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Original Article

HIV infection is associated with upregulated circulating levels of the inflammaging miR-21-5p

Javier Meseguer-Donlo [a,b], Jade Soldado-Folgado [a,c], Juan Du [d], Alicia González-Mena [d], Fabiola Blasco-Hernando [d], Esperanza Cañas-Ruano [d], Xavier Nogués [c,e], Hernando Knobel [d], Natalia Garcia-Giralt [e,*], Robert Güerri-Fernández [a,b,d,f]

[a] *Universitat Autònoma de Barcelona, Barcelona, Spain*
[b] *Department of Medicine and Life Sciences (MELIS), Universitat Pompeu Fabra, Barcelona, Spain*
[c] *Department of Internal Medicine, Hospital del Mar, Barcelona, Spain*
[d] *IMIM (Hospital del Mar Research Institute), Parc de Salut Mar, Department of Infectious Diseases, Barcelona, Spain*
[e] *IMIM (Hospital del Mar Research Institute), Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES), ISCIII, Barcelona, Spain*
[f] *Centro de Investigación Biomédica en Red Enfermedades Infecciosas, CIBERINFEC, Instituto de Salud Carlos III, Madrid, Spain*

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Abstract *Background:* HIV infection produces a chronic inflammation which leads to early aging of people living with HIV. Even though antiretroviral treatments (ART) have significantly increased HIV patient survival, an underlying chronic inflammation persists leading to HIV-related comorbidities. In this context, changes in microRNAs (miRNAs) expression may contribute to this inflammatory response. This study aims to detect differential expression of circulating miRNAs in treatment-naïve HIV-infected individuals compared to uninfected controls and evaluation of altered miRNAs after one year of ART.
Methods: Serum from patients and controls was collected at baseline and after 48-weeks on ART in HIV-treated patients. Circulating miRNAs were analysed using next generation sequencing.
Results: A total of 32 HIV patients and 10 controls were recruited. Of HIV+ individuals, 7 were long-term non-progressors (elite controllers), a group of HIV-infected individuals that spontaneously control the infection. Higher circulating levels of miR-21-5p, and lower levels of miR-

* Corresponding author. IMIM (Hospital del Mar Research Institute), CIBERFES, PRBB, Barcelona, 08003, Spain.
E-mail address: ngarcia@imim.es (N. Garcia-Giralt).

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6503-3p and miR-3135b were detected in HIV+ progressors. After one year of ART, these miRNAs remain altered. Moreover, miR-21-5p and miR-6503-3p were also altered in elite controllers compared to control group. *In silico* analyses showed that miR-21-5p target pathways are related to inflammation mechanisms and immune system.
Conclusion: miR-21-5p circulating levels are involved in inflammation and oxidative stress mechanisms in HIV patients even after one year of ART or in elite controllers.

Introduction

The estimated number of people living with HIV (PLWH) in 2019 was 38 million, 36.2 of whom were adults.[1] The development of antiretroviral therapy (ART) and its early start has led a life expectancy of PLWH close to the general population.[2] However, aging of PLWH has given rise new comorbidities such as neurocognitive disorders and liver, cardiovascular and bone diseases.[3,4] These pathologies affect typically advanced age patients, but in PLWH appear at a younger age. ART induces side effects related to drug toxicity and moreover it is not able to stop chronic inflammation and continuous immune activation which leads to early aging of PLWH.[4] This chronic inflammation is reflected by levels of IL-6, C-reactive protein (hsCRP), cystatin C and D-dimers which remain high with ART.[5]

