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Damage of Gut Junctional Complexes Features HIV-Infected Immunological Non Responders

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1. Introduction

HIV infects and replicates within CD4+ T-cells of the GI compartment *(Veazey 1998, Mehandru 2004, Mehandru 2007)* accounting for mucosal activation *(Mehandru 2006)*, CD4+ T-cell depletion *(Schneider 1995, Veazey 1998, Guadalupe 2003, Brenchley 2004)*, and impairment of epithelial barrier function *(Lim 1993, Keating 1995)*. These events may result in the translocation of microbial bioproducts from the gut lumen to the systemic circulation *(Brenchley 2006, Estes 2010)*, ultimately leading to immune activation *(Brenchley 2006, Estes 2010)*, known to represent a main driving force of CD4+ T-cell failure in untreated HIV disease *(Liu, 1996, Giorgi, 1999, Deeks 2004, Hazenberg AIDS 2010)*.

Long-term HAART accounts for partial restoration of the HIV-mediated immunological and anatomical defects within the GI mucosa *(Mehandru 2007, Epple 2009, Ciccone 2010)*, leading to decreases in microbial translocation *(Brenchley 2006)* and peripheral immune activation *(Hunt 2003)*.

Immunological Non Responders (INR), display lack of CD4+ T-cell recovery regardless of efficient HIV RNA control *(Piketty 1998)*. In keeping with the evidence of the gut mucosa as an important site of HIV pathogenesis, INR present high levels of peripheral immune activation *(Marchetti 2006)* which may be partially driven by microbial translocation *(Marchetti 2008)*.

Epithelial barrier impairment in the GI tract was investigated as an underlying cause of microbial translocation and T-cell activation in INR.

2. Methods

2.1 Study Patients Subjects were recruited at the Clinic of Infectious Diseases and Tropical Medicine, San Paolo Hospital, University of Milan. Patients had to be on HAART for ≥ 12 months, have a CD4+ cell nadir <350/mmc and HIV RNA <40 cp/mL. Patients with current CD4+ count <350/mmc and/or delta CD4 change from baseline <30% were defined as INR. Subjects with current CD4+ count >350/mmc and/or delta CD4 change from baseline >30% were defined as FR.

2.2 Flow cytometry Fresh peripheral blood was stained with CD4, CD8, CD38, CD45R0, CD45RA and CD127.

2.3 Microbial translocation Plasma levels of sCD14 and EndocAb were measured by ELISA test. Circulating levels of lipopolysaccharide (LPS) were assessed using the LAL test.

2.4 Urinary Lactulose/Mannitol (LAC/MAN) fractional excretion ratio
Patients drank a solution containing 5 g of lactulose and 1 g of mannitol. Urine collected in the following 5 hours was frozen and stored for HPLC analysis.

2.5. Colonoscopy 8 INR and 13 FR with no history and/or symptoms of GI disease underwent colonoscopy. Biopsies were performed in colon and distal ileum.

2.6. Immunohistochemical (IHC) staining Intestinal junction proteins were analyzed by cadherin (CDh1) and zonulin. For T lymphocyte sub-populations, CD3, CD4 and CD8 were used. Proliferation index was determined by Ki67 antibody.

2.6 Electron microscopy (EM) Ileum and colon biopsies from 3 INR and 4 FR were routinely processed for EM examination.

2.7 Fecal microbial population analysis PCR amplification of the total bacterial community in feces was performed. A nested approach was used to detect Bacteroides. Banding patterns of DGGE profiles were analyzed and a sequence analysis was performed for species identification.

2.8 Virological studies CD4+ T-cells were isolated by negative selection from PBMCs obtained by Ficoll procedure. A pellet of $1x10^6$/CD4 and gut biopsies were digested and the lysate was used directly in a nested PCR to quantify both HIV and CD3 gene copy numbers.

3. Results

3.1 Patient population

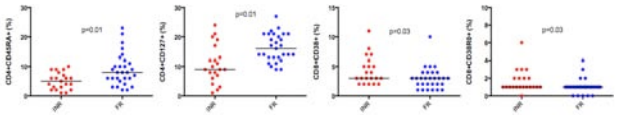
21 INR and 29 FR were enrolled. Subjects were comparable in term of demographic characteristics, HIV duration and months on HAART (Table 1). No major differences were registered between study groups in terms of endoscopic and histological evaluation (not shown).

Table 1. Clinical characteristics of study patients

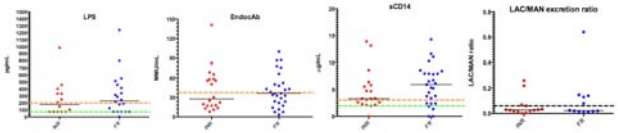
	INR, n=21	FR, n=29
Age, years (IQR)	51 (43-66)	51 (43-66)
Gender, no females (%)	4 (19)	7 (24)
Ongoing antibiotic prophylaxis, n (%)	6 (29)*	0 (0)
Risk factor for HIV infection		
Heterosex, n (%)	13 (62)	13 (45)
MSM, n (%)	4 (19)	12 (41)
IV drug use, n (%)	4 (19)	4 (14)
HCV-Ab positivity, n (%)	2 (10)	2 (7)
Duration of HIV infection, mths (IQR)	74 (42-216)	70 (60-96)
Duration of HAART, mths (IQR)	68 (37-87)	56 (39-68)
AIDS diagnosis, n (%)	13 (62)*	7 (24)
Nadir CD4+ T-cells		
Absolute counts, mmc (IQR)	94 (39-145)†	230 (78-278)
Percentage (IQR)	8 (5-15)†	16 (10-23)
Current CD4+ T-cells		
Absolute counts, mmc (IQR)	261 (194-323)†	468 (429-624)
Percentage (IQR)	18 (13-25)†	27 (22-30)
Zenith HIV RNA load, log 10 cp/mL (IQR)	5 (5-6)	5 (5-6)
Ongoing HAART regimen, no patients (%)		
NNRTI-based	4 (19)	10 (35)
PI-based	13 (62)	17 (59)
Other	4 (19)	2 (7)

