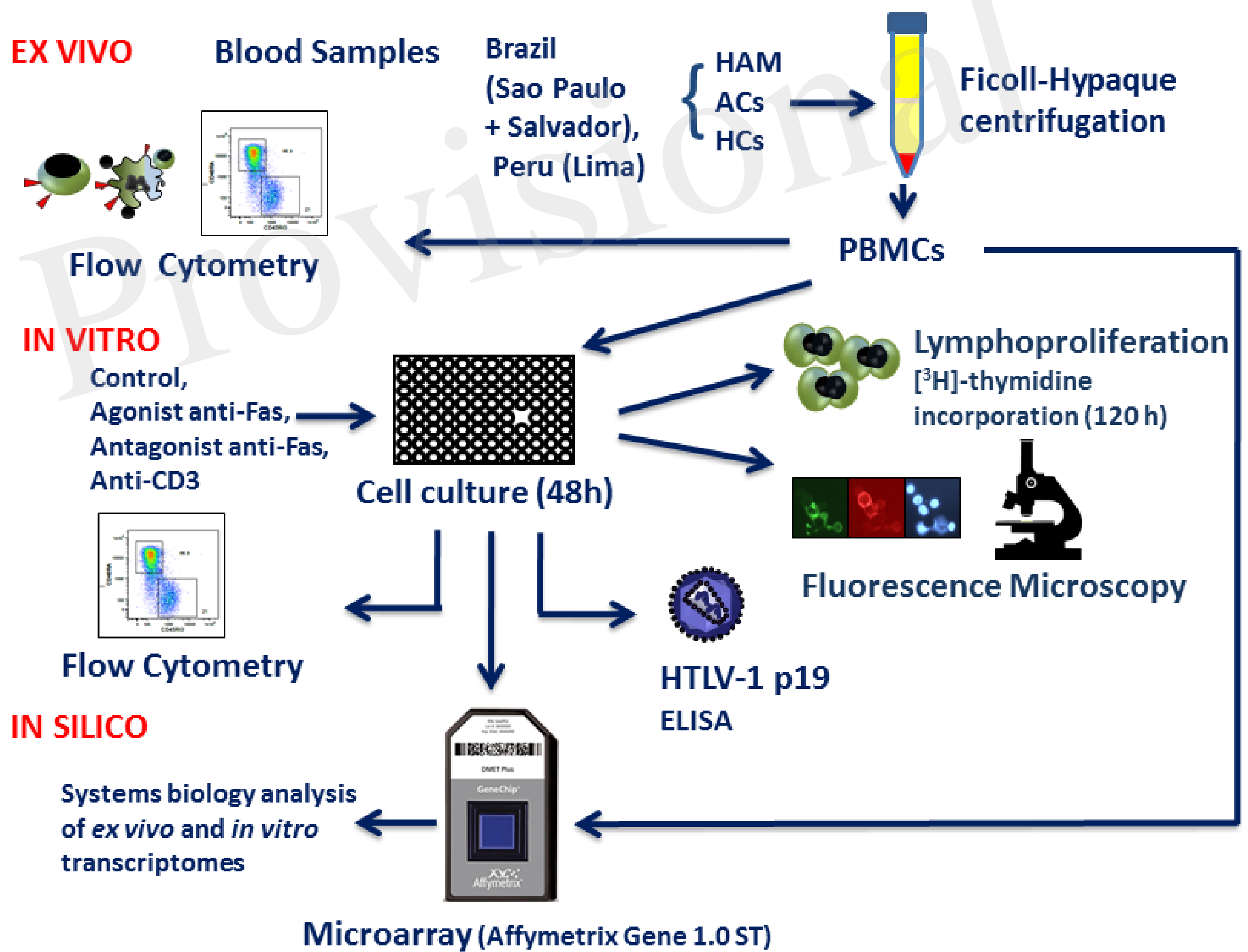
716 **Authorship:** JVW designed research; SMM, FEL, TD, RicardoK, DD, GSS, GL and JVW
717 performed research; SVS, DFN, JV and AMV contributed to data analysis; FEL, RamonK,
718 CA, MT, EG, DB, RL and BGC provided patient samples; SMM and JVW analyzed data and
719 wrote the paper.

