ncRNA PROFILE IN EXTRACELLULAR VESICLES REVEALS A POTENCIAL MECHANISM OF HIV-CONTROL

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I. Background

• Recent findings have pointed out that HIV can package its own material into extracellular vesicles (EVs) to enhance infection and pathogenesis. However, EVs could be candidates for therapeutic strategies against HIV infection given their capacity to transfer HIV restriction factors, including mRNAs and non-coding RNAs (ncRNAs), to nearby cells.

• Herein, we analyzed HIV RNAs and human ncRNAs content carried by EVs from plasma of patients with different degrees of HIV virologic control.

II. Methods

• Three groups of HIV patients were included according to plasma viral load (pVL): elite controllers (EC); cART-treated patients (TT) and cART-naive with detectable pVL (NT). Uninfected controls (UC) were included as reference (**Table 1**).

• For each subject, EVs were isolated from plasma by Size Exclusion Chromatography, and were quantified and visualized by Nano Tracking Analysis (Nanosight) and Transmission Electronic Microscopy (TEM).

• Viral RNAs and human ncRNAs were evaluated by RNAseq in a Miseq System. Identification of RNAs sequences was carried out by mapping them to HIV-HXB2 sequence and to human ncRNA database.

• Differential enrichment analysis of the identified human ncRNAs was conducted with EdgeR, and then, target genes for the differently expressed ncRNAs were predicted with miRDB database.

Table 1. Characteristics of patients included in the study

Characteristic	Study Group EC	TT	NT	UC	p-valor[1]	p-valor[2]
n	10	10	10	10	NA	NA
Age (years)	42 [35-48]	47 [44-51]	37 [31-44]	40 [34-51]	0,09	0,11
% Male	50	90	90	50	0,05	0,07
Viral load (HIV-RNA copies/mL)	<50	<50	61.607 [28.717-81.635]	NA	<0,00	NA
CD4 counts (cells/µL)	872 [634-982]	988 [585-1.469]	599 [518-832]	NA	0,35	0,60
Time since HIV diagnosis (years)	12 [4-14]	13 [11-16]	5 [2-5]	NA	0,00	0,23
Lenght of EC status (years)	6 [3-12]	NA	NA	NA	NA	NA
Lenght of treatment (years)	NA	12 [9-16]	NA	NA	NA	NA

Kruskal Wallis test; except sex comparisons, evaluated with χ2 test); p-value² comparison between EC and TT groups (Mann Whitney test). Sex comparisons evaluated with χ2 test. * RNAseq analysis was unsuccessful in 1 EC patient, 1 NT patients and 2 UC subjects. NA : not apply.

III. Results

Table 2. Proportion (%) of patients in each study group with EVs carrying viral RNAs

Viral RNA	Proportion (%) of patients in whom viral RNA has been detected TT	CE	NT	p-valor[1]	p-valor[2]
3'LTR	0	0	33	0.04	0.03
5'LTR	10	22	33	0.45	0.63
INS	0	22	22	0.21	0.59
Psi elements Ψ	30	11	55	0.21	0.19
tat	10	0	0	0.43	1.00
nef	0	0	11	0.37	0.35
vif	0	0	33	0.04	0.03
vpr	0	0	33	0.04	0.03
env	0	0	22	0.13	0.11
rev	0	0	22	0.13	0.11
pol	0	0	77	0.00	0.00
gag	10	0	55	0.01	0.01

Proportion (%) of patients in whom each viral RNA has been detected. p-value[1]: comparison between the three groups of patients (χ2 test); p-value[2]: comparison between patients with controlled viral replication (EC+TT) and patients with active viral replication (NT) (χ2 test).
LTR: Long Terminal Repeat ; INS: Inhibitory/Instability RNA sequences; Psi elements: Ψ RNA packaging signal.

▪ Higher proportion of Non-controller (NT) patients carried HIV-RNAs in their EVs when compared to controller patients (EC+TT): *vif* (p=0.03), *vpr* (p=0.03), *pol* (p=0.00) and *gag* (p=0.01) mRNAs; and 3'LTR-RNA (p=0.03) (**Table 2**).

▪ A total of 109 cellular ncRNAs were differently expressed between NT patients and UC subjects, mostly (89%) upregulated in NT patients. In contrast, only 14 ncRNAs in EC and 9 ncRNAs in TT were differently expressed respect to UC subjects, mostly upregulated in EC and TT patients (**Figure 1**).

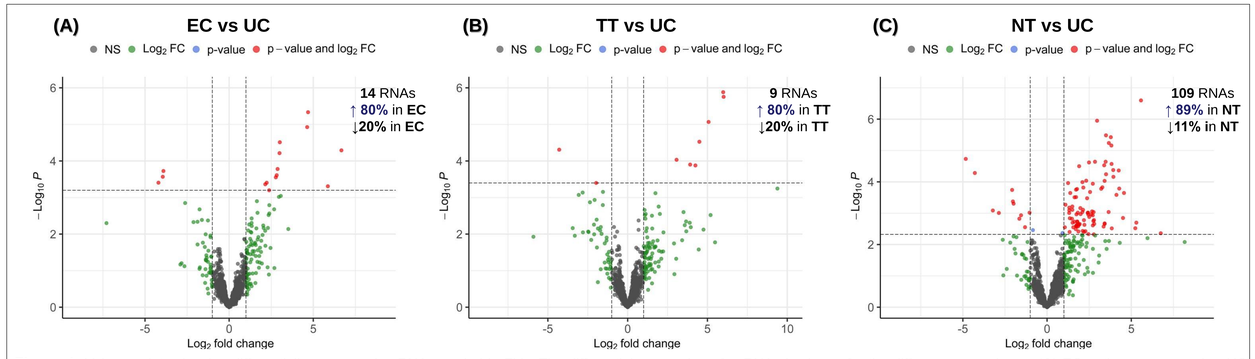

Figure 1. Volcano plots showing differentially expressed ncRNAs carried in EVs. The differential expression of ncRNAs is shown for the different comparisons: (**A**) EC patients compared to UC subjects; (**B**) TT patients compared to UC subjects; and (**C**) NT patients compared to UC subjects. Red dots show significant differentially expressed ncRNAs between groups of subjects (log2 FC>1 or log2 FC<-1, and FDR-adjusted p-value <0.05).