HIV infection results in a multitude of changes in infected cells which impairs the host. During periods of active viral replication these changes affect RNA and protein expression as well as microRNAs (miRNAs) expression.[6,7] Hence, miRNAs constitute an additional class of molecules to study in the context of HIV infection and ART. miRNAs take part in multiple biological processes, such as cell differentiation, proliferation and death, metabolic regulation, silencing of transposons, and antiviral defence.[8] Extracellular miRNAs can be found in vesicles or associated with proteins. Unlike cellular RNAs, these characteristics make them highly stable, resisting degradation at room temperature up to 4 days. They interact with target cells and can act as autocrine, paracrine or endocrine regulators of cell metabolism.[9] In the HIV pathogenesis, miRNAs play a complex role involving several aspects of HIV infection, affecting viral replication, host immune response, and ultimately disease management.[10] Interestingly, miRNAs are able to regulate both host and viral mRNA. For instance, miR-21 and miR-222 take part in the development of apoptosis resistant CD4 T cells infected with HIV.[11] Other miRNAs, such as miR-29a, miR-128, and miR-223 can produce a decrease in HIV replication.[12,13] In this line, miR-29 inhibited HIV replication by approximately 60%, while miR-133b, miR-138, miR-326, miR-149, and miR-92a reduced HIV viral replication by 40%.[14] These findings suggest that miRNAs could be involved in HIV progression or inhibit viral replication. Thereby, identification of miRNAs related to different phases of HIV infection could help us to understand the underlying mechanism involved in the chronic inflammatory status.[15,16]

For all mentioned above, we entertain the hypothesis that HIV infection may alter the expression of miRNAs related to chronic inflammation and immune response. The aim of this project is to study the different profiling of circulating miRNAs in ART naïve people living with HIV compared to uninfected individuals and the influence of ART on their expression. The objectives are to better understand the role of miRNAs in HIV chronic infection.

Methods

Patients

A longitudinal case—control study was conducted with HIV-infected individuals naïve to ART aged over 18 years recruited consecutively between January 2017 to June 2018 from the Infectious Diseases Department in Hospital del Mar, Barcelona, Spain and with HIV-negative controls recruited from a Primary Care Center in Barcelona, Spain. Control individuals were healthy people with similar epidemiological characteristics than HIV-infected individuals (HIV+ progressors) that shared their same environment. Naïve to ART HIV-infected individuals were followed-up for 48-weeks after starting first-line recommended ART (DTG-3TC-ABC or Elvitegravir/cobicistat-TDF-FTC). A subset of long-term non-progressors (elite controllers) controlled in the Hospital del Mar was also included. This group was defined as patients without previous ART and plasma HIV RNA levels below 500 copies/mL for the last 24 months.[17] In this case, all patients who met the inclusion criteria and were willing to participate were included in the study. Patients with history of co-infection with chronic hepatitis B or hepatitis C, advanced liver disease, neoplasia, chronic kidney disease and inflammatory diseases were excluded. All participants had a baseline visit at recruitment and at 48 weeks after ART in HIV participants, where demographic and anthropometric variables, full medical history and blood samples were collected.

Ethics statement

The study protocol was approved by the Parc de Salut Mar Ethics Committee (2016/6793/I) in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization. Participants gave written informed consent. Privacy and confidentiality of subjects were respected at all times.

Laboratory tests

Blood samples were obtained by phlebotomy at fasting at 8:00 AM and were stored at −80 °C until analysis. Serum levels of the following markers were obtained: high sensitivity C-reactive protein (hsCRP) [Chemiluminescent immunoassay (CLIA) (Immulite 2000, Siemens], beta-2 microglobulin [CLIA (Immulite 2000, Siemens)], D-dimer [immunoturbidimetry (ACL TOP300)], erythrocyte sedimentation rate (ESR), and fibrinogen [Clauss method (ACL TOP300)]. In HIV patients, CD4 cell count and viral load (VL) were also measured.