720 **Meetings presented at:**

721 16th International Conference on Human Retrovirology: HTLV and Related Retroviruses, 26-
722 30 June 2013, Montreal, Canada.

Provisional

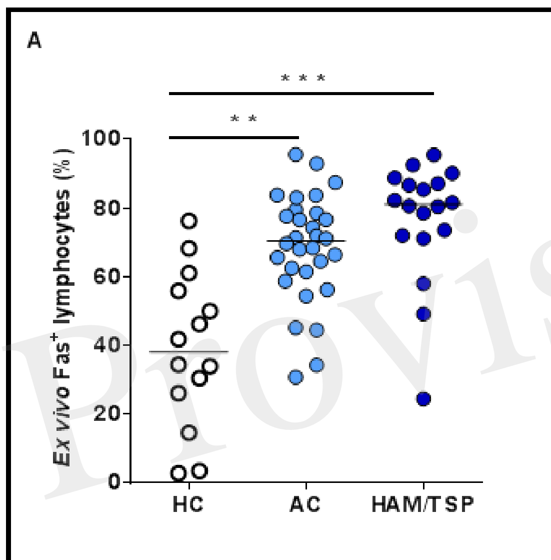
Figure 01.TIF

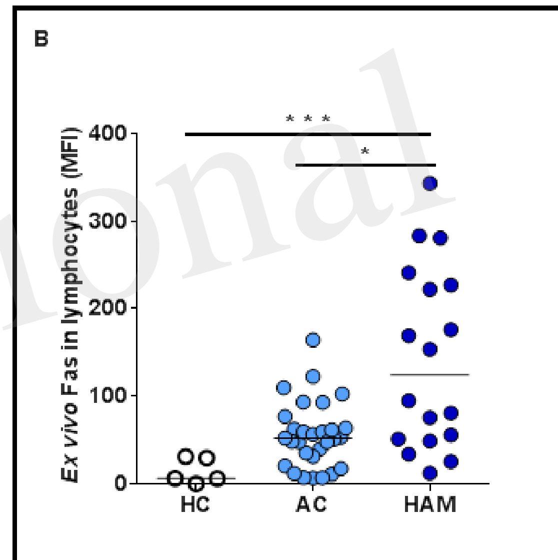