▪ Twenty-eight ncRNAs were differentially expressed between EC and TT patients: 19 ncRNAs upregulated in EC and 9 ncRNAs upregulated in TT (**Figure 2A**). ncRNAs upregulated in EC were mainly piRNAs, LncRNAs and tRNAs, while ncRNAs up-regulated in TT patients were mainly snoRNAs (**Figure 2B).**

▪ Target prediction analysis showed that ncRNAs upregulated in TT could inhibit expression of HIV restriction factors (**Figure 3A**), whereas ncRNAs upregulated in EC could inhibit expression of cellular targets related to promotion of HIV infection and replication (**Figure 3B**).

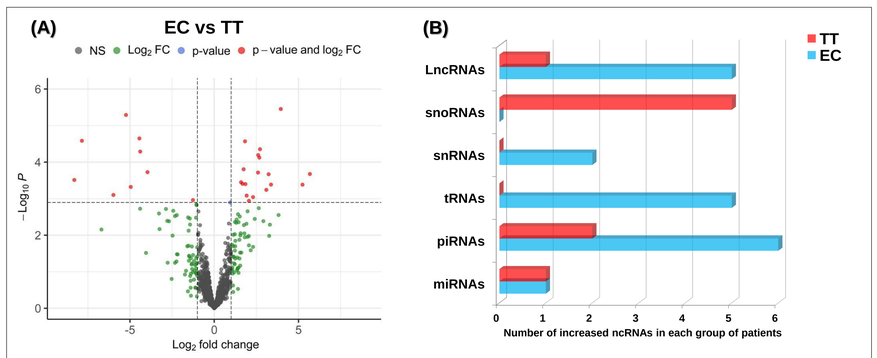

Figure 2. ncRNAs differentialy expressed between EC and TT patients: (**A**) Volcano plot showing in red dots the significant differentially expressed ncRNAs (log2 FC>1 or log2 FC<-1, and FDR-adjusted p-value <0.05) between EC and TT pantients; (**B**) bars graph showing the number of each type of ncRNA differentially expressed between EC and TT patients.

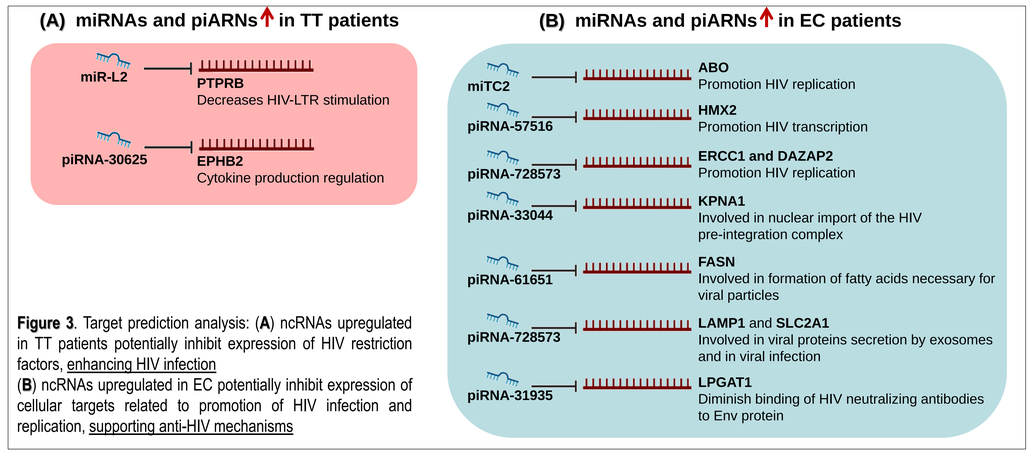

Figure 3. Target prediction analysis: (**A**) ncRNAs upregulated in TT patients potentially inhibit expression of HIV restriction factors, enhancing HIV infection
(**B**) ncRNAs upregulated in EC potentially inhibit expression of cellular targets related to promotion of HIV infection and replication, supporting anti-HIV mechanisms

IV. Funding / Acknowledgements

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V. Conclusions

✓ Our results show that HIV infection induces the packaging of different HIV RNAs in EVs and promotes deregulation in the profile of packaged human ncRNAs in EVs, which could modify gene expression in recipient cells. This deregulation is considerably diminished when considering the ncRNA profile of EVs from patients with viral replication control (regardless of the type of control, spontaneous or cART-mediated), revealing the role of active viral replication in the packaging modulation of ncRNA into EVs.

✓ However, the ncRNA profile differs depending on the viral control (spontaneous or cART-mediated): EC patients showed higher levels of ncRNA that could inhibit HIV replication and enhance the immune response against the virus. In contrast, ncRNA cargo into EVs from TT patients appears to induce proviral mechanisms, showing that cART-mediated suppression of viral replication is not sufficient to promote ncRNA packaging with anti-HIV actions in EVs , but it is the type of control (spontaneous or cART-mediated) that makes the difference: "*spontaneous control of HIV could be mediated by EVs content*".