miRNA isolation, library construction and sequencing

RNA was extracted from serum samples using miRNeasy Serum/Plasma kit (QIAGEN). miRNA quantity and purity were determined on Qubit miRNA assay and RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). Small RNA libraries were prepared by processing 16 and 14 samples per batch (conditions well distributed) using QIAseq miRNA Library Kit (QIAGEN) according to the manual QIAseq miRNA Library Kit Handbook for Illumina® (QIAGEN, Version 1.0, 12/18). The suggested parameters for serum/plasma samples were: 5 ul of initial sample, different dilutions of adaptors/primers and 22 PCR cycles. Quality control of the libraries was done using Bioanalyzer DNA High Sensitivity and Qubit DNA High Sensitivity, 29 of 30 libraries passed the quality control and subsequently were sequenced. Library quantifications were performed with KAPA Library Quantification kit on ABI Fast 7500 RT-PCR instrument. All libraries were pooled and sequenced on the Illumina NextSeq 500 platform for single read 75 cycles with an expected coverage of 14 M per sample.

miRNA sequence data processing and statistical analysis

QIAGEN adapters were removed (allowing 1 mismatch) and unique molecular identifier (UMI) sequences were retained with UMI tools version 0.5.4 (extract module). Reads shorter than 15 bp were removed with Cutadapt version 2.1. Reads in the fastq files were mapped with STAR version 2.6.0[18] against miRBase v22.1. The table of counts was obtained with Feature Counts function in the package subread, version 1.6.4[19] and posterior counting of unique UMIs with UMI tools version 0.5.4 (count, default method "directional"). Genes having less than 3 counts in at least 2 samples were excluded from the analysis. Raw library size differences between samples were treated with the weighted trimmed mean method (TMM)[20] implemented in the edgeR package.[21] The normalized counts were used in order to make unsupervised analysis, principal component analysis and clusters. For the differential miRNA expression analysis, read counts were converted to log2-counts-per-million (logCPM) and the mean–variance relationship was modelled with precision weights using voom approach in limma package version 3.44.1[22] using R version 4.0.0. Comparisons of expression for each miRNA between groups were made with moderated t-test and adjusted with the false discovery rate (FDR) method. Results with an adjusted $P < 0.05$ and a $|\log2FC| > 1$ were considered statistically significant.

miRNA pathway analysis

Pathway analysis of miRNAs was done using DIANA-miRPath v4.0 (using the target gene data from miRTarBase 2022),[23] MiRSystems, miTALOS2, miRTar, and miRTargetLink. The enrichment analysis method selected was Fisher's exact test with FDR correction. Pathways with a $P < 0.05$ were selected.

Statistical analysis of patient characteristics

Statistical analysis of clinical and analytical variables was done using SPSS23. Chi squared, Mann Whitney U, and T-Tests were used to make pairwise comparisons between groups of qualitative and quantitative variables respectively. Results with $P < 0.05$ were considered statistically significant.

Results

Patient distribution, clinical and analytical variables

A total of 42 subjects were included in the study: 10 controls and 32 HIV+ individuals. Of HIV+ individuals, 7 were elite controllers, a group of HIV-infected individuals that spontaneously control the infection. Clinical and analytical characteristics of participants are shown in Table 1 and Table 2. The main analysis was performed comparing the HIV+ progressors versus non-HIV+ individuals (control group). No differences in terms of age, sex or anthropometric characteristics were found between controls and HIV+ progressors (Table 1). As expected, HIV-infected individuals showed higher levels of inflammatory markers (hsCRP, ESR, D-dimer) than controls. At 48-week after ART initiation, all HIV+ individuals had undetectable viral load and CD4+ T-cell count raised from 375 (254–615) to 547 (393–720); $p < 0.001$) (Table 1). However, not all inflammatory parameters were restored and were comparable to control group, i.e. ESR was still significantly higher in treated HIV-infected individuals compared to controls: 7 mm/h (3–14) in treated HIV vs 3 mm/h (2–7) in controls; $p = 0.009$.