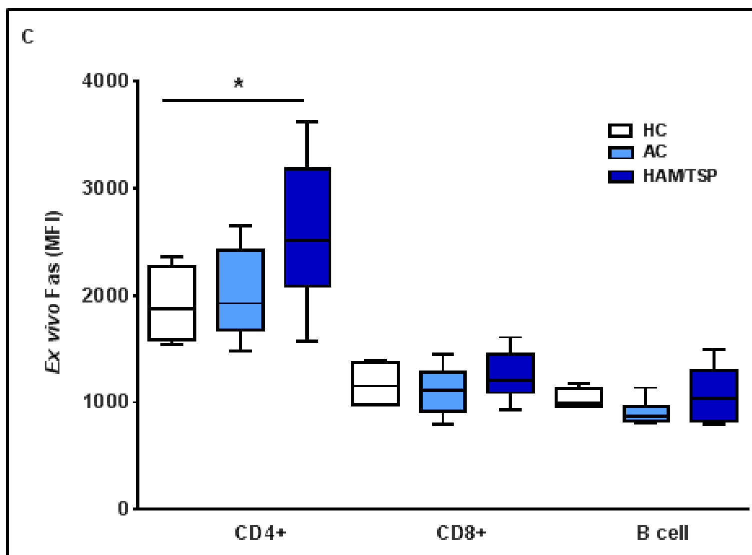
Provisional

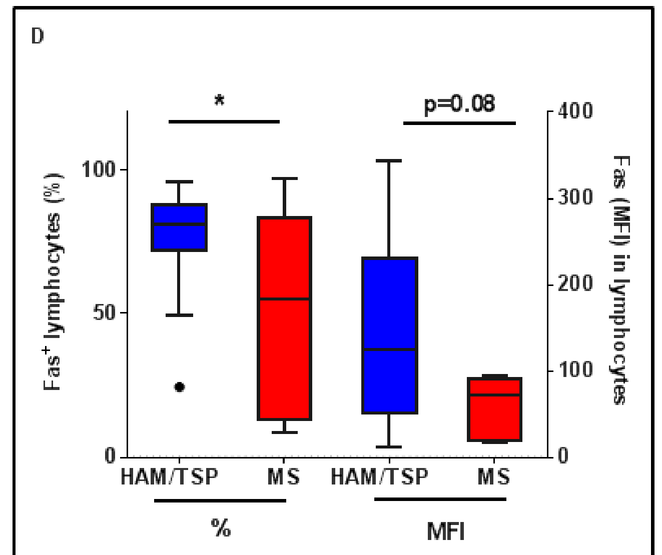
Figure 02.TIF

Figure 2





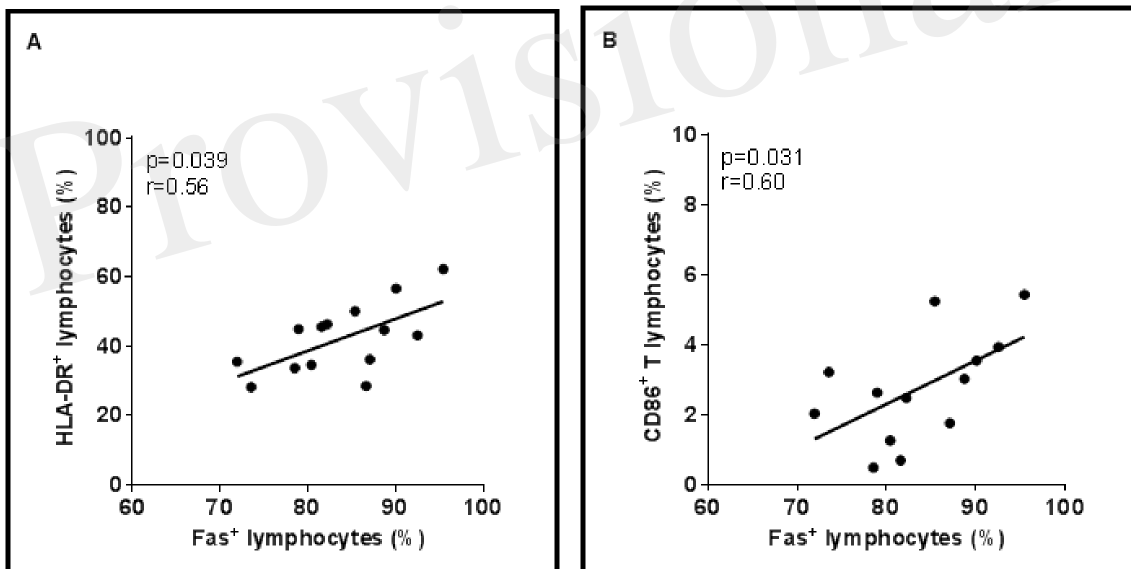




Provisional

Figure 03.TIF

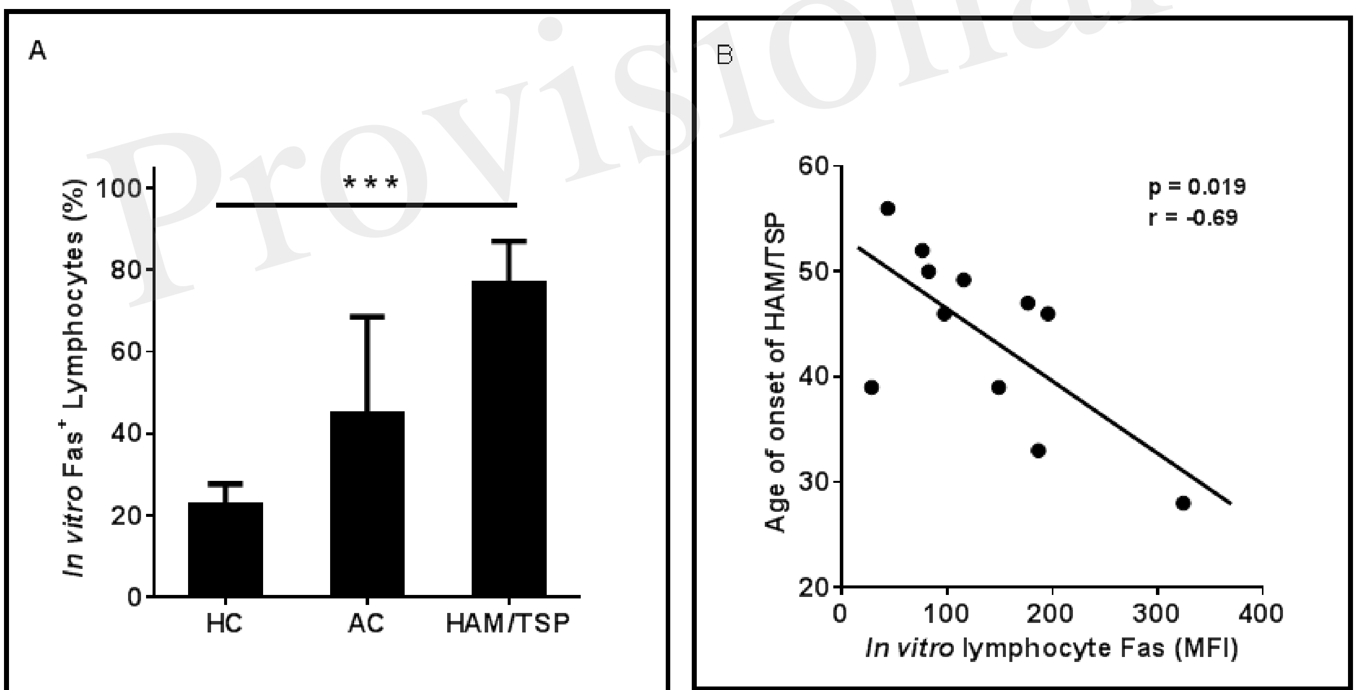
Figure 3



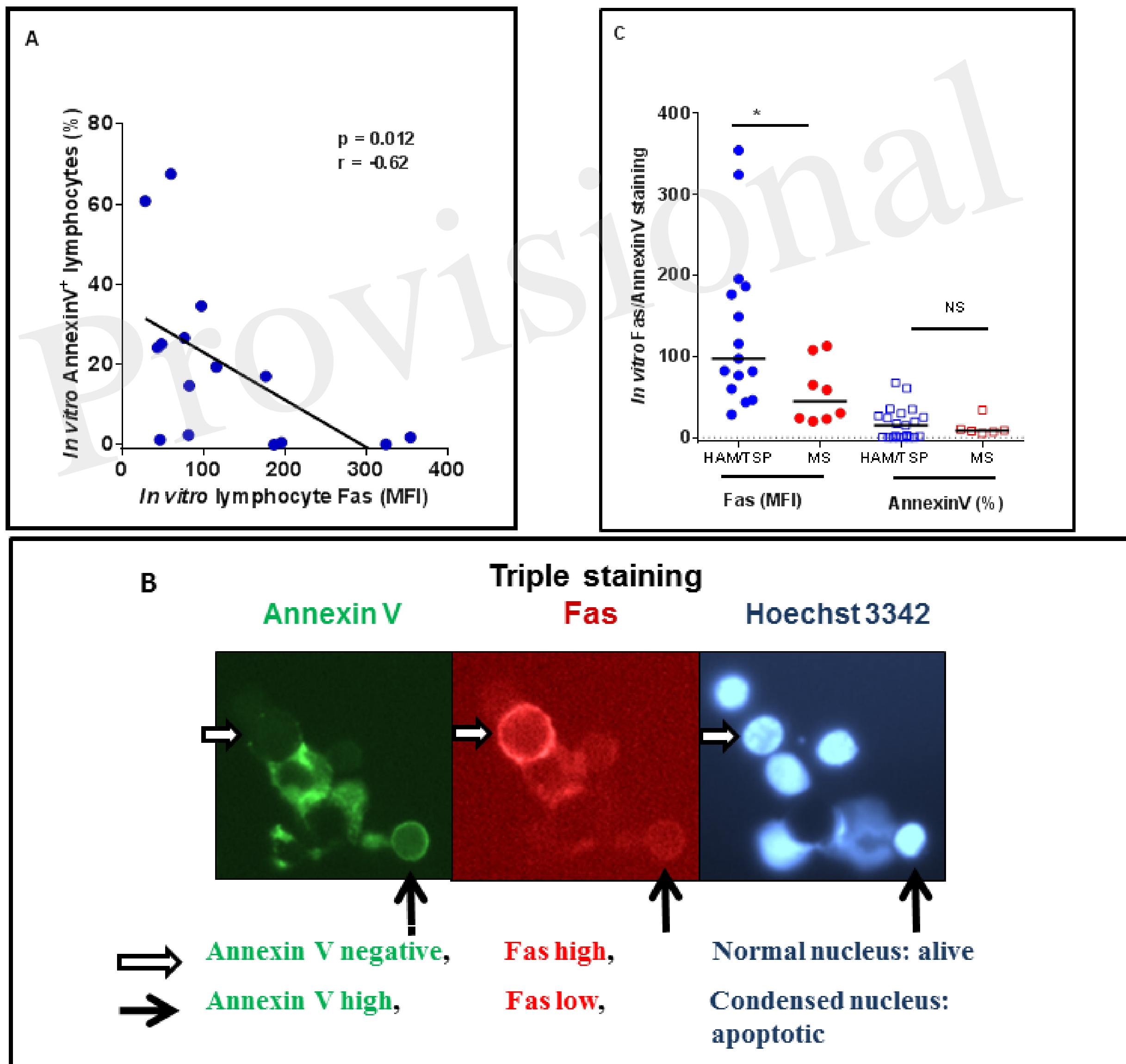