* p<0.05 for INR vs FR (Chi-squared test): † p<0.05 for INR vs INR (Mann-Whitney test)

3.2 INR patients show contraction of the CD45RA+ and CD127+ CD4+ pools and expansion of CD38+ and CD38+R0+ CD8+ subsets



3.3 Both INR and FR patients show increased microbial translocation and intestinal permeability (LAC/MAN fractional excretion ratio)



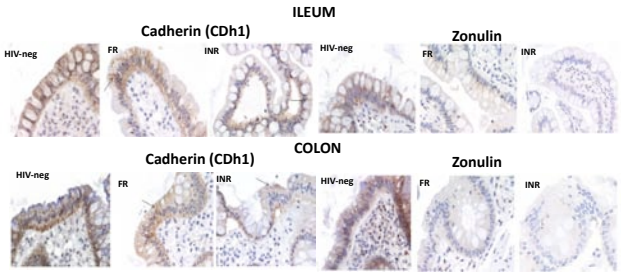
---- median values obtained by our lab in a large cohort study on 488 untreated patients (Marchetti et al. 16th IWHOD, Athens, 2012): LPS 204 pg/mL, EndocAb 36.5 MMU/mL, sCD14 2.83 ug/mL; ---- median values obtained by our lab in HIV-negative controls (Merlini et al. PlosOne, 2011); ---- indicates normal values: ≤0.032.

3.4 INR and FR show comparable intestinal CD4 reconstitution and Ki67 levels

IHC analysis of ileum and colon biopsies showed:

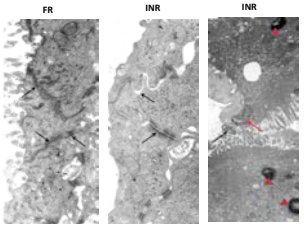
- comparable CD4 in ileum biopsies (High Power Field, HPF, 40x) INR: 105, IQR 84-207; FR: 98, 67-152, p=0.61
- comparable CD4 in colon biopsies (HPF) INR: 107, IQR 100-144; FR: 128, IQR 79-186, p=0.7
- comparable Ki67 in ileum: INR: 25%, 10-35; FR: 20, 15-28%; p>0.05
- comparable Ki67 in colon: INR: 35%, 30-40; FR: 25%, 9-30; p>0.05

3.5 INR patients show damage of gut junctional complexes



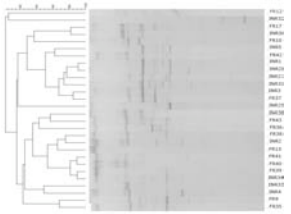
HIV-negative: strong diffuse basal and lateral positivity of enterocytes. **FR**: arrows point to focal lateral positivity between enterocytes; **INR**: CDh1, arrows point to rare weak lateral positivity between enterocytes; zonulin, the positivity of reaction is only in some nuclei of inflammatory cells. 40X original magnification

3.6 EM demonstrated focally wider intercellular spaces between adjacent cells in INR



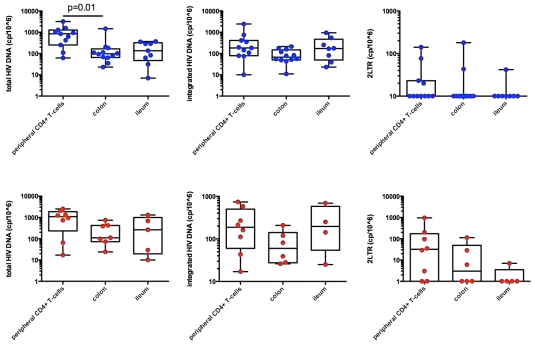
FR: arrows point to normal intercellular space. **INR**: arrows point to dilated intercellular space. **INR (further right)**: intestinal (ileocolic junction) biopsy, arrow points to an extracellular bacterium among microvilla, red arrow points to intracellular bacterium, arrow heads point to partly digested intracytoplasmic bacteria.
30,000 x original magnification

3.7 DGGE profiles of fecal microbiota showed partial clustering of patients belonging to the 2 groups. The discriminating band was compatible with *B. uniformis*



A hierarchical cluster analysis of the DGGE profiles of Bacteroides/Prevotella group in INR and FR. Dendrogram constructed using Pearson's correlation coefficients and the UPGMA algorithm of DGGE gels obtained analyzing the V2–V3 region of all fecal samples. Amplicons for DGGE analysis of the Bacteroides-Prevotella group were obtained using a nested-approach.

3.8 Lower reservoir size in colon biopsies vs peripheral CD4+ T-cells in FR, but no differences in HIV DNA content between study subjects



4. Conclusions

We show that INR display i) CD4 immune reconstitution in the GI tract despite poor CD4+ T-cell gain in the periphery; ii) severe dysfunction of ileum and colon junctional complexes.
Ongoing replication at mucosal sites may represent an underlying mechanism of epithelial barrier impairment in INR.