miRNA differences in HIV patients (progressors) versus controls

A total of 645 miRNAs were detected in serum samples in overall participants (GSE217795). We compared miRNA expression levels between controls and HIV+ progressors at baseline and we observed three significantly differentially expressed miRNAs (Table 3). Of them, two were downregulated in HIV participants, miR-3135b and miR-6503-3p, whereas miR-21-5p was upregulated. After 48 weeks on ART, these 3 miRNAs, although in lesser extension, remained significantly altered in HIV patients in comparison

Table 1 Clinical and analytical characteristics of participants: non-HIV+ individuals (Controls) and HIV+ patients. HIV+ patients were evaluated previous ART (Naïve HIV+) and after 48 weeks on ART (HIV+ 48-w).

	Control n = 10	Naïve HIV+ n = 25	p-value	HIV+ 48-w n = 25	p-value[1]
Age	34 (31—39)	37 (32—43)	0.506	-	
Sex (% males)	5 (50%)	20 (80%)	0.076	-	
Smoking	5 (50%)	14 (56%)	0.748	-	
Alcohol use	0 (0%)	2 (8%)	0.357	-	0.748
Recreative drug users	1 (10%)	6 (24%)	0.350	-	0.357
IVDU	0 (0%)	1 (4%)	0.521	-	0.350
BMI (kg/m^2)	22.3 (19.78—25.30)	24.22 (21.84—26.72)	0.068	25.4 (21.9—27.1)[a]	0.521
Nadir CD4 cell count (cells/μl)	-	374 (231—526)		-	
Nadir CD4/CD8	-	0.38 (0.18—0.67)		-	
Zenit viral load (copies/ml)	-	4.65 (3.65—4.98)		-	
Opportunistic infections	-	5 (20%)		-	
CD4 cell count (cells/μl)	-	375 (254—615)		**547 (393—720)**	**<0.001**
Plasma viral load (Log copies/ml)	-	4.6 (3.7—4.9)		**0**	**<0.001**
Beta-2 microglobulin (mg/l)	1.40 (1.37—1.43)	2.14 (1.68—2.83)	**0.002**	**1.77 (1.53—2.05)**[a]	**<0.001**
hsCRP (g/dl)	0.04 (0.01—0.24)	0.29 (0.11—0.59)	**0.009**	**0.12 (0.04—0.29)**[a]	**<0.001**
ESR (mm/h)	3 (2—7)	18 (11—30)	**0.028**	**7 (3—14)**[2]	**<0.001**
Fibrinogen (mg/dl)	279 (197—300)	247 (202—275)	0.568	269 (233—312)[a]	0.518
D-dimer (ng/ml)	97 (62—141)	190 (105—595)	**0.036**	**143 (72—220)**[a]	**0.009**

[a] Non-significant compared to controls.

n: sample size, IVDU: intravenous drug users, BMI: body mass index, hsCRP: high sensitivity C-reactive protein, ESR: erythrocyte sedimentation rate. For qualitative variables data is shown as frequencies and percentages and for quantitative variables as median and interquartile range. P value was calculated with X^2 test for qualitative variables and with Mann Whitney U test for quantitative variables. P < 0,05 was considered statistically significant and is shown in bold.

p-value comparing controls vs Naïve HIV+.

[1]p-value comparing HIV+ at baseline vs week 48 post ART.

[2]p-value<0.05 comparing 48-weeks vs. controls.

Table 2 Clinical and analytical characteristics of elite controllers. Parameters were compared to controls (non-HIV+ individuals).