Provisional

Figure 04.TIF

Figure 4



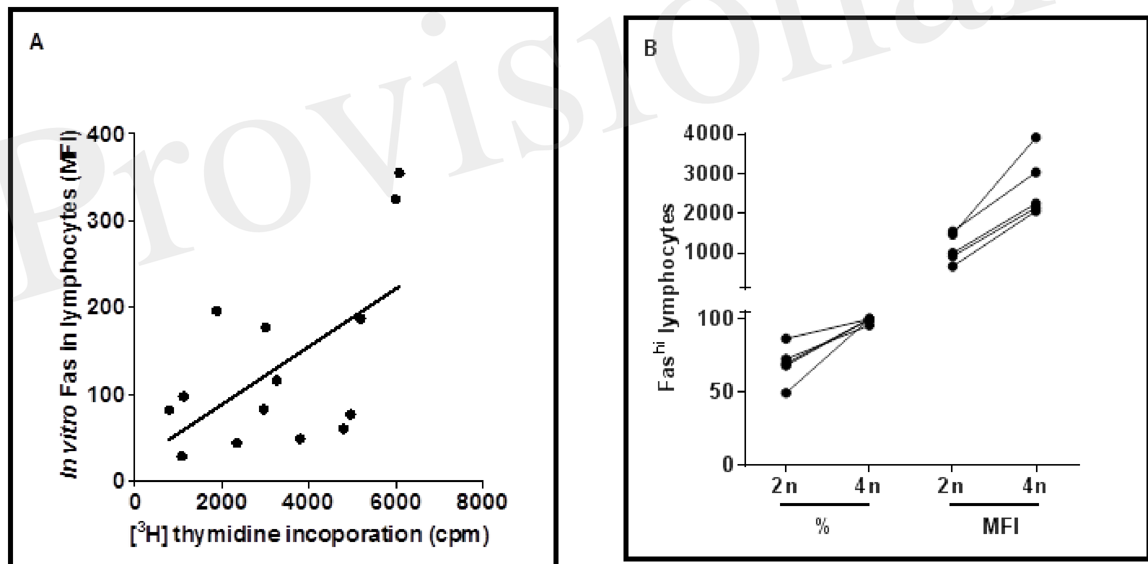
Provisional



Provisional

Figure 06.TIF

Figure 6



Provisional