	Control n = 10	Elite controllers n = 7	p-value
Age	34 (31—39)	54 (45—55)	**0.005**
Gender (% males)	5 (50%)	4 (57%)	0.772
Smoking	5 (50%)	4 (57%)	0.772
Alcohol use	0 (0%)	3 (42,9%)	**0.023**
Recreative drug users	1 (10%)	1 (14.3%)	0.787
IVDU	0 (0%)	1 (14.3%)	0.218
BMI (kg/m^2)	22.3 (19.78—25.30)	25.6 (23.25—29.01)	**0.025**
Nadir CD4 cell count (cells/μl)	—	417 (341—548)	—
Nadir CD4/CD8	—	0.638 (0.439—1.244)	—
Zenit viral load (copies/ml)	—	27.5 (20—122)	—
Opportunistic infections	—	0 (0%)	—
Actual CD4 cell count (cells/μl)	—	444 (356—660.2)	—
Actual CD4/CD8		0.533 (0.463—1.123)	
Actual Viral load (copies/ml)	—	66 (39—145)	—
Beta-2 microglobulin (mg/l)	1.40 (1.37—1.43)	1.63 (1.41—2.17)	0.164
hsCRP (g/dl)	0.04 (0.01—0.24)	0.19 (0.05—0.34)	0.193
ESR (mm/h)	3 (2—7)	11 (10—22)	0.296
Fibrinogen (mg/dl)	279 (197—300)	307 (251—320.5)	0.277
D-dimer (ng/ml)	97 (62—141)	280 (210—822.5)	**0.038**

n: sample size, IVDU: intravenous drug users, BMI: body mass index, hsCRP: high sensitivity C-reactive protein, ESR: erythrocyte sedimentation rate. For qualitative variables data is shown as frequencies and percentages and for quantitative variables as median and interquartile range. P value was calculated with X2 test for qualitative variables and with Mann Whitney U test for quantitative variables. P < 0,05 was considered statistically significant and is shown in bold.

Table 3 Significant differentially expressed serum miRNAs in Naïve HIV+ patients vs Controls and in HIV+ 48 weeks on ART vs Controls.

miRNA	Log2FC Naïve vs Control	Adjusted P value Naïve vs Control	Log2FC 48 w ART vs Control	Adjusted P value 48 w ART vs Control
miR-3135b	−1.58	0.032	−1.28	0.042
miR-6503-3p	−1.38	0.014	−1.05	0.041
miR-21-5p	1.33	<0.001	1.10	<0.01

Log2FC: log2 fold change. P value was calculated with moderated t-test and adjusted with the false discovery rate (FDR) method. Results with an adjusted $P < 0.05$ and a $|log2FC| > 1$ were considered statistically significant.

with controls. In this way, no significant change of these miRNA levels was detected in HIV+ individuals after ART: Log2FC 48 w ART vs Naïve for the miR-21-5p was −0.24 (p value = 0.7); Log2FC 48 w ART vs Naive for the miR-3135b was 0.30 (p value = 0.78), and the Log2FC 48 w ART vs Naive for mir-6503 was 0.33 (p-value = 0.7). Hence, ART was not able to restore normal levels of these miRNAs during this follow-up time, despite CD4 reconstitution and viral suppression (Table 3).

miRNA differences in elite controllers versus controls

In order to rule out an ART effect on miRNA levels, a subset of HIV+ individuals who naturally are controlling the infection (elite controllers) was also analysed. Elite controllers were older than controls, they had higher BMI and elevated D-dimer levels (Table 2).

In elite controllers, miR-21-5p and miR-6503-3p showed altered levels compared to control individuals, better levels than HIV+ Naïve patients, and similar levels than HIV+ individuals after ART (Table 4).

miRNA pathway analysis

Target genes with strong evidence of miR-21-5p are shown on Fig. 1 using miRTargetLink tool (https://ccb-web.cs.uni-saarland.de/mirtargetlink/network.php?type=miRNA&qval=hsa-miR-21-5p). Among the top 20 KEGG pathways (the most significant FDR p-values) for miR-21-5p obtained in miRPath v4.0 (using experimentally validated data), several are related to inflammation and infection mechanisms: MAPK signaling pathway, Hepatitis B, Human cytomegalovirus infection, Epstein–Barr virus infection, FoxO signalling pathway, Primary immunodeficiency, Human immunodeficiency virus 1 infection, etc.

Overall, using the different pathway enrichment tools, the most representative pathways with strong supportive evidence involving hsa-miR-21-5p were: MAPK signalling, Cytokine–cytokine receptor interaction, TGF beta signalling, HIV-1 Nef: Negative effector of Fas and TNF-alpha, and P53 signalling.

No experimentally validated pathways and target genes were found for miR-3135b and miR-6503-3p in miRPath v4.0 and TarBase v8.0. Enriched pathways with supportive evidence were not found using the different miRNA analysis tools.

Discussion

Next generation sequencing was applied to characterize differentially expressed serum miRNAs between treatment-naïve HIV-infected individuals and uninfected controls. We report that HIV infection upregulates circulating levels of miR-21-5p and downregulates miR-6503-3p and miR-3135b compared to controls. Moreover, we detected a persistent miRNA profiling alteration after 48-weeks on ART, suggesting that these miRNAs were impaired because of the chronic inflammation status underlying HIV infection. The miR-21-5p and miR-6503-3p were also upregulated and downregulated, respectively in HIV+ patients who did not receive ART because they did not show signs of progressive disease.

No previous literature was found about miR-3135b and miR-6503-3p related to HIV infection or inflammation mechanisms. Downregulation of both miRNAs has unclear consequences since there are no known validated target genes and pathways. Further studies are needed in order to replicate our results and to elucidate the role of these miRNAs in HIV infection.

On the other hand, higher miR-21-5p levels in circulating extracellular vesicles of HIV-positive individuals on ART

Table 4 Significant differentially expressed serum miRNAs in Elite controllers vs Controls and comparison to HIV+ Naïve and after 48 weeks on ART.

miRNA	Log2FC Elite controllers vs Control	P value Elite controllers vs Control	Log2FC Elite controllers vs Naïve	P value Elite controllers vs Naïve	Log2FC Elite controllers vs 48 w ART	P value Elite controllers vs 48 w ART
miR-6503-3p	−1.19	0.007	0.20	0.61	−0.14	0.72
miR-21-5p	0.80	0.016	−0.54	0.07	−0.30	0.3

Log2FC: log2 fold change. P value was calculated with moderated t-test. Results with an adjusted $P < 0.05$ and a $|log2FC| > 1$ were considered statistically significant.