Figure 07.TIF

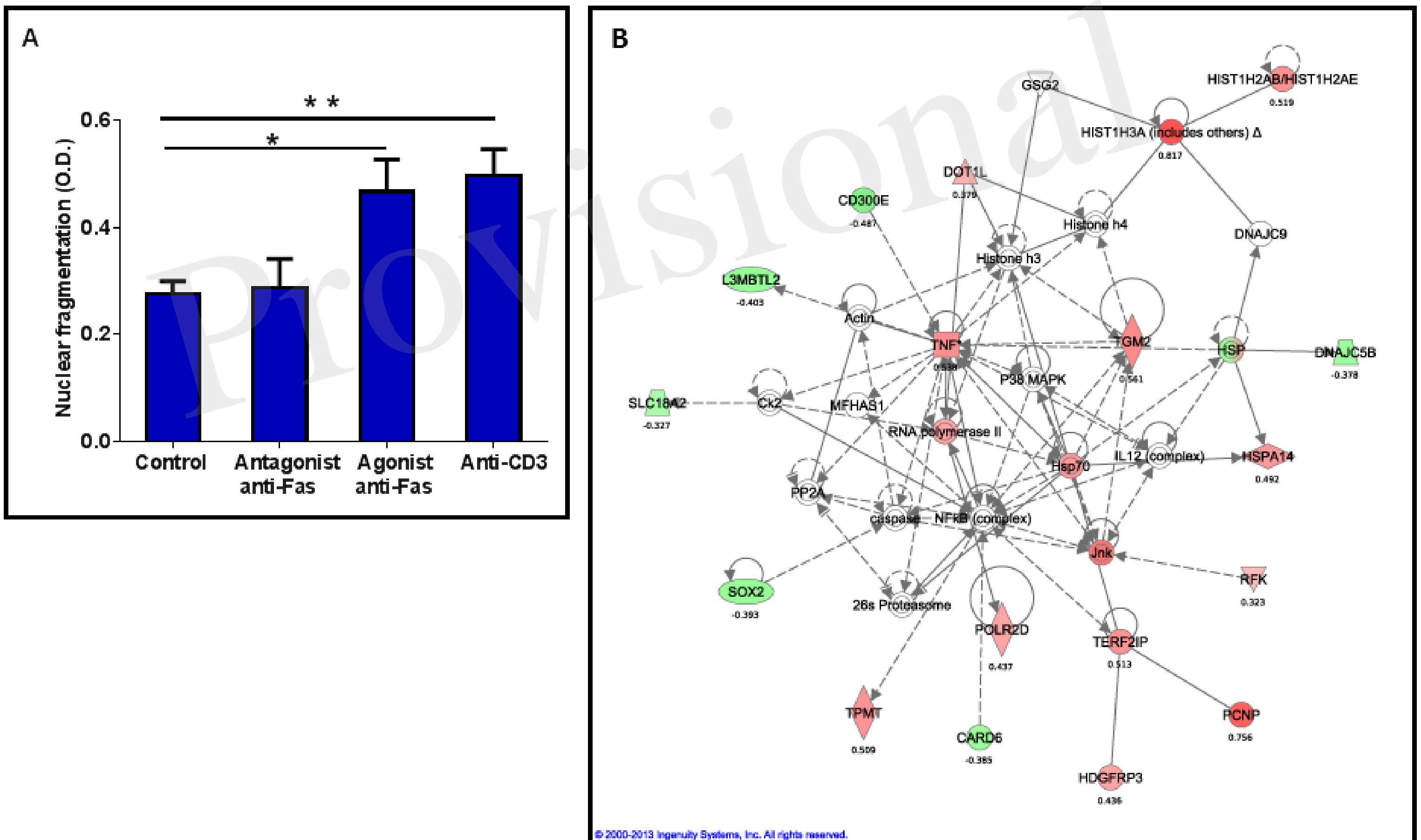
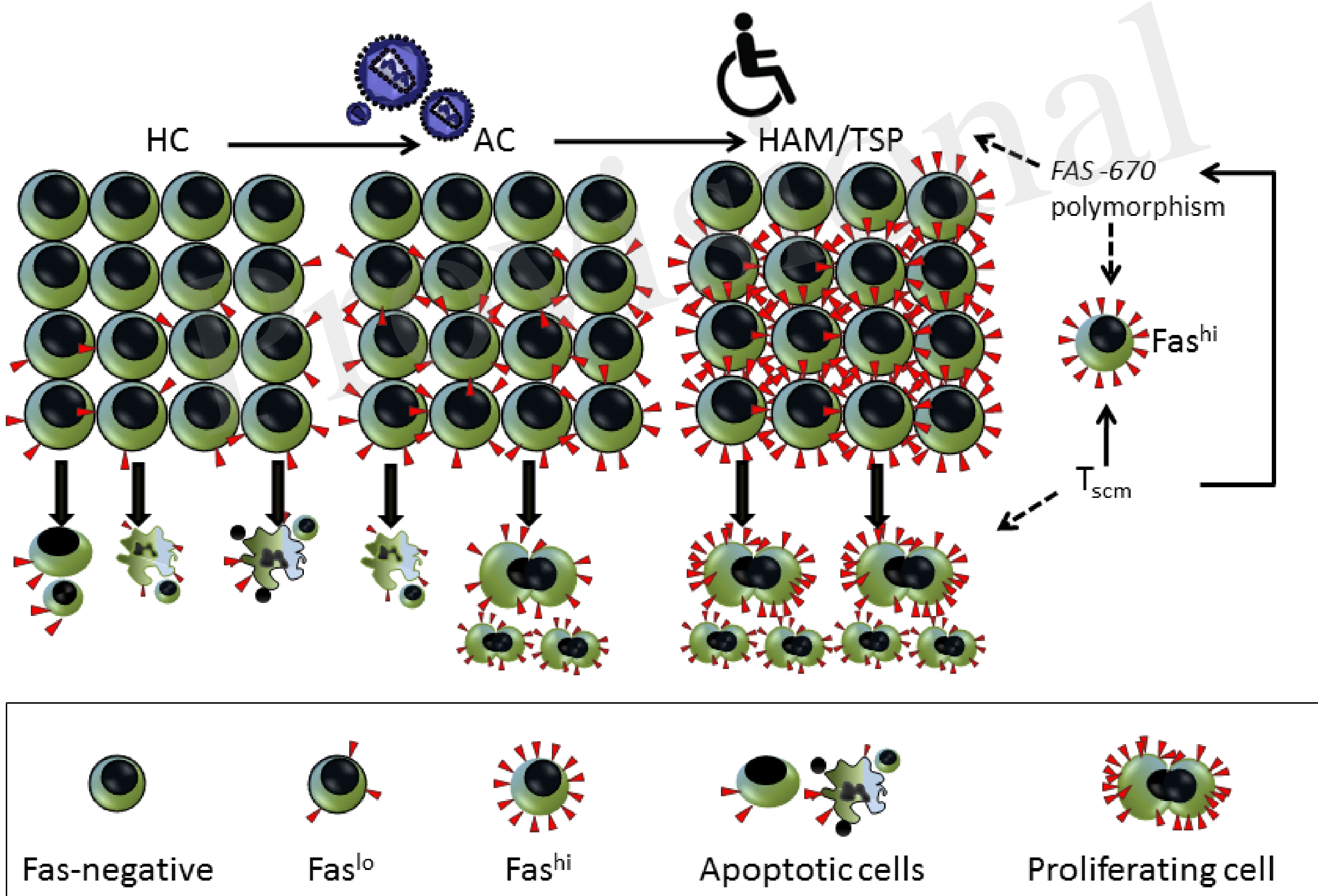


Figure 08.TIF

Figure 8



Provisional