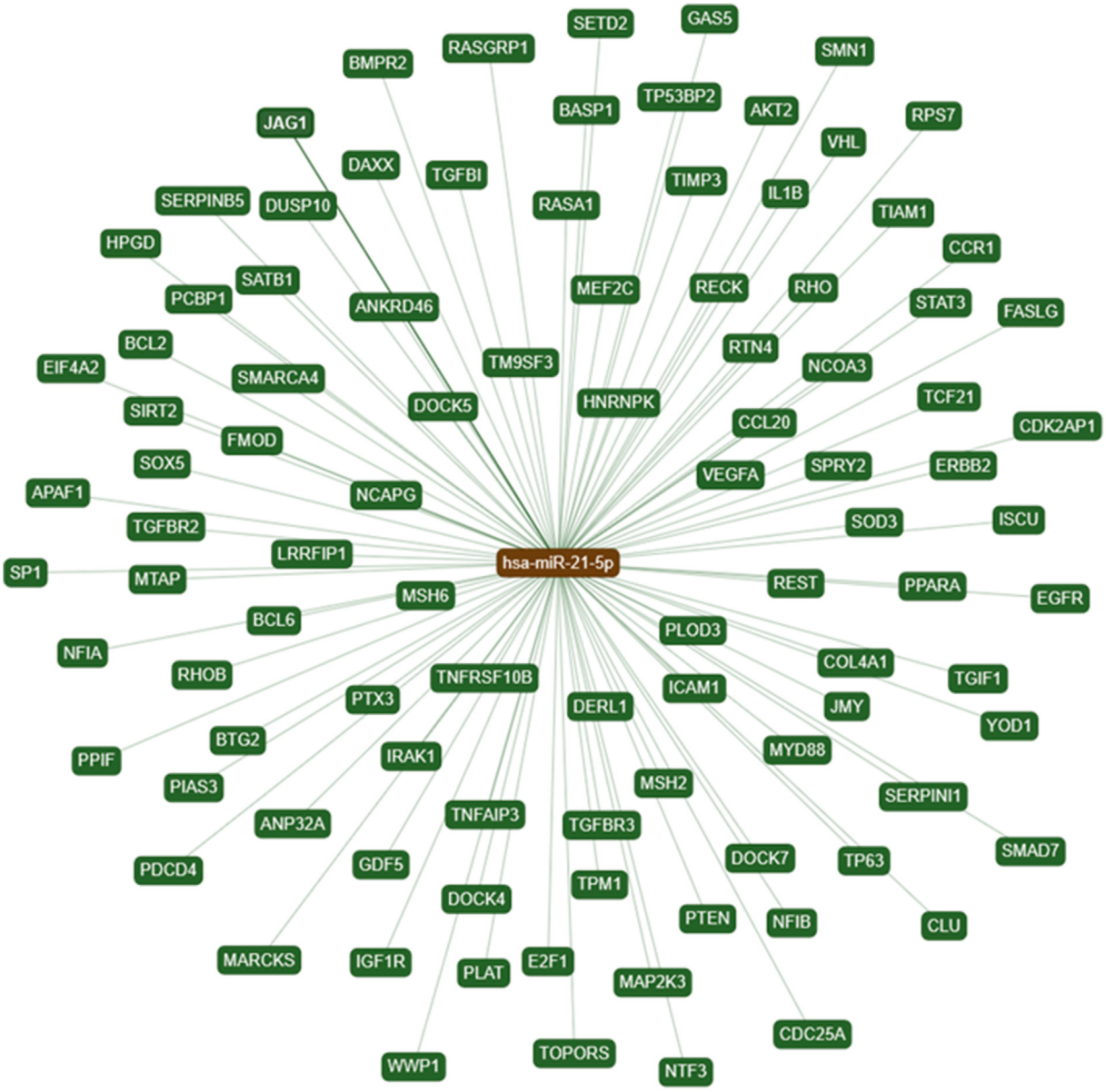

Figure 1. Predicted targets for miR-21-5p using Chair for Bioinformatics at the University of Saarland.

have been previously reported by Chettimada et al.[24] They found that levels of miR-21-5p correlated with metabolites of the cysteine metabolism pathway (cysteine, cystine, oxidized cys—gly, cysteine s-sulfate) which are markers of oxidative stress. Furthermore, there is evidence of higher levels of circulating miR-21-5p related to aging.[25] Also, lower circulating levels of this miRNA have been observed in centenarian subjects compared to healthy 80 years old individuals, suggesting that lower levels of miR-21-5p are beneficial for longevity.[26] These findings have made some researchers to propose this miRNA as a biomarker of pathological aging or inflammaging,[27] and thus, it might contribute to the accelerated ageing observed in PLWH. Other diseases where higher serum levels of miR-21-5p have been reported are autoimmune diseases like ulcerative colitis, systemic lupus erythematosus and myasthenia gravis.[28—30] Furthermore, in the case of myasthenia gravis patients, circulating miR-21-5p levels were reduced following immunosuppressive therapy.[30] Additionally, several target genes and pathways are described for miR-21-5p related to inflammation, infection mechanisms and apoptosis regulation such as oxidative stress response, Toll-like receptor signalling, TGF beta signalling and Notch signalling.[24]

Existing evidences demonstrate that serum levels of miR-21-5p are related to chronic inflammation and continuous immune activation. All these data give rise to the hypothesis that this miRNA could have an important role in the pathophysiology of early aging in HIV patients. Importantly, ART would not be able to normalize the miRNA levels after 48 weeks, and therefore, also unable to totally restore inflammatory parameters.

Additionally, long-term non-progressors are a special group of HIV+ patients due to their ability to suppress HIV

replication without treatment. Despite naturally controlling the infection, many studies have found that elite controllers present an abnormal pattern of immune activation and therefore they had worse clinical outcomes in terms of cardiovascular diseases, non-AIDS-defining infections, and increased rates of all-cause hospitalization.[31,32] Of note, elite controllers were around two decades older than controls and therefore, we cannot rule out an ageing effect on miR-21-5p levels. Hence, it is plausible that miRNAs involved in ageing and/or related to a permanent inflammatory status were also altered in this group of patients.

A small trend toward normalization of miR-21-5p and miR-6503-3p levels was observed after ART. We hypothesize that this trend could mean some immune reconstitution after the virus is controlled. In elite controllers, the Log2FC of these miRNAs versus the control group is also smaller than naïve HIV-infected individuals, corroborating this immune reconstitution. However, miRNA levels remained significantly altered in both HIV+ groups, which correlates with persistent inflammation detected in these patients.[33] Otherwise, altered miRNA levels would involve the host response against the virus or alternatively as a mechanism for the virus to facilitate viral replication and infection or to maintain latency, both of which decrease following ART and in elite controllers.[10] Functional studies should be addressed in order to explore these hypotheses.

The main limitation of the study is that results are obtained from a single centre study with a limited number of patients and restricted to 48 weeks of follow-up, and therefore our findings should be replicated in other cohorts. In addition, elite controllers are older than control and HIV+ progressors, which should be considered while making comparisons. Control individuals were selected to be representative of a healthy population, and similar to HIV+ progressors. On the other hand, elite controllers are a very rare HIV-infected population and all individuals who met inclusion criteria were included. Our work has some strengths that have to be considered. We applied next generation sequencing which allows to identify most of the small RNAs present in our samples, rather than other approaches like microarrays or quantitative PCR that only detect pre-defined miRNAs included in the chip. Hence, as majority of circulating miRNAs have been analysed, we were able to find new evidence, such as downregulated circulating levels of miR-6503-3p and miR-3135b in HIV patients. Another strength is that our patients were naïve for the ART and therefore we have been able to isolate the impact of the HIV infection on miRNA expression without the interference of ART. But, more interestingly, we replicated the analysis in the same patients one year after ART and in a subset of elite controllers, and these microRNAs were consistently altered. This is an intra-cohort validation, showing that these miRNAs were affected by the presence of HIV itself and its associated chronic inflammation and not HIV replication.

In conclusion, treatment-naïve HIV patients showed upregulated circulating levels of miR-21-5p and downregulated levels of miR-6503-3p and miR-3135b compared to a healthy control population. miR-21-5p target genes and pathways are implicated in inflammation and immune activation. We found upregulation of this miRNA independently of ART, since it was altered before and 48 weeks after starting ART, and in elite controllers. These findings suggest that expression of miR-21-5p could be consequence of chronic inflammation and oxidative stress levels associated with HIV infection. This study contributes to new insights on miRNA dysregulation in HIV pathogenesis, but the application of miRNAs as diagnostic and interventional medicine should be assessed in future investigations.

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Author contributions

N.G.G., R.G.F. conceived the project. J.S.F., A.G.M, E.C.R., and F.B.H. visited the patients and collected the patients' data and samples. J.M.D., N.G.G., and R.G.F. designed the analysis. N.G.G. and J.M.D. performed the experiments. J.M.D., N.G.G., J.D., and R.G.F. performed the analyses and discussed the results. X.N. and H.K. supervised the project and made a critical revision. J.M.D., N.G.G., and R.G.F. wrote the manuscript. All authors contributed to the article revision and approved the submitted version.

Conflicts of interest

The authors declare no conflict of interest